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ecotoxicological aspects of dithiocarbamates

by

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Chapter 1

General Introduction

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Chapter 1

General Introduction

The legal framework for combatting inland water pollution in The Netherlands is provided by the 1970 Pollution of Surface Waters Act and the implementary regulations pursuant to it (Water Action Programme, 1980). The Act was revised in 1982 in order to allow for the adoption of a number of international conventions including the convention on the protection of the Rhine against chemical pollution (1976) and the EC directive on pollution caused by certain dangerous substances which are discharged into the aquatic environment (Council Directive, 1976). The convention and the directive decree that member states should take all appropriate measures to terminate pollution by substances appearing on list 1 (the so-called black list) and to reduce pollution by substances on list 2 (the so-called grey list).

However, given the wide variety of actual discharges and other forms of water pollution, the various properties of the substances involved, the different conditions and functions of the receiving waters, it is obvious that pollution cannot be reduced by the mere prescribing of a neat, uniform procedure. First, not all substances need to be restricted to the same extent: as indicated above, black-listed substances are to be tackled in such a way as to eliminate the pollution they cause, while the targets for the other substances, i.e. other than those on the black list, are generally less stringent. Second, there are different ways and instruments available for reducing water pollution. In fact, the EC directive referred to above, formalises a line of thinking that already existed, namely that there are two avenues for implementing policy:

- the 'direct emission approach', i.e. a straigtforward reduction in discharges, particularly of black-listed substances and
- the 'water quality approach', i.e. a reduction in discharges combined with the water quality objectives, mainly relating to the other substances.

An essential feature of the Dutch Pollution of Surface Waters Act is decentralization. Central government is responsible for the management and quality of the major watercourses such as the rivers Rhine, Meuse and Scheldt and of Lake IJssel and the Wadden Sea. The provincial authorities are essentially responsible for the remaining watercourses but they may in turn delegate responsibility to the Water Boards. This decentralization means that ways must be found of ensuring the necessary coordination and uniformity of planning, licensing and levy procedures. An important instrument in this coordination is the 'Water Action Programme', which is drawn up every five years by the Minister of Transport and Public Works and the Minister of Housing, Physical Planning and Environment. The Pollution of Surface Waters Act places at the disposal

of the authorities responsible for pollution control a number of instruments by which their polices may be put into effect. The most important instruments are permits for all discharges into surface water of polluting or harmful substances and the possibility to prohibit or limit discharges of certain substances. To meet the cost of pollution abatement levies are imposed on those companies discharging oxygen-consuming substances and certain heavy metals. The Institute for Inland Water Management and Waste Water Treatment[1] is among other things charged with an advisory task in the execution of the Pollution of the Surface Waters Act and related policy preparation.

The efforts to combat water pollution have made rapid progress since the introduction of the Act. Attention has mainly been focussed on the reduction of discharges of oxygen-consuming wastes, heavy metals and organochlorine pesticides. As a result of considerable investments in industrial and sewage purification plants both at the national and the international level, water quality has drastically improved (Water Action Programme, 1980). Nevertheless, due to the absence of geochemical and ecotoxicological realism in the setting of quality criteria for heavy metals and certain persistent lipophilic compounds, contamination of sediments has reached unacceptable levels. Moreover, many organic micropollutants escape attention due to the lack of adequate analytical methods, to their complex environmental behaviour, their unknown impact on aquatic ecosystems and, sometimes due to political interference resulting in inaccuracies in setting priorities. These points all apply to dithiocarbamates (DCs). Fortunately, however, biomonitoring of the waste water of DC-manufacturing companies has revealed their high toxicity, and drawn attention to this group of pesticides. This made them one of the main topics of the Institute for Inland Water Management and Waste Water Treatment, not only because of their high toxicity but also because of their large production and wide use:

- Dutch annual DC-production is about 14,000 tonnes; an estimated 400 tonnes per year are directly discharged into the aquatic environment;
- DCs are industrially applied as heavy metal scavengers in waste water treatment, as biocides in water-cooling systems, as slimicides in sugar, pulp and paper manufacturing and as vulcanization accelerators in the rubber industry (Wing and Rayford, 1982; Wilholm, 1982; Thorn and Ludwig, 1962; EPA, 1982);
- DCs constitute one of the most important classes of fungicides currently used in agricultural practice, both in the Netherlands and abroad (EPA, 1982; Curatorium Landbouwemissie, 1979).

DCs are synthesized from amines, carbon disulfide and sodium hydroxide. The resulting sodium derivatives are either oxidized to thiuramdisulfides or reacted with metal sulphates, and the desired organic complex is precipitated (Thorn and Ludwig, 1962; Commission of the European Communities, 1979). DCs can be devided into two subgroups according to their chemical structure:
a) N,N'-ethylenebisdithiocarbamates (BDCs) and b) N-substituted monoalkyldithiocarbamates (MDCs) and dialkyldithiocarbamates (DDCs). The former group is

[1] The former Government Institute for Sewage and Waste Water Treatment.

derived from primary amines and the latter from secondary amines. This chemical distinction is, among other things, reflected in their different routes of degradation.

On exposure to moisture, acids and high temperatures DCs decompose into a large variety of degradation products (Klisenko and Vekshtein, 1970; Siegel and Sissler, 1977). Although the primary degradation processes may proceed at high rates, ultimate biodegradation, i.e. mineralization in soils, hydrosoils and water in particular, is relatively slow (Schlagbauer and Schlagbauer, 1972; Strufe, 1968; Fig.1). Hence, in drawing conclusions about the environmental impact of DCs, the ecotoxicological profiles of their degradation products should also be considered.

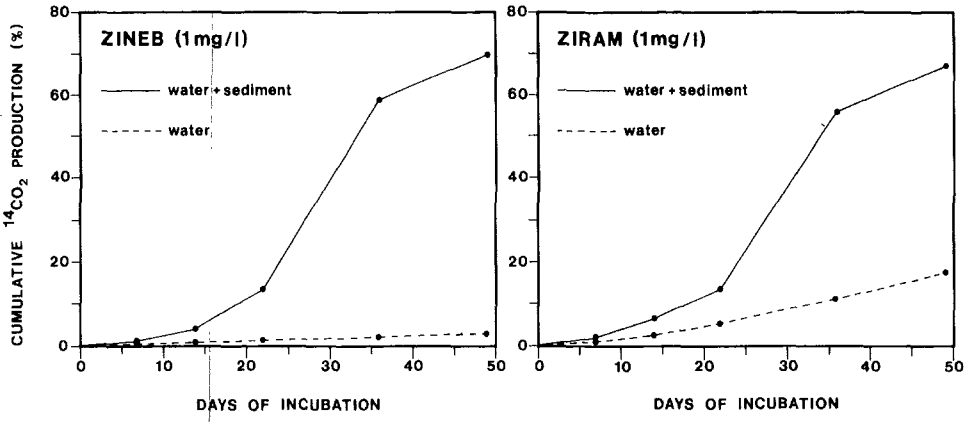

Fig. 1. Biodegradation of [^{14}C-ethylene] zineb and [^{14}C-methyl] ziram in surface water with and without bottom sediment. At the end of the incubation period approximately 23% of the total ^{14}C-activity in the mud was recovered as bound residue [14].

DCs are effective against a broad spectrum of fungi and other organisms (Thorn and Ludwig, 1962; Owens, 1969). They disturb many cellular processes. Pronounced alterations in the intermediary metabolism of ethanol caused by tetraethylthiuram disulfide (disulfiram) constitute the most illustrative example of this; its effectiveness as an anti-alcoholic drug has been discovered casually by two Danish physicians. These persons who had taken disulfiram in the course of an investigation of its potential anthelmintic usefulness became ill at a cocktail party and were quick to realize that disulfiram had altered their response to alcohol.

The toxicology of BDCs and MDCs/DDCs is generally treated separately, probably mainly because of their different degradation pathways. Ethylenethiourea (ETU), a degradation product of BDCs, which has drawn much attention because of its goiterogenic, mutagenic, carcinogenic and teratogenic properties (Seiler, 1974; Van Leeuwen et al., 1982), has played an important part in this distinction. A closer look at their degradation schemes reveals some relevant similarities. Both groups degrade into thiourea derivatives and carbon disulfide. The latter compound may contribute considerably to the toxicological similarities between the two groups as it reacts with nucleophilic groups and degrades into highly reactive atomic sulfur. DC-like structures may arise through reaction of CS_2 with aminogroups. This may be one of the reasons

for the close similarity in toxic symptomatology of CS_2 and DCs (Brieger and Teisinger, 1967; Beauchamp et al., 1983; W.H.O., 1979; De Bruin, 1976; Fishbein, 1976).

A large body of information concerning the toxicity of DCs to mainly warm-blooded animals has been reviewed by Fishbein (1976). Their acute oral toxicity is low, with rat-LD_{50} values varying from 0.4 to 7 g/kg. In longer-term exposure studies many functions, such as neurological, immunological, hormonal, gonadal, renal, haematological and visual functions may be affected. Reports have appeared on their mutagenicity, carcinogenicity, teratogenicity and their dysmorphogenetic effects on regeneration processes (Fishbein, 1976; Hedenstedt et al., 1979; Arias and Zavanella, 1979). On the basis of these effects the ADIs of various DCs for man have been lowered to 0.005 mg/kg b.w. (Vettorazzi, 1979). However, information on their aquatic toxicity is scarce (Thorn and Ludwig, 1962; EPA, 1982), which prevents an adequate evaluation of their aquatic ecotoxicological impact. It is the aim of this thesis to furnish these data.

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Chapter 2

Aquatic toxicological aspects of dithiocarbamates and related compounds. Short-term tests

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Abstract

Short-term toxicity of 26 dithiocarbamates (DCs) and related compounds was determined in tests with guppies (*Poecilia reticulata*), water fleas (*Daphnia magna*), green algae (*Chlorella pyrenoidosa*) and bacteria (*Photobacterium phosphoreum*). A screening test for the inhibition of nitrification was also conducted. It was found that both ethylenebisdithiocarbamates and dialkyldithiocarbamates were toxic to the organisms tested. Experiments were carried out to determine the *n*-octanol/water partition coefficient of a number of substances. In the case of DCs and their degradation products (metal derivatives were not considered) it was possible to draw up quantitative structure-activity relationships for the various test species. Various aspects of tests conducted on these fungicides and their degradation products (hydrolysis, biodegradation, polymerization and volatilization) are discussed, as are a number of biochemical mechanisms of toxicity. It is concluded that DCs are cytotoxic substances and therefore must be regarded as broad-spectrum biocides.

Chapter 2

Aquatic toxicological aspects of dithiocarbamates and related compounds. Short-term toxicity tests

Introduction

Dithiocarbamates (DCs) are a well-known group of pesticides which have been used since 1934 to control a number of species belonging to taxonomically different groups e.g. bacteria, fungi, nematodes and molluscs (Thorn and Ludwig, 1962; Strufe, 1968; McEwen and Stephenson, 1979). They possess insecticidal properties (Gretillat, 1962) and are also used as rodent repellent to protect young planting (McEwen and Stephenson, 1979; CBS, 1981). Besides these agricultural applications DCs are also used in industry, for example as vulcanization accelerator and antioxidant in rubber (Thorn and Ludwig, 1962), as slimicides in sugar, pulp and paper mills and to control algal, bacterial and fungal growth in water-cooling systems (EPA, 1982; Rus, 1978). Because of their chelating properties these substances are used as reagents for heavy metal analysis, as heavy metal scavengers in waste water treatment, and in human medicine (e.g. in cases of nickel poisoning). The DC disulfiram is used in treating chronic alcoholism (Thorn and Ludwig, 1962; Wilholm, 1982; Wing and Rayford, 1982).

According to their structure the DCs can be roughly divided into two main groups: the dialkyldithiocarbamates (DDCs) and ethylenebisdithiocarbamates (BDCs; Fig. 1). Many other structural analogues are known, however (Thorn and Ludwig, 1962). The structure of the salts of bisdithiocarbamic acid and bivalent metal ions is less defined. They have a highly polymeric character and coordinative structures have been proposed (Vonk, 1975).

DCs, and BDCs in particular, are manufactured and formulated on a large scale. The Dutch production is estimated at about 14,000 tonnes per year. Until now little is done to purify the waste water resulting from this production. This, together with the fact that these substances are used as slimicides, biocides in cooling systems and heavy metal scavengers, lead to the assumption that surface waters must be directly contaminated with these substances on a relatively large scale. Diffuse pollution from agricultural activities in this country and elsewhere may also contribute to such contamination. (CEC, 1979; Kuenen, 1980; EPA, 1982). However, there are no figures on the actual concentrations of DCs to be found in surface water, suspended matter or aquatic organisms. Information on the toxicity of these substances to aquatic organisms is in equally short supply. Therefore short-term toxicity tests were carried out with guppies (*Poecilia reticulata*), water fleas (*Daphnia magna*) and the unicellular green algae (*Chlorella pyrenoidosa*). Tests with *Photobacterium phosphoreum* (the microtox test) and nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*) were also conducted.

Materials and methods

Test substances

Information about the compounds used in the toxicity experiments is given in Table I. Structural formulas of some of these compounds are given in Fig. 1. Mancozeb and metiram contained 36% and 38.5% CS_2 respectively, and PETD contained 3% ETU and 0.1% DIDT. The dimethylamine used was a 40% solution in water. Labelled

TABLE I

Test compounds

Compound	Abbreviation	Supplier[a]	Purity (≥ %)
disodium ethylenebisdithiocarbamate	nabam	Lamers & Indemans	99
manganese ethylenebisdithiocarbamate	maneb	ICN	90
zinc ethylenebisdithiocarbamate	zineb	ICN	95
the polymer of maneb and zinc	mancozeb	Lamers & Indemans	-[b]
the polymer of zineb and ethylenethiuram-disulfide	metiram	Lamers & Indemans	-[b]
polymeric ethylenethiuramdisulfide	PETD	Pennwalt	-[b]
5,6-dihydro-3H-imidazo (2,1-c)-1,2,4-dithiazole-3-thion	DIDT	TNO	98
ehtylenethiourea	ETU	EGA	99
ethyleneurea	EU	EGA	97
ethylenediamine	-	Baker	-[b]
disodium sulfide	-	Baker	99
carbon disulfide	-	Baker	99
sodium dimethyldithiocarbamate	NaDMDC	Fluka	97
zinc dimethyldithiocarbamate	ZnDMDC	ICN	95
ferric dimethyldithiocarbamate	ferbam	ICN	95
tetramethylthiurammonosulfide	thiram	ICN	98
tetramethylthiurammonosulfide	TMTM	ICN	95
tetramethylthiourea	TMTU	Riedel de Haen	97
tetramethylurea	TMU	Riedel de Haen	98
sodium diethyldithiocarbamate	NaDEDC	Fluka	99
zinc diethyldithiocarbamate	ZnDEDC	ICN	90
tetraethylthiuramdisulfide	disulfiram	Fluka	97
tetra-*n*-propylthiuramdisulfide	TPTD	TNO	95
tetra-*n*-butylthiuramdisulfide	TBTD	ICN	95
dimethylamine	-	Fluka	98
diethylamine	-	Fluka	99

[a] The chemicals were obtained from Lamers & Indemans B.V. ('s-Hertogenbosch, The Netherlands), Baker Chemicals B.V. (Deventer, The Netherlands), Pennwalt Holland B.V. (Rotterdam, The Netherlands), TNO, Institute for Applied Chemistry (Utrecht, The Netherlands), Fluka AG (Buchs, Switzerland), EGA (Steinheim, F.R.G.), Riedel de Haen (Hannover, F.R.G.) and ICN Pharmaceuticals Inc. (Montreal, Canada).
[b] See Materials and Methods.

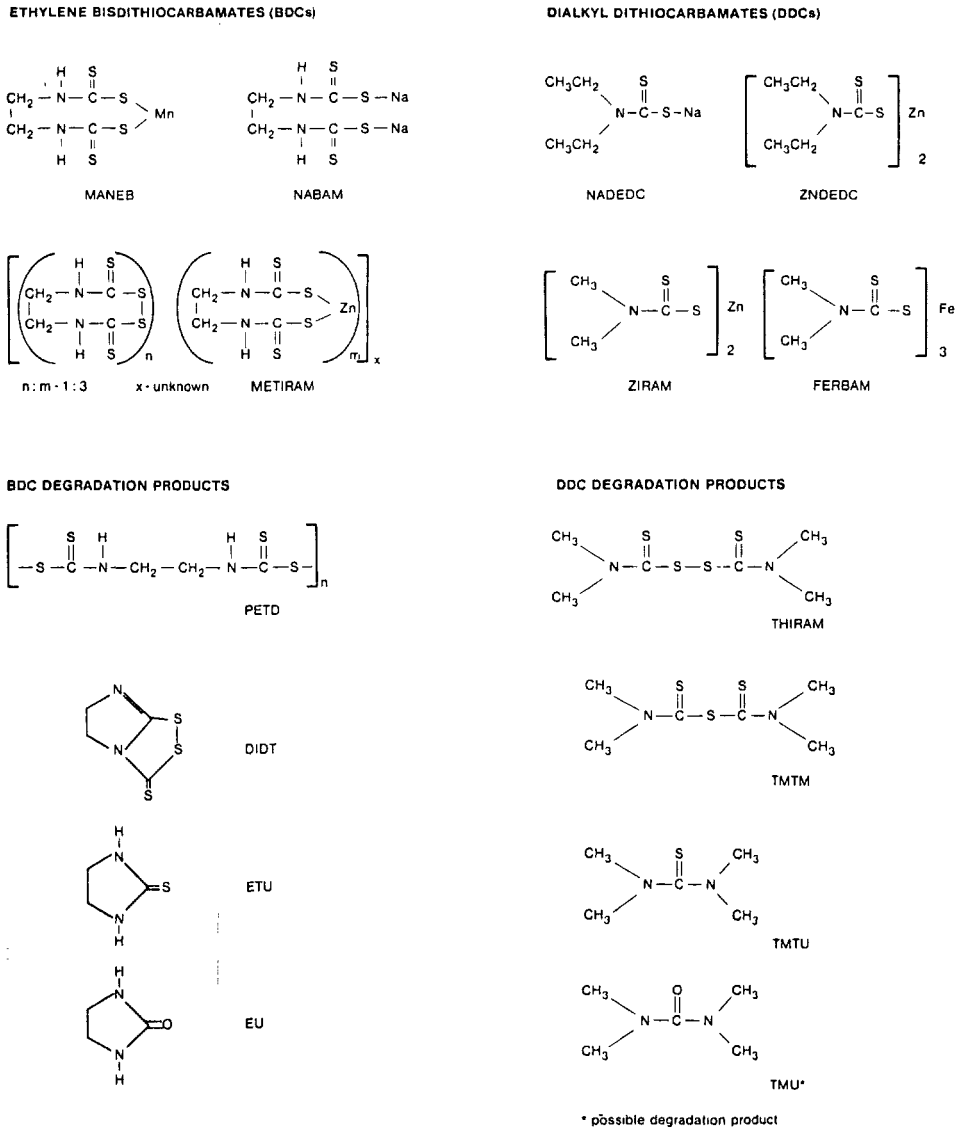

Fig. 1. Structural formulas of dithiocarbamates and several degradation products.

substances, [^{14}C]$NaHCO_3$ (sp.act. 52.3 mCi/mmol; r.c.p. 95%) and [^{14}C]ETU (sp.act. 22 mCi/mmol; r.c.p. 98%), were obtained from the Radiochemical Centre, Amersham (England).

Chemical tests

The half-conversion time of the hydrolysis reactions of the sodium dialkyldithiocarbamates was measured in relation to the pH. In accordance with OECD guideline 111

(1981), the tests were carried out in duplicate in phosphate buffer solutions with pHs of 3.8, 5.7, 7.0, 8.0 and 9.0 respectively. The concentrations of NaDMDC and NaDEDC in water were determined spectrofotometrically using a Perkin Elmer Hitachi 2000 at 252.5 and 257.0 nm.

The determination of the partition coefficient (*n*-octanol/water; P_{oct}) of some substances was conducted in triplicate in accordance with OECD guideline 107 (1981). [^{14}C]ETU was measured by liquid scintillation counting (LKB/WALLAC 1215 RACKBETA). Disulfiram, thiram, TMTM, TMTU and DIDT were determined by means of a Dupont HPLC 830 with UV detection at 240-280 nm (Guard column: Reversed Phase Pellicular; column: Chrompack CP/spher C18, 250 x 4.6 mm; eluent: methanol-water (70/30 v/v); pressure: 700 psi; temperature: 20°C).

Toxicity tests with P. reticulata and D. magna

Acute toxicity tests with *P. reticulata* (96 h) and *D. magna* (48 h) were carried out according to OECD guidelines 203 and 202, with slight modifications. The stock solutions were prepared fresh each day. The test solutions were renewed daily. Detailed information about the chemical composition of the media is given elsewhere (Van Leeuwen and Maas, 1985). The daphnids were fed on $1x10^8$ cells/l *C. pyrenoidosa*. The tests with Na_2S, CS_2 and the amines were conducted in sealed vessels. LC_{50} values and their 95% confidence intervals were calculated according to the Litchfield and Wilcoxon (1949) method.

Toxicity tests with C. pyrenoidosa

The algal bioassays were conducted in a temperature controled room at 20 ± 1°C. The test solutions with an initial cell density of approximately 10^8 cells/l (100 ml) were added to 200 ml Erlenmeyer flasks, which were stoppered with a cotton plug and placed on a mechanical shaker under fluorescent light (7.5 W/m²). The cells were counted in a model Z_BI Coulter Counter, using a 70 μm aperture. The tests were carried out in triplicate. The composition of the medium used is given elsewhere (Van Leeuwen and Maas, 1985). The tests with Na_2S, CS_2 and the amines were carried out in infuse bottles. In order to determine the algal density, while minimizing the loss of test substance, samples were taken using syringes. In order to compare EC_{50} values when similar tests are analysed in different ways, the values were calculated both for the average specific growth rate (μ), as defined in OECD guideline 201 (1980) and for the effects on the inoculum (*i*), the reproduction rate (*r*) or the yield (*y*), or combinations of these, as defined by Kooyman et al. (1983).

Effects on photosynthesis were studied in radiometric assays with ziram, thiram, maneb, PETD and DIDT. Hereto 100 ml flasks were filled with a 40 ml suspension of *C. pyrenoidosa* ($5x10^8$ cells/l), 50 μl $NaH^{14}CO_3$ (33.34 μCi/ml) and the requisite amount of test solution. Incubation was carried out according to the procedure described above. After 4 h, incubation was stopped by adding 50 μl of

a lugol solution (50 g I_2/l and 50 g KI/l) and 8 ml samples were taken. These were acidified with 100 µl 1N HCl, degassed for 25 minutes after which 12 ml of scintillation fluid (Insta-gel, Packard) was added and radioactivity was measured with a LKB/WALLAC 1215 RACKBETA liquid scintillation counter. These measurements were carried out in triplicate. The proportional inhibition of bicarbonate uptake was then calculated and the EC_{50} determined graphically on log-probability paper.

Inhibition of respiration was studied with the same compounds. A 50 ml *C. pyrenoidosa* suspension of 10^{10} cells/l together with 1.5 ml of a 33.34 µCi/ml solution of labelled sodium bicarbonate was incubated for 4 h. Incubation was carried out according to the procedure described above. In order to remove dissolved $NaH^{14}CO_3$ after incubation, this suspension was centrifugated (3500 rpm for 15 minutes) and the pellet resuspended in inactive *Chlorella* medium for three consecutive times. Microscopic observations with an inactive control did not reveal any damage to the cells. The ^{14}C-labelled algae were added to 100 ml flasks containing 39 ml of the test solution made up in *Chlorella* medium to give a final algal density of $5x10^8$ cells/l. These flasks were incubated in the dark for 16 h after which lugol was added and the remaining radioactivity was measured.

Toxicity tests with P. phosphoreum

The microtox test (Beckman, model 2055) and the calculation of the EC_{50} values (the concentrations which reduce the bacterial luminescence by 50%) were carried out in accordance with the procedure described in the Beckman Instruments Manual (1982).

Toxicity tests with nitrifying bacteria

The effects on the nitrification process were studied with the Blok (1981) screening test, which is based in principle on the conversion of ammonia via nitrite into nitrate. This reaction can be observed using a pH-indicator mixture. After 1 to 3 h a change in colour can be observed and the lowest effect concentration (MIC; minimum inhibiting concentration) determined.

QSAR studies

Quantitative structure-activity relationships (QSARs) and correlation coefficients were calculated using a computer program based on the method of least squares. The statistical evaluation of the quality of the QSARs was based on a comparison of the standard deviations using a *F*-test as described by Sokal and Rohlf (1981) and Hansch (1973).

Results

Chemical tests

Hydrolysis of sodium dialkyldithiocarbamates provides a perfect example of a (pseudo) first order reaction. The results together with the data from Klisenko and Vekshtein (1971) and Vonk (1975) are given in Table II. The reactions occur very rapidly at low pHs. Extraction of the NaDMDC buffer solution with chloroform revealed the presence of thiram. It can be concluded that the chemical stability of dithiocarbamates in water is determined by the pH value and the metal ion with which they form a complex. Thiuramdisulfides are relatively stable. The results of the partition coefficient determinations are shown in Table III.

TABLE II. Half-conversion times (h) of the hydrolysis reaction of some dithiocarbamates[a] in water at different pH values.

Test compound	pH				
	3.8	5.7	7.0	8.0	9.0
NaDMDC	0.04	2.4	62	483	2667
Ziram[b]	0.25	9.1	121	433	-
Thiram[b]	9.5	108	1123	3316	-
NaDEDC	0.02	0.67	20	200	1117
Maneb[b]	0.08	4.0	60	265	-
Zineb[b]	0.15	6.5	96	405	-

[a] Nabam decomposed completely mainly into DIDT and ETU within 1 day (Vonk, 1975; see also Fig. 2).
[b] Data taken from Klisenko and Vekshtein (1971).

TABLE III. Log P_{oct} values of the compounds used in QSAR calculations.

No	Compounds	Log P_{oct}
1	TPTD	6.06[a]
2	Disulfiram	4.00
3	Thiram	1.82
4	DIDT	1.62
5	TMTM	1.17
6	TMTU	0.49
7	TMU	0.20[b]
8	ETU	-0.67
9	EU	-0.96[b]

[a] Calculated from the experimental value of thiram and f (CH_2) from Rekker (1977).
[b] Calculated from the experimental values of the thioureas and △ (=S/=O) from Rekker (1977).

Toxicity tests with P. reticulata and D. magna

Table IV shows that the toxicity of the BDCs is more or less the same. However, PETD and DIDT are more toxic than the original compounds, whereas ETU and EU are less so. In the case of the DDCs (Table V) the sodium derivatives are less toxic than the iron and zinc derivatives and their thiuramdisulfides (thiram and disulfiram). Consecutive desulphuration of thiram into TMTM, TMTU and TMU is accompanied by an increased hydrophilicity and a reduced toxicity (Tables III and V). Sulfide and CS_2, degradation products of both BDCs and DDCs, were rather toxic; toxicity was observed in the low ppm range.

Toxicity tests with P. phosphoreum

The results of these tests (Table IV and V) again reveal roughly the same picture as the tests with the other species, with exception of the nitrifying bacteria. The high EC_{50} value for nabam in particular should be noted. Toxicity of nabam, however, increases with time (Fig. 2). Therefore it is likely that the toxicity of this compound is caused mainly by its degradation products. The high EC_{50} value of CS_2 should be attributed to the fact that the microtox test is not suitable for testing relatively volatile compounds.

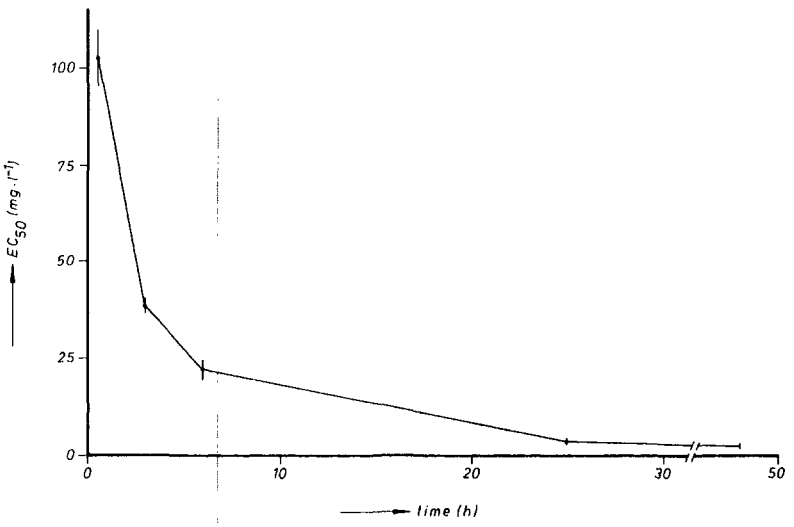

Fig. 2. Changes in toxicity of nabam in time as measured with a series of tests with *Photobacterium phosphoreum*. Bars represent 95% confidence limits.

TABLE IV. Results of short-term toxicity studies (mg/l) with ethylenebisdithiocarbamates and related compounds.

Compound	96-h LC_{50} (95% C.L.) *P. reticulata*		48-h LC_{50} (95% C.L.) *D. magna*		96-h EC_{50}[a] *C. pyrenoidosa*		15-min EC_{50} (95% C.L.) *P. phosphoreum*		3-h MIC *Nitrosomonas/ Nitrobacter*
Nabam	5.8	(4.0-8.5)	0.44	(0.37-0.52)	2.4	(1.8;r)	102	(96-110)	32
Maneb	3.7	(3.2-5.6)	1.0	(0.8-1.3)	3.2	(3.6;r)	1.2	(1.2-1.3)	56
Zineb	7.2	(5.0-10.3)	0.97	(0.56-1.80)	1.8	(1.1;i)	6.2	(4.8-8.0)	18
Mancozeb	2.6	(2.1-3.3)	1.3	(1.2-1.5)	1.1	(1.1;y)	0.08	(0.07-0.08)	32
Metiram	6.4	(4.0-10.4)	2.2	(1.8-2.8)	1.8	(0.87;i)	0.37	(0.35-0.39)	32
PETD	0.88	(0.56-1.0)	0.28	(0.24-0.31)	0.56	(0.29;y)	0.06	(0.05-0.06)	5.6
DIDT	0.49	(0.32-1.0)	0.21	(0.18-0.24)	0.18	(0.19;r)	0.03	(0.02-0.04)	5.6
ETU	7500	(5600-10,000)	26.4	(21.6-32.2)	6600	(860;y)	2100	(1500-1800)	1.0
EU	13,000	(10,000-18,000)	5600	(3200-10,000)	16,000	(6800;i)	3300	(2600-4200)	1000
Ethylenediamine	275	(180-560)	26.5	(20.4-34.4)	100	(61;y)	20.4	(18.1-23.0)	3.2
Na_2S	15.0	(7.7-29.1)	2.1	(1.0-3.2)	75	(6.7;i)	4.3	(2.9-6.3)	32
CS_2	4.0	(3.0-5.8)	2.1	(1.9-2.2)	21	(10.6;y)	341	(260-448)	28

[a]The first value represents the EC_{50} for μ. The values in parentheses represent the results of the parametric analysis; in case EC_{50} values could be determined for more than one criterium the lowest value is given. Abbreviations: see Materials and Methods.

TABLE V. Results of short-term toxicity studies (mg/l) with diakyldithiocarbamates and related compounds.

Compound	96-h LC_{50} (95% C.L.) *P. reticulata*		48-h LC_{50} (95% C.L.) *D. magna*		96-h EC_{50}[a] *C. pyrenoidosa*		15-min EC_{50} (95% C.L.) *P. phosphoreum*		3-h MIC *Nitrosomonas/ Nitrobacter*
NaDMDC	2.6	(2.1-3.2)	0.67	(0.52-0.86)	0.8	(0.24;r)	0.51	(0.40-0.66)	26
Ziram	0.75	(0.56-1.0)	0.14	(0.10-0.18)	1.2	(1.4;r)	0.15	(0.12-0.19)	100
Ferbam	0.09	(0.06-0.18)	0.09	(0.07-0.10)	2.4	(0.33;i)	0.20	(0.14-0.28)	10
Thiram	0.27	(0.22-0.33)	0.21	(0.17-0.27)	1.0	(0.03;i)	0.10	(0.08-0.11)	18
TMTM	5.3	(4.2-6.8)	2.9	(2.3-3.6)	1.0	(0.1;i)	1.9	(1.7-2.1)	32
TMTU	1800	(1000-3200)	770	(630-948)	1800	(450;y)	72.1	(63.5-81.8)	560
TMU	1800	(1000-3200)	2900	(1200-7300)	3200	(1600;y)	1100	(1000-1300)	1000
NaDEDC	6.9	(5.5-8.5)	0.91	(0.71-1.06)	1.4	(0.84;r)	1.22	(0.91-1.64)	43
ZnDEDC	0.49	(0.40-0.61)	0.24	(0.20-0.29)	1.1	(1.1;r)	1.70	(1.33-2.17)	> 320
Disulfiram	0.32	(0.24-0.43)	0.12	(0.10-0.14)	1.8	(1.4;r)	1.21	(0.91-1.61)	> 320
TPTD	3.6	(2.4-5.4)	0.23	(0.16-0.33)	> 32		> 100		> 320
TBTD	> 10		> 0.56[b]		> 10		> 60		> 100
Dimethylamine	210	(127-349)	50.0	(44.2-56.5)	30	(22;r)	26.8	(20.8-34.5)	180
Diethylamine	130	(100-180)	56.0	(32.0-100)	56	(78;r)	21.8	(19.0-25.1)	320

[a]The first value represent the EC_{50} for μ. The values in parentheses represents the results of the parametric analysis; in case EC_{50} values could be determined for more than one criterium the lowest value is given. Abbreviations: see Materials and Methods.
[b]See Results.

Toxicity tests with C. pyrenoidosa

The results of the experiments using *C. pyrenoidosa* correspond largely with those for guppies and daphnids, at least in respect of the effects on the average specific growth rate. However, parametric analysis of the measurements reveals considerable differences, particularly when the substances have effects other than on the reproduction rate.

For example, thiram (Fig. 3) induced acute mortality relative to its concentration, resulting in an increase of the time-lag until maximum population growth (EC_{50}:0.03 mg/l). Considering the effects on the specific growth rate, an EC_{50} of 1.0 mg/l could be calculated. Carbon disulfide caused an effect both on reproduction rate (EC_{50}:23.1 mg/l) and yield (EC_{50}:10.6 mg/l). The first effect is shown by the decrease in steepness of the log phase growth of the algae and the second effect by the decrease in the maximum density of the algal cells (Fig. 4). These results show that differences in the EC_{50} values arise when the same toxicity tests are analysed in different ways, i.e., when different parameters are studied.

For all compounds studied, inhibition of photosynthesis was shown over a broad range of concentrations (2 decades). The concentration of the compounds studied which inhibited assimilation, were either without effect or produced a slight acceleration of respiration (Table VI). An EC_{50} value could not be calculated. When the EC_{50} values for assimilation are compared with those for exponential growth it can be concluded that the compounds studied inhibit algal growth by means of inhibiting carbon fixation.

TABLE VI. Inhibition of the average specific growth rate (μ), assimilation and respiration of *C. pyrenoidosa*.

Compound	EC_{50} (mg/l)		
	μ	Assimilation	Respiration
Ziram	1.2	5.1	> 10
Thiram	1.0	4.0	> 10
Maneb	3.2	9.6	> 32
PETD	0.56	0.75	> 10
DIDT	0.18	0.33	> 10

Toxicity tests with nitrifying bacteria

The toxicity of the substances to nitrifying bacteria differs from that found in other tests (Table IV and V). Hydrophilic substances such as ETU and ethylenediamine inhibit nitrification quite considerably, but this is also true for DIDT, a relatively

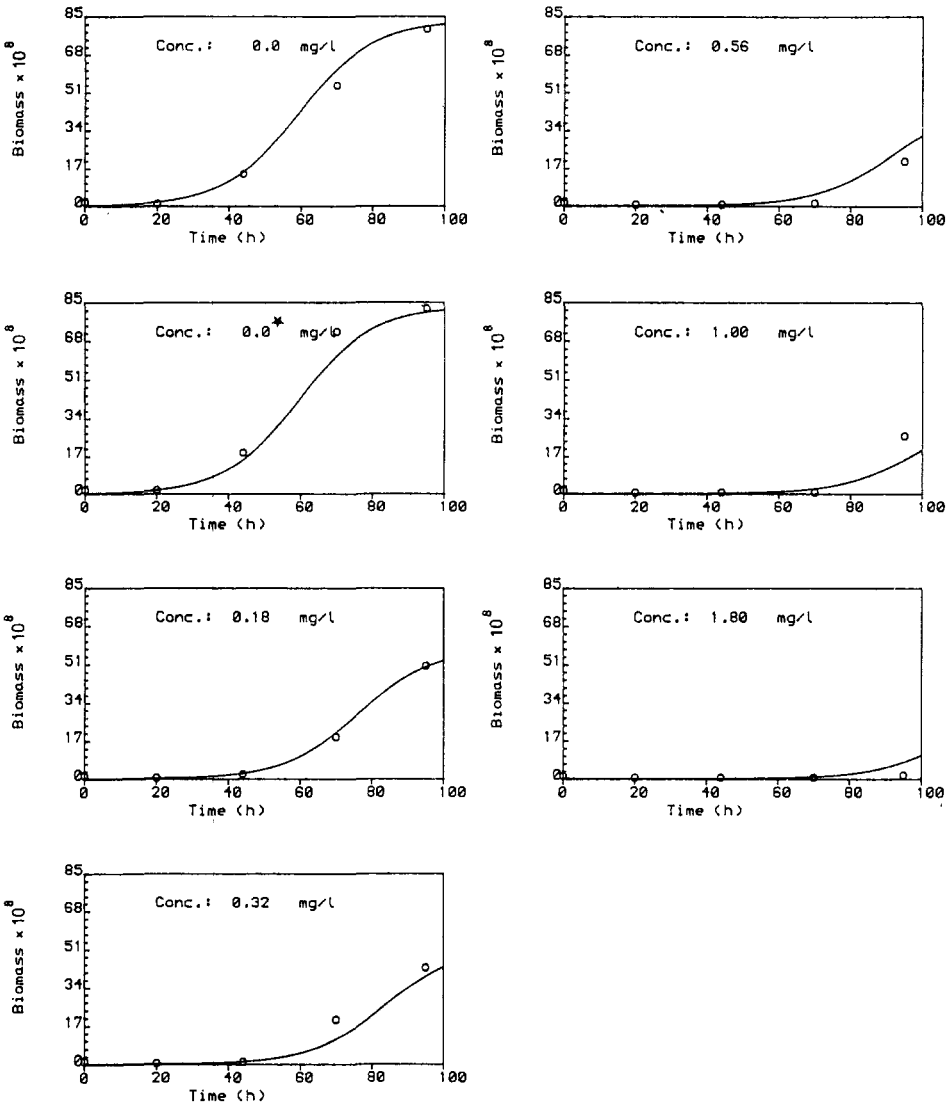

Fig. 3. Effects of thiram on the inoculum of *C. pyrenoidosa* populations. Circles represent the observed and lines the expected number of algae/l. Conc.: 0.0* is the solvent control.

hydrophobic compound. Inhibition markedly decreased when the sulphur atom disappeared from the molecule (ETU, EU; thiram, TMTM, TMTU and TMU). This screening test is not applicable to the testing of volatile compounds. It can be concluded that the nitrification process may be disturbed if DCs or their degradation products are discharged into the environment.

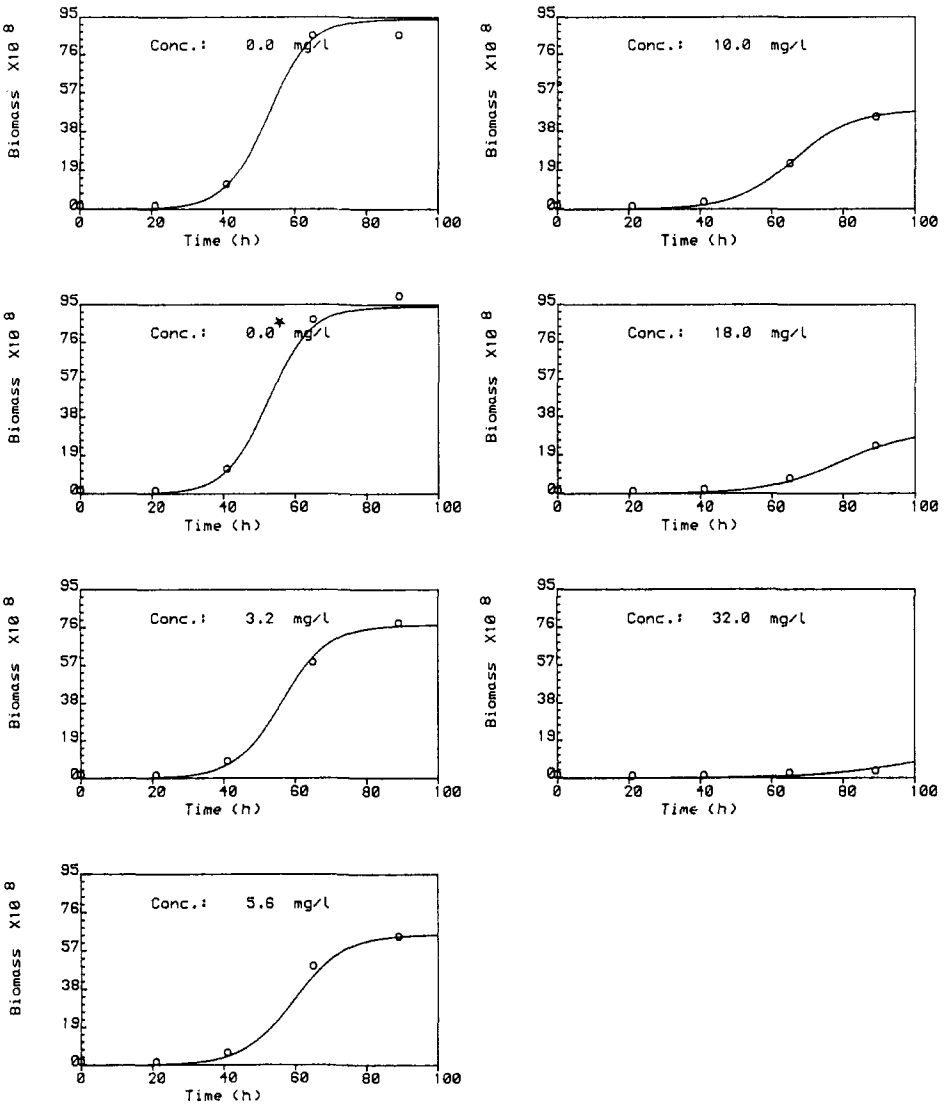

Fig. 4. Effects of CS_2 on both reproduction rate and yield of *C. pyrenoidosa* populations. Circles represent the observed and lines the expected number of algae/l. Conc.: 0.0* is the solvent control.

QSAR studies

The compounds used in the QSAR calculations are shown in Table III. Metal derivatives and polymers were excluded from the calculations. The studies revealed that the *n*-octanol/water partition coefficient explained nearly all variation in toxicity (Table VII). Effects on nitrification, however, could not be explained by this parameter.

TABLE VII. QSARs and correlations for the compounds shown in Table III. Linear relationships[a].

QSAR equation			log 1/C= a log P_{oct} + b			
C(μmol/l)	No.	a	b	n	s	r
LC_{50} 96-h *P. reticulata*	1[b]	1.93	-3.91	7	0.71	0.955
LC_{50} 48-h *D. magna*	2[b,c]	2.01	-3.81	6	0.88	0.935
EC_{50} 96-h *C. pyrenoidosa*	3[b]	1.98	-3.89	7	0.91	0.932
EC_{50} 15-min *P. phosphoreum*	4[b]	2.01	-3.25	7	0.72	0.957

[a] No.: equation number; n: number of data points; s: standard error of the estimate and r: correlation coefficient.
[b] QSAR calculated without disulfiram and TPTD.
[c] QSAR calculated without ETU.

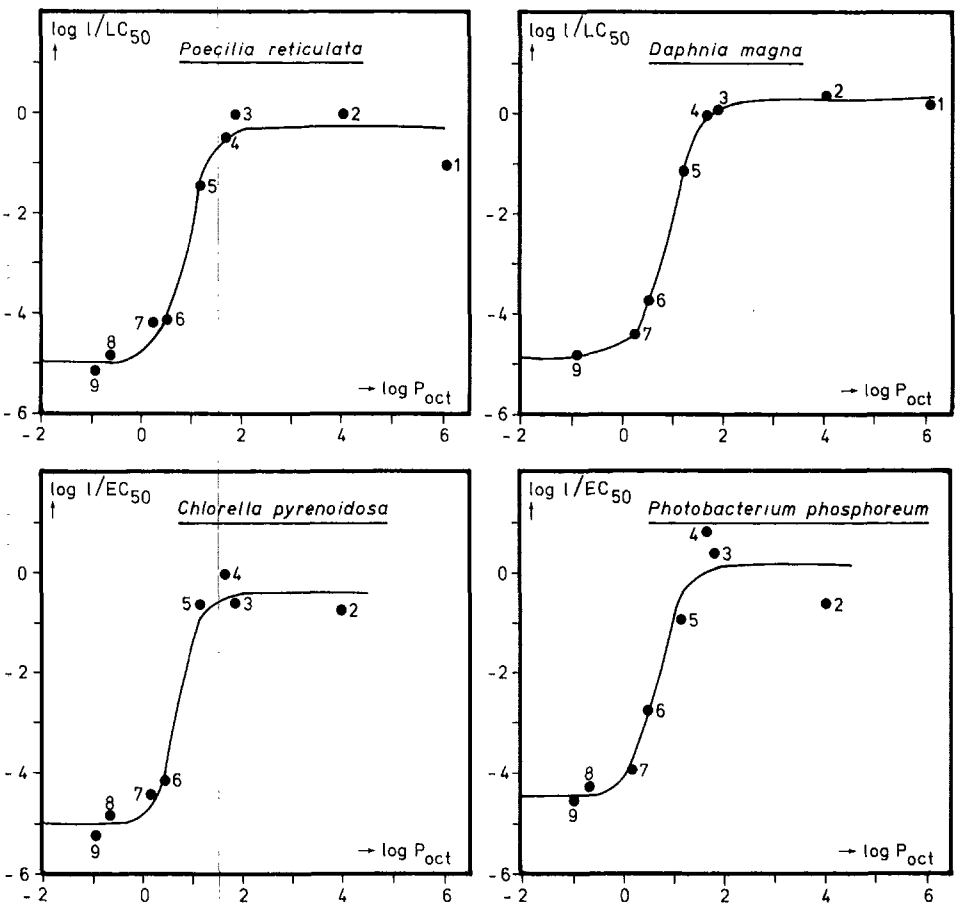

Fig. 5. QSARs for DCs and related compounds. Circles represent the observed and lines the expected values based on equations presented in Table IX. Numbers correspond to those of Table III.

The relationship between toxicity and log P_{oct} is shown in Fig. 5. In all cases the linear equations improved as disulfiram and TPTD were eliminated. This was particularly noticeable in the case of *P. reticulata* and *P. phosphoreum* ($P < 0.05$). The aqueous solubility of TBTD was extremely low. The calculated log P_{oct} was 8.18 and appropriate testing with *D. magna* was impossible because the neonates stuck to the organic surface layer formed, and died as a consequence. In absence of these effects no mortality occurred. In the QSARs for *D. magna*, ETU appeared to be an outlier.

The introduction of a $(\log P_{oct})^2$ term (Table VIII) improved the quality of the QSARs significantly in all cases ($P < 0.05$) in comparison with the linear relationships. Equation 9 was an exception to this rule. The deviations from linearity, however, were in opposite direction. Therefore we calculated QSARs in two log P_{oct} ranges in which only one deviation from linearity was observed. The QSARs 5, 7, 9 and 11, i.e. the equations with a positive $(\log P_{oct})^2$ term, were calculated excluding disulfiram and TPTD. The QSARS 6, 8, 10 and 12, i.e. the equations with a negative $(\log P_{oct})^2$ term, were calculated excluding ETU and EU.

TABLE VIII. QSARs and correlations for the compounds shown in Table III. Parabolic relationships[a].

QSAR equation	$\log 1/C = a(\log P_{oct})^2 + b(\log P_{oct}) + c$						
C(μmol/l)	No.	*a*	*b*	*c*	*n*	*s*	*r*
LC_{50} 96-h *P. reticulata*	5[b]	0.68	1.34	-4.47	7	0.41	0.988
	6[c]	-0,41	3.05	-4.78	7	0.73	0.945
LC_{50} 48-h *D. magna*	7[b,d]	0.75	1.31	-4.37	6	0.53	0.983
	8[c]	-0,38	3.07	-4.67	7	0.78	0.948
EC_{50} 96-h *C. pyrenoidosa*	9[b]	0.55	1.49	-4.34	7	0.85	0.953
	10[c]	-0,86	4.64	-5.58	6	0.67	0.964
EC_{50} 15-min *P. phosphoreum*	11[b]	0.62	1.47	-3.76	7	0.51	0.983
	12[c]	-0,90	4.68	-4.89	6	0.36	0.989

[a] See footnote a in Table VII.
[b] QSAR calculated without disulfiram and TPTD.
[c] QSAR calculated without ETU and EU.
[d] QSAR calculated without ETU.

TABLE IX. QSARs for the compounds shown in Table III. Sigmoidal relationships[a].

QSAR equation	$\log 1/C = ab\{a+(b-a)\exp(-k \log P_{oct})\}^{-1} + c$						
C(μmol/l)	No.	*a*	*b*	*c*	*k*	*r*	*s*
LC_{50} 96-h *P. reticulata*	13	0.18	4.62	-4.94	3.80	9	0.44
LC_{50} 48-h *D. magna*	14[b]	0.23	5.11	-4.83	3.44	8	0.08
EC_{50} 96-h *C. pyrenoidosa*	15	0.11	4.59	-5.00	5.00	8	0.40
EC_{50} 15-min *P. phosphoreum*	16	0.34	4.62	-4.48	3.73	8	0.63

[a] See footnote a in Table VII.
[b] Calculated without ETU.

Sigmoidal relationships between the partition coefficient and toxicity are shown in Table IX and Fig. 5. In case of *D. magna* this QSAR improved significantly ($P < 0.05$) as compared to the parabolic ones.

Discussion

Aspects of testing dithiocarbamates

Toxicological investigation of DCs is hindered by the fact that they are unstable. It is known that BDCs break down during storage. This means that the compounds tested, may be contaminated with degradation products. The stability of DCs in water depends to a large extent on the pH and the metal ion with which they form a complex. The hydrolysis experiments, however, were conducted under sterile conditions, ruling out the possibility of biodegradation.

According to the 'Verdrängungsreihe' established by Eckert (1957), the stability of DEDC complexes increases in the following order: Mn, As, Zn, Sn, Fe, Cd, Pb, Co, Ni, Cu, Ag, Hg. This means that the metal in a dithiocarbamate complex is replaced by that which follows it in the series. The sodium derivatives which are soluble in water are highly dissociated. A complex mixture of polymeric structures of unknown size is formed with bivalent heavy metal ions during synthesis of BDCs. These polymers are hardly soluble in water and most organic solvents. They are not expected to be of great importance toxicologically, because of their molecular size.

DCs are both chemically and biologically degradable. The degradation process may cause the formation of a series of products, amongst others relatively volatile compounds such as CS_2 and H_2S, which are reactive and toxic. Information on hydrolysis in water, microbial degradation, metabolism in plants and animals and the products that are released is given in Fishbein (1977), Kaars Sypesteyn et al. (1977), Kaufman (1977), Klisenko and Vekshtein (1971), Vonk (1975) and Woodcock (1977).

In other words, research was conducted with a group of substances whose chemical structure was unclear in the case of the BDCs. The concentrations of the DCs were liable to alter in the course of the experiments due to complexation and (bio)degradation. This made it extremely difficult to interpret the test results and determine the ultimate toxic agent, and therefore to establish standards.

QSAR studies

Hydrophilic compounds such as ETU and ethylenediamine were relatively toxic to nitrifying bacteria. A clear relation between toxicity and lipophilicity was absent. As these substances can form complexes with metals, and metallo-enzymes play an important role in nitrification (Perrin, 1979; Wood et al., 1981), it may be concluded that either the cell wall is highly permeable or the enzyme system is localized at an easily accessible place.

In fungitoxicity studies activity declined ascending the homologous series of both the

sodium dialkyldithiocarbamates and tetraalkylthiuramdisulfides (Thorn and Ludwig, 1962). Particularly noticeable was the sharp decline in activity between the ethyl and propyl derivatives. These results correspond to the toxicity tests with *P. reticulata, C. pyrenoidosa* and *P. phosphoreum*. Deviations from linearity in case of high log P_{oct} values can be explained both by supraoptimal hydrophobicity (Hansch, 1971), and molecular size. Deviations from linearity in case of low log P_{oct} values may be related to diffusion of small hydrophilic molecules through aqueous membrane pores (Hermens, 1983).

If QSARs from Table VII are compared with those for substances with 'minimum toxicity' (Könemann, 1981; Hermens, 1983; Slooff et al., 1983) both the slope and the intercept are greater in all cases. This means that DCs and related compounds are more toxic than those which are assumed to possess 'minimum toxicity' - in other words they show a more specific mode of action.

Toxicity of DDCs

Uncharged DDC complexes with zinc or iron, are hardly dissociated. They are therefore among the relatively hydrophobic species of DDCs, as are the thiuram-disulfides. This explains why these substances are relatively toxic and why toxicity increases in the order of the sodium-, zinc- and iron-complexes. The intrinsic toxicity of the DDCs is partly attributable to the fact that thiuramdisulfides and the heavy metal dithiocarbamates undergo radical interchange reactions with thiol groups involving, for example, enzymes and coenzymes (Owens, 1969). Dithiocarbamate ions are released upon this reaction. As NaDMDC is converted into thiram in water and NaDEDC can be expected to convert into disulfiram, it is highly probable that sodium derivatives also interfere with cellular processes by interacting with thiol groups. Chelation of free metals or complex formation with metallo-enzymes may disrupt many biochemical processes as well (Owens, 1969).

Toxicity of BDCs

The toxicity of BDCs is ascribed to their degradation products. The theory of isothiocyanate formation and sulfhydryl-interaction has been postulated but there is insufficient evidence to support it (Morehart and Crossan, 1965). These authors come to the conclusion that ethylenethiuramdisulfide is responsible for the toxic action of BDCs. In water, however, polymeric ethylenethiuramdisulfide can be converted into ethylenethiuram monosulfide (ethylene bisdiisothiocyanato sulfide) the structure of which has been revised and later described as DIDT (Engst and Schnaak, 1970; Vonk, 1975; Yoshida et al., 1978). In water, nabam breaks down largely into DIDT and ETU within a day (Vonk, 1975; see also Fig. 2). DIDT is relatively stable in water in a pH range from 5 to 8, but can quickly be converted into ETU by microbes; ETU itself is relatively resistant to microbial degradation (Vonk, 1975).

As other BDCs can be expected to degrade in an analogous manner, at least qualitatively, it might be tentatively concluded that DIDT is 'the ultimate toxic agent'. DIDT is relatively lipophilic. Its intrinsic toxicity is probably caused by its reaction with thiol groups (Yoshida et al., 1978), and the subsequent formation of dithioacidic groups. As a consequence of this reaction, metal complexation can be expected. BDCs are known to interfere with metallo-enzymes (Owens, 1969).

A comparison of BDC and DDC toxicity

Although there are differences in the degree of toxicity and degradation pathways of BDCs and DDCs, it is probable that both (including the thiuramdisulfides) are toxic from the fact that either they or their degradation products undergo radical interchange reactions with SH-groups and interfere with metallo-enzymes. The latter is illustrated by thiram and DIDT which show similar activity (Tables IV and V). This may be expected on the basis of their liposolubility and comparable mode of action. Moreover, the intracellular formation of CS_2, a degradation product of both BDCs and DDCs, can be expected to play an important part in view of its affinity for nucleophilic groups (Vasák and Kopecky, 1967). The oxydative desulphuration of CS_2, which liberates reactive elementary sulphur (Beauchamp et al., 1983) may be an important factor as well. It might therefore be postulated that DCs, and in particular their relatively hydrophobic degradation products and complexes, should only be regarded as optimum chemical structures for bringing CS_2 or elementary sulphur to the site of action.

As a consequence of these mechanistic similarities, BDCs and DDCs cannot be as readily separated as is done on the basis of their chemical differences.

Specificity

DCs are toxic to highly toxic to the organisms tested. Taken over the entire range of substances the sensitivity of the organisms decreases in the following order: *D. magna, P. phosphoreum, C. pyrenoidosa, P. reticulata* and *Nitrosomonas/Nitrobacter.* Based on the mechanisms of toxicity described, inhibition of metabolism must occur at many sites and in many processes. BDCs and DDCs must therefore be regarded as cytotoxic substances. This explains their polytoxic nature. DCs can therefore be regarded as broad-spectrum biocides.

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Chapter 3

Sublethal effects of tetramethylthiuram disulfide (thiram) in rainbow trout (Salmo gairdneri)

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Abstract

In short-term toxicity experiments with rainbow trout (*Salmo gairdneri*), thiram (tetramethylthiuram disulfide) was found to lower the concentrations of protein-bound SH in the liver, and to raise the concentration of non-protein-bound SH. The lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities increased. The glucose content of the blood fell, while an increase in total lipid content of the liver was found. Thiram also interfered with some haematological indices; it induced leucopenia, a fall in haemoglobin content and an increase in the osmolarity of the blood. The observed effects appear to agree well with those reported in other test systems and can be ascribed to the cytotoxic properties of thiram and/or its metabolites.

Chapter 3

Sublethal effects of tetramethylthiuram disulfide (thiram) in rainbow trout (Salmo gairdneri)

Introduction

Relatively much is known about the toxicological mechanisms of dithiocarbamates (DCs), and their resemblance to carbon disulfide intoxication (Thorn and Ludwig, 1962; Owens, 1969; W.H.O., 1979). The chelating effect of DCs and DC-metabolites on various trace metals essential for the prosthetic groups of many enzymes may reduce their activity (Melson and Weigelt, 1967; Owens, 1969; Fig. 1).

Reactions with nucleophilic groups (Owens, 1969; Fig. 1) can change the structure of e.g. proteins in such a way that their activity or function is modified. Their ability to interact with sulfhydryl groups is of particular importance in this respect, as the latter are considered essential for the activity of many enzymes, particularly oxidoreductases (dehydrogenases) and kinases, as well as cofactors such as coenzyme A and lipoic acid (De Bruin, 1976). Because CS_2 reacts with amino groups, giving rise to the formation of dithiocarbamic acids (Vasák and Kopecky; Fig. 1), and DCs metabolize into CS_2 (De Bruin, 1976), there are similarities in their mode of toxic action. Formation of atomic sulfur due to oxydative desulfuration of CS_2 (Beauchamp et al., 1983; Fig. 1) may be an important mechanism of toxicity both for DCs and CS_2 (cf. Savolainen et al., 1977; Neal and Halpert, 1982). Due to the affinity of CS_2 to amino groups, the symptoms of CS_2 intoxication have been connected with vitamin B_6 deficiency (Vasák and Kopecky, 1967) and impairment of nicotinic acid metabolism (W.H.O., 1979).

DCs, and dialkyldithiocarbamates in particular, are toxic to aquatic organisms. In short-term experiments with guppies (*Poecilia reticulata*), lethality was observed below ppm-level (Van Leeuwen et al., 1985). Their bioaccumulative potential, however, is low. This emerged from a study with ^{14}C-labelled DCs (Van Leeuwen et al., submitted). The present work was undertaken to study in detail some sublethal effects of thiram after acute treatment of rainbow trout (*Salmo gairdneri*). The results suggest that thiram interferes with many cellular processes, which may account for its broad-spectrum biocidal activity.

Materials and methods

Chemicals

Thiram (tetramethylthiuram disulfide; chemical purity > 98%) was supplied by Fluka AG (Buchs, Switzerland). Acetone (chemical purity 99%), used as solvent for

thiram, was supplied by Baker Chemicals B.V. (Deventer, The Netherlands). Chemicals for the determination of lipids, glycogen, sulfhydryl, and red and white blood cells were obtained from Merck (Darmstadt, F.R.G.). Other reagents were obtained from Sigma Chemical Company (St. Louis, USA).

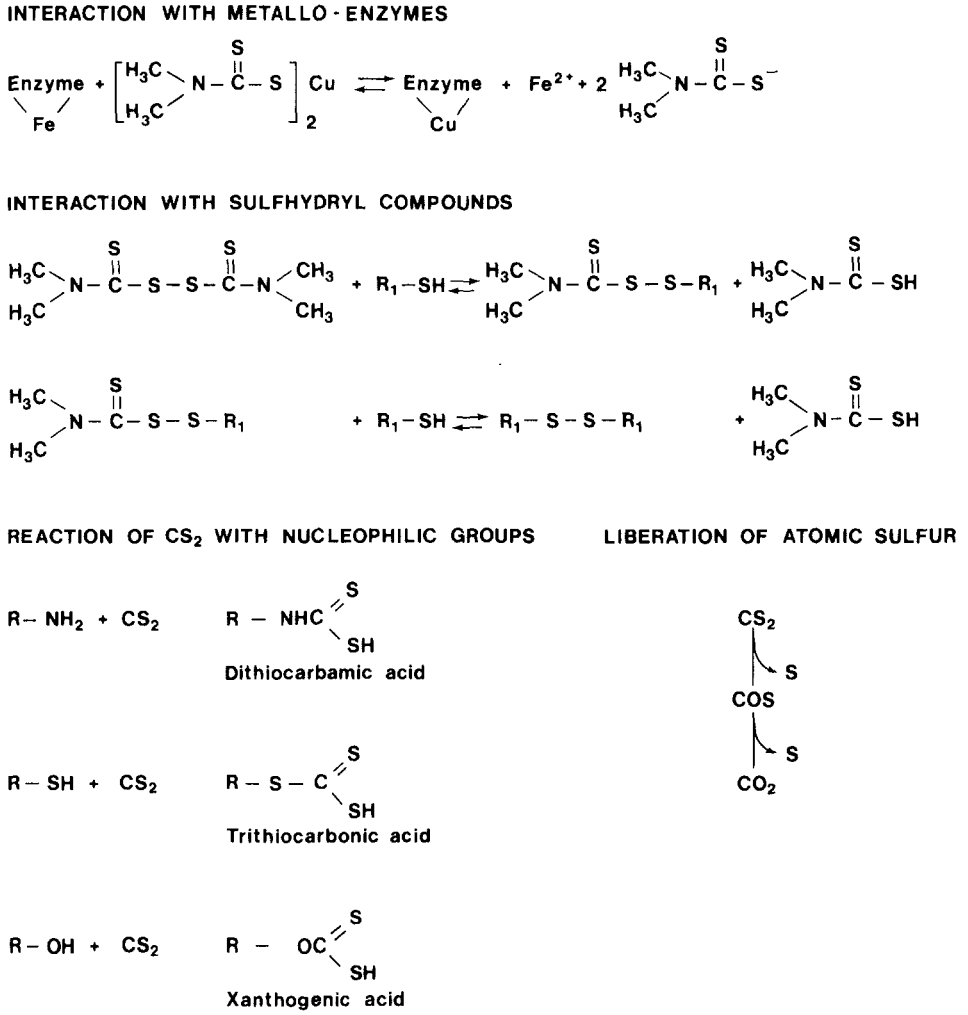

Fig. 1. Reaction mechanisms of dithiocarbamates and carbon disulfide.

Experimental animals

Rainbow trout (*Salmo gairdneri*), were obtained from Fijge Trout Farm at Vaassen (The Netherlands). They were acclimatized at the laboratory in a 400-l tank with standard water at 15±1°C (approximately 100 fish per 400 l) for 5 to 7 days prior to ex-

perimentation. Fish were exposed to a 12-h photoperiod and fed daily with trout pellets (Trouvit, Trouw & Co. N.V., The Netherlands). At the start of the experiments the fish had an average weight of 34.0±4.7 g, and a length of 15.3±0.7 cm. A few experiments were performed with fish weighing 46.7±8.8 g and measuring 16.8±1.1 cm. Standard water was prepared according to Alabaster and Abram (1965), with a pH of 7.8 and a hardness of 50 mg/l (as $CaCO_3$).

LC_{50} experiments

In order to determine an experimental concentration for thiram, a 24-h LC_{50} test was carried out. During this test, groups of 5 fish were placed in 10-l tanks. The ratio of concentrations was 1.3. Fish were fasted for 24 h prior to and during the test. LC_{50} values were calculated according to Litchfield and Wilcoxon (1949).

Sublethal toxicity experiments

A group of 10 fish was exposed for 24 h to thiram (0.18 mg/l) and a solvent control, under conditions described above for the LC_{50} studies. The concentration of acetone, was 18 µl/l. At the end of exposure period fish were anesthetized at high concentrations of buffered tricaine methane sulphonate (MS 222 Sandoz, Basel). Immobilization was generally completed after 30 sec. Various determinations (Table I) were carried out in different tissues sampled immediately after immobilization. Blood was collected from the caudal vein with a syringe rinsed with anticlotting fluid. The liver was homogenized with a Potter-Elvehjem homogenizer in a homogenization fluid (Table I). To measure the adenylate energy charge (AEC), fish were killed by a blow on the head. A piece of caudal muscle tissue was rapidly removed and transferred into liquid nitrogen within 20 sec; pulverized in a mortar and put into perchloric acid.

Statistics

Outliers were detected with Dixon's test (Sokal & Rohlf, 1981) and not included in the calculations of the mean values. Normality was verified by means of the Kolmogorov-Smirnov test (Sokal and Rohlf, 1981). Mean values were compared with one another by means of the Student's *t*-test. For this purpose, similarity of standard deviations was tested with the *F*-test (Nie et al., 1975).

Results and discussion

LC_{50}-experiments

The 24-h LC_{50} of thiram for *S. gairdneri* with an average weight of 34.0 g was 0.26 mg/l. The lower and upper 95% confidence limits were 0.24 and 0.32 mg/l, respectively. A somewhat arbitrary test concentration of 0.18 mg/l was selected on the basis of this value. As the toxic response is inversely related to body size (Anderson and Weber,

TABLE I. Parameters and methods of determination.

Parameter	Tissue/ organ	Anticlott./ homogen. fluid	Method
Lactate dehydrogenase (LDH)	Liver	EDTA[a]	Tech.Bull.Sigma/ No.340-UV(1977)
Alcohol dehydrogenase (ADH)	Liver	EDTA[a]	Bergmeyer (1974)
Glycogen	Liver	TCA[b]	Montgomery (1957)
Glucose	Blood	Heparin[c] + NaF[d]	Tech.Bull.Sigma/ No.15-UV(1983)
Lipids	Liver	EDTA[a]	Zöllner and Kirsch (1962)
AEC	Muscle	Liquid N_2 + PCA[e]	Sklar and McKee (1984)
Total SH content (T-SH)	Blood/ liver	EDTA[a]	Sedlak and Lindsay (1968)
Protein-bound SH content (PB-SH)	Blood/ liver	EDTA[a]	Sedlak and Lindsay (1968)
Non-protein-bound SH content (NP-SH)	Blood/ liver	EDTA[a]	Sedlak and Lindsay (1968)
Glucose-6-phosphate dehydrogenase	Blood	EDTA[a]	Tech.Bull.Sigma/ No.345-UV(1984)
Osmolarity	Blood	EDTA[a]	Knauer osmometer
Haemoglobin	Blood	EDTA[a]	Tech.Bull.Sigma/ No.525(1982)
Haematocrit	Blood	EDTA[a]	Blaxhall and Daisley (1973)
Erythrocytes	Blood	EDTA[a]	Blaxhall and Daisley (1973)
Leucocytes	Blood	EDTA[a]	Blaxhall and Daisley (1973)

a) Ethylenediaminetetraacetic acid (EDTA) concentration 0.02 M.
b) Trichloroacetic acid concentration 5% (w/v).
c) Heparin concentration 35 U/ml blood.
d) Sodium fluoride (NaF) concentration 10 mg/ml blood.
e) Perchloric acid (PCA) concentration 4% (v/v).

1975; Tsai and Chang, 1981), the experiment was repeated for fish of an average weight of 46.7 g. The 24-h LC_{50} (0.30; 0.18-0.50 mg/l) did not increase significantly, so there was no need to adjust the test concentration.

Sublethal toxicity experiments

Effects of thiram on several sublethal parameters are summarized in Table II. Thiram significantly changed both the blood glucose level and the G-6-PDH activity. A change in protein-bound and non-protein bound SH-levels was also observed (cf. Fig. 1). These results may point to a stimulated pentose phosphate cycle (HMP-shunt), probably to meet a demand for NADPH, i.e. reducing power, due to the interference thiram and/or its metabolites with sulfhydryl groups. Sinet et al. (1982), in their study on the effects of diethyldithiocarbamate in human erythrocytes, also found increased HMP-shunt activity. Impairment of the citric acid cycle at the level of lipoic acid and/or coenzyme A,

TABLE II. Effects of thiram on some biochemical and haematological parameters in rainbow trout (*Salmo gairdneri*) after exposure to 0.18 mg/l for 24 h. Results given are averages ± S.E. of the number of observations in parentheses.

Parameter	Tissue/ organ	Unit	Thiram concentration (mg/l) 0	0.18
LDH	Liver	U/g	0.23± 0.07(10)	0.40±0.05(9)[b]
ADH	Liver	U/100g	1.62± 0.36(10)	1.76±0.41(9)
Glycogen	Liver	mg/g	3.49± 0.97(9)	3.15±1.38(10)
Glucose	Blood	mg/100ml	76.20±17.26(10)	48.90±8.66(10)[b]
Lipids	Liver	%	1.14± 0.09(10)	1.27±0.13(9)[a]
AEC	Muscle	-	0.71± 0.04(4)	0.65±0.11(4)
T-SH	Blood	mmolSH/100ml	2.19± 0.34(9)	1.99±0.29(10)
T-SH	Liver	mmolSH/100g	1.71± 0.15(10)	1.74±0.09(9)
PB- H	Blood	mmolSH/100ml	2.03± 0.33(9)	1.83±0.28(10)
PB-SH	Liver	mmolSH/100g	1.39± 0.09(9)	1.28±0.08(9)[a]
NP-SH	Blood	mmolSH/100ml	0.17± 0.02(10)	0.16±0.01(10)
NP-SH	Liver	mmolSH/100g	0.36± 0.04(10)	0.46±0.04(10)[b]
G-6-PDH	Blood	U/ml	1.22± 0.30(9)	1.59±0.27(9)[a]
Osmolarity	Blood	mosmol	289± 7.5 (10)	306±4.2 (10)[b]
Haemoglobin	Blood	g/100ml	6.83± 1.27(10)	6.08±0.85(10)
Haematocrit	Blood	%	34.9 ± 3.1(6)	38.3 ±3.1(9)
Erythrocytes	Blood	$x10^6/\mu l$	1.14± 0.30(10)	1.15±0.20(10)
Leucocytes	Blood	$x10^3/\mu l$	12.6 ± 9.3(10)	6.7 ±2.1(10)

a) Significant at $\alpha < 0.05$.
b) Significant at $\alpha < 0.001$.

may also account for the hypoglycemic action of thiram in trout, as well as for the enhanced LDH-activity (Tabel II). Similar effects in mammals are known to ensue from toxic dosage of DCs (De Bruin, 1977). The thiram-mediated increase of the lipid content of the liver (Table II) may point at fatty degeneration of hepatocytes. Fatty dystrophy has been reported both for thiram-treated rats (Fishbein, 1977) and workers hospitalized because of chronic CS_2 poisoning (WHO, 1979).

The thiram-mediated fall of haemoglobin in blood (11%; $P=0.138$) may be due either to accelerated haeme degradation (Järvisalo et al., 1978) or inhibition of δ-aminolaevulinic acid synthetase, a pyridoxal-phosphate dependent and rate-limiting enzyme involved in haeme sythesis. Inhibition of this enzyme by ethylenebis-diisothyocyanatosulfide, a degradation product of ethylenebisdithiocarbamic acid, has been reported (Yoshida and Neal, 1978). In this respect it might be relevant to note that anaemia is a prominent phenomenon in hypovitaminosis B_6 (Goodman Gilman et al., 1980). The decrease in the mean number of leucocytes (47%; Table II) was almost significant ($P = 0.064$). Leucopenia has also been demonstrated in experiments with zineb and maneb in rats (Fishbein, 1977). The osmolarity of the blood

of thiram-treated fish was slightly but significantly higher than in the corresponding controls. This is possibly due to a disturbed osmoregulatory function. Korablev and Evets (1977) have reported on the anti-diuretic effect of some DCs (including thiram) in rats within 4 h after administration, the diuresis being 1.6-4.5 lower and the excretion of Na and K being 1.2-11 times lower than the controls. It may therefore be concluded that thiram is effective at many levels and should be regarded as a cytotoxic chemical.

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Chapter 4

The use of cohorts and populations in chronic toxicity studies with Daphnia magna. I. A cadmium example

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Abstract

Two semistatic life-table experiments with *Daphnia magna* were carried out on reconstituted and Lake IJssel water. The 'nontoxic concentrations' for cadmium with respect to the intrinsic rate of natural increase, derived from age-specific survival and fecundity were 1 and 3.2 μg/l, respectively. Body length appeared to be a sensitive parameter. A third intermittent-flow experiment was started with small, exponentially growing populations. These populations had a stable age distribution, were composed of cohorts of different ages and showed an almost perfect logistic growth. Cadmium was shown to reduce the upper numerical limit (carrying capacity) for *D. magna* and was inversely related to this parameter: Log $Y = 2.85 - 0.20 \log [Cd]$; $r = -0.99$. A 'nontoxic concentration' could not be established. Based on the 'background' concentration of cadmium, a freshwater quality criterion of 0.1 μg/l is proposed. The results are used to discuss several shortcomings of the current methods. Finally it is stated that the introduction of the concepts of population dynamics in reproduction tests with *D. magna* is a realistic step towards ecotoxicology.

Chapter 4

The use of cohorts and populations in chronic toxicity studies with Daphnia magna. I. A cadmium example

Introduction

For the assessment of water quality criteria for chemicals and for the hazard evaluation of waste discharges to the aquatic environment, traditionally acute fish toxicity data have been used in which the measured biological response was mortality occurring within an exposure period of 96 h (Sprague, 1976). It is well recognized that this type of information is insufficient to identify concentrations that protect against adverse effects in aquatic ecosystems (Sprague, 1971; Stephan and Mount, 1973). Therefore chronic toxicity tests are carried out to assess pollutant effects on growth, survival and reproduction over long periods of exposure, with several species which are representative of different trophic levels (Calamari et al., 1979). Quality criteria based upon several single species tests certainly are an improvement but they are no more than an approximation of the 'nontoxic concentration' at the ecosystem level, as complicated biological processes and interactions occur in aquatic ecosystems (Buikema et al., 1982; Cairns, 1981). Still, they are preferred from a practical as well as an economic point of view because of the common inability to replicate baseline characters in laboratory microcosm systems (Brungs and Mount, 1978).

Daphnia species are among the most sensitive species in aquatic toxicology (Buikema, 1980). In extensive studies on the effects of cadmium on aquatic life *Daphnia magna* has been found to be the most sensitive test species (Environmental Protection Agency (EPA), 1980). Because of its short life cycle, the small water volumes required, ease in handling, and the good correlation obtained between 21-day chronic *D. magna* data and chronic fish toxicity data, Maki (1979) concluded that these tests are an attractive alternative to long-term fish toxicity studies. The toxicity criteria used in chronic tests with daphnids generally are survival and reproduction. As far as reproduction is concerned, normally attention is paid only to the total number of young after 21 days (NEN 6502, 1980; APHA, 1980).

From an ecological point of view, however, it is more realistic to study the adverse effects on the level of populations. In toxicity tests with cohorts (isolated generations) separate measures of age-specific survival and fecundity rates may be linked together and used to estimate the intrinsic rate of natural increase under given environmental conditions according to the formula of Lotka (1913):

$$\sum_{x=0}^{\infty} l_x m_x e^{-r_m x} = 1. \qquad (1)$$

In this equation l_x is the probability of surviving to age x, m_x represents the number of female offspring per female of age x, born during the time interval x to $x+1$ and r_m is the growth constant of an exponentially increasing population, i.e., the intrinsic rate of natural increase. In populations having a stable age distribution, r_m may also be calculated from the equation describing population growth in time in an unlimited environment (exponential growth):

$$N_t = N_0 e^{r_m t} \tag{2}$$

Here N_t and N_0 represent the size of the population at time t and at time zero. The stable age distribution is calculated from Eq. 3:

$$c_x = [l_x e^{-r_m x}][\sum l_x e^{-r_m x}]^{-1}. \tag{3}$$

In this equation c_x is the proportion of the total population in the xth age class. When the environment becomes limiting, population growth can be described by the logistic equation:

$$N_t = K[1+e^{-r_m(t-t_0)}]^{-1}. \tag{4}$$

K is the carrying capacity or upper numerical limit for population growth (see Pielou (1977) for a thorough treatment).

The life-table approach has only been used in a limited number of pollution studies with daphnids (Marshall, 1962; Winner, 1976; Winner and Farrel, 1976; Winner et al., 1977; Van Urk, 1979); Bertram and Hart, 1979; Daniels and Allen, 1981) and other crustaceans (Daniels and Allen, 1981; Allen and Daniels, 1982; Gentile et al., 1982), whereas toxicity studies with populations have been used by Marshall (1978a,b).

The objective of our research was to determine whether short-term life-table and population growth experiments with *D. magna* could be used to derive 'nontoxic levels' for an environmental pollutant. The results are used to discuss the current methods used for this purpose.

Materials and methods

Chemical Aspects

The stock solutions were prepared from analytical grade cadmium solutions (BDH) and made up in demineralized water. Actual test concentrations, before and after 0.45-μm filtration were only verified chemically in an intermittent-flow experiment by atomic absorption spectrophotometry. Oxygen concentration and pH were measured at regular intervals.

Organisms

The organism used was the freshwater crustacean *D. magna* cultured in the laboratory according to the Dutch standard NPR 6503 (1980). The culture medium used was Lake IJssel water. Daphnids were fed with cells of the unicellular alga *Chlorella pyrenoidosa* (strain: Wisconsin 2005; Culture Centre of Algae and Protozoa, Cambridge).

Test media

A synthethic test medium was prepared as described in NPR 6503 (1980). This medium had a hardness of 200 mg/l (as $CaCO_3$) and a pH of 8.4 ± 0.1. In subsequent tests 50-μm filtered Lake IJssel water was used, after treatment with a WEDECO M/2-6 UV sterilization apparatus. The chemical composition of Lake IJssel water (February 1983) is shown in Table I.

TABLE I. Chemical composition of Lake IJssel water (February 1983).

Compound	Concentration (mg/l)
Na	59.2
K	6.9
Ca	72.0
Mg	10.7
Mn	0.015
Fe	0.162
Si	4.1
NH_4-N	0.13
NO_3-N	4.46
NO_2-N	0.08
o-PO_4-P	0.28
SO_4^{2-}	78.0
HCO_3^-	159
Cl-	133
Hardness (as $CaCO_3$)	224
Chlorophyll *a*	0.002
TOC	4.5
pH	8.1

Test procedures

The experiments were carried out in constant temperature rooms having a temperature of 20 ± 0.5°C and a 12-h photoperiod. Three types of toxicity experiments with daphnids were carried out:

Experiment A was begun with newborn (< 24 h) daphnids. For each concentration, 20 neonates were used, one animal per jar containing 40 ml of NPR 6503 medium, enriched with cells of *C. pyrenoidosa* according to NEN 6502 (1980). The medium was renewed daily. The test was terminated after 21 days.

Experiment B was also started with newborn daphnids. Ten daphnids were placed in each jar, containing 500 ml Lake IJssel water. The experiment was replicated five times. The medium contained 3 x 10^8 *C. pyrenoidosa* cells/l and was renewed daily. At the end of the experiments (Day 21) the length of the daphnids was determined from the top of the head to the base of the spine using an ocular micrometer.

Experiment C was performed in an intermittent-flow system equipped with electric valves. The water was aerated before algal cells and toxicant solutions were added. The algal suspension and toxicant solutions were delivered separately by a peristaltic pump and mechanical injectors, respectively. The water flow through the 20-l test vessels was 667 ml/h. The *C. pyrenoidosa* concentration was 3 x 10^8 cells/l. An initial dose of $CdCl_2$ was added to the test vessels half an hour before the experiment started. The test vessels consisted of four compartments, each containing one population. The test was initiated with exponentially growing populations of 20 daphnids composed of cohorts of different ages. The stable age distribution was calculated from Eq. 3. The l_x and r_m values were derived from preceeding static cohort experiments (r_m = 0.3 and l_{21} = 1.0) thus giving the following stable age distribution: 5, 4, 3, 2, 2, 1, 1, 1 and 1 daphnid(s) aged 0-1, 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, 7-8 and 8-9 day(s), respectively. The total number of daphnids in each test compartment (biomass) was counted at regular intervals.

Calculations and Statistics

The intrinsic rate of increase from the cohort experiments was calculated by successive approximation from Eq. 1. The LC_{50} values, confidence limits, and χ^2 fit were determined by the method of Litchfield and Wilcoxon (1949). When necessary, corrections were made for mortality in the controls. The r_m values and mean size of daphnids from treatments and controls were tested using Williams' procedure (1971, 1972). Population growth was analyzed according to Kooyman et al. (1983).

Results and discussion

Experiment A

In this test, as well as in the other experiments, no problems occurred with regard to pH and oxygen concentration. The results of this test are shown in Figs. 1 and 2 and Table II.

The LC_{50} values gradually declined with time until a constant level was reached after about 2 weeks (Fig. 2). Daphnids started to reproduce after 8 days. The number of off-

spring at different levels of exposure are depicted in Fig. 3. Cadmium is shown to reduce the number of offspring and to delay reproduction.

This reproductive impairment should be ascribed to the 'teratogenic' effects of cadmium as many aborted eggs and embryos were found on the bottom of the test vessels after each ecdysis. This prenatal mortality was also observed by Marshall (1978a). These effects increased with time. Reproduction was completely inhibited at concentrations exceeding 3.2 μg/l. At 3.2 μg/l Cd, only one daphnid reproduced (at Day 11). As time-dependent survival and reproduction (r_m) were significantly reduced at 1.8 μg/l Cd ($\alpha < 0.01$), 1 μg/l Cd is considered to be the 'no-effect level' for this parameter.

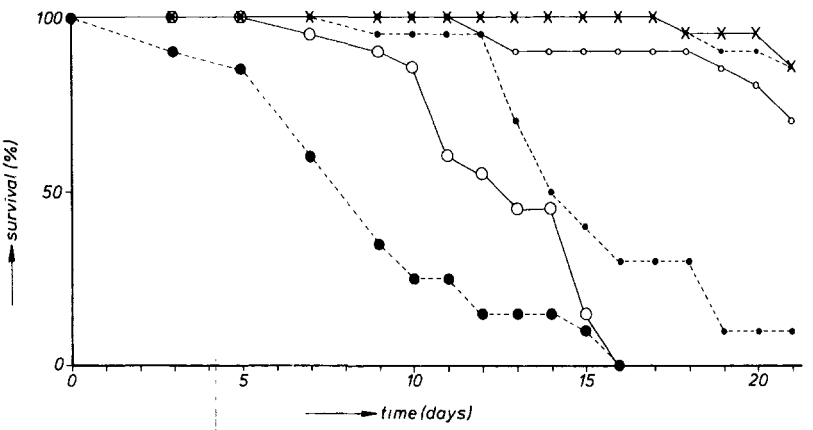

Fig. 1. Survival of *D. magna* exposed to different concentrations of cadmium; x, control; •, 1μgl; ○, 1.8 μgl; ●, 3.2 μgl; ○,5.6 μg/l; and ●, 10 μg/l.

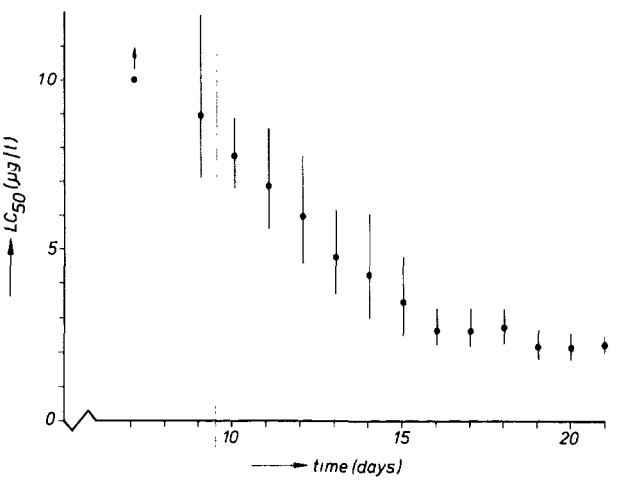

Fig. 2. The relation between LC_{50} and time for *D. magna* and cadmium.

TABLE II. Results of a semistatic life-table experiment with *D. magna* in cadmium-contaminated reconstituted water (Experiment A).

Nominal Cd Concentration (μg/l)	Survival after 21 days (%)	$\Sigma m_x \pm SE$	$r_m \pm SE$
0	85	63 ± 25	0.308 ± 0.086
1.0	85	40 ± 19[a]	0.289 ± 0.069
1.8	70	15 ± 13	0.203 ± 0.103[a]
3.2	10	0	0
5.6	0	0	0
10	0	0	0

[a]Lowest rejected concentration tested ($\alpha = 0.01$).

The population statistic r_m was chosen as a parameter because it integrates age-specific survival and reproduction ((first) time of reproduction, reproduction frequency, brood size and reproduction longevity). Generally, in chronic toxicity tests only the number of offspring per surviving female in a certain time interval (m_x) is calculated, summarized over the entire test period (Σm_x) and treated as a reproduction parameter. As only surviving daphnids reproduce and mortality within three weeks under normal test conditions is the end point of toxicant stress, Σm_x might be derived from relatively toxicant-resistant individuals. Therefore Σm_x as a single reproduction parameter has to be considered as incomplete. Moreover this parameter is statistically weak because it might be derived from only several surviving and reproducing daphnids, i.e., theoretically no discrimination is made between 50 surviving and reproducing daphnids and 1 surviving and reproducing daphnid. This could possibly offer an explanation for the difference in the LC_{50} and EC_{50} reproductive values published by Canton and Slooff (1982) for *D. magna* exposed to cadmium, these being 0.67 and 4.2 μg/l, respectively. Generally another important aspect is not included either: time.

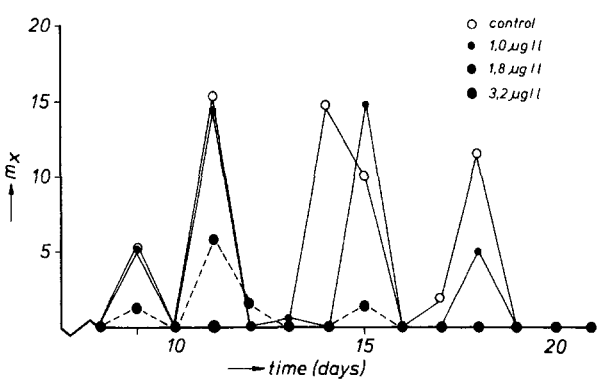

Fig. 3. Temporal changes in reproduction of *D. magna* exposed to different concentrations of cadmium.

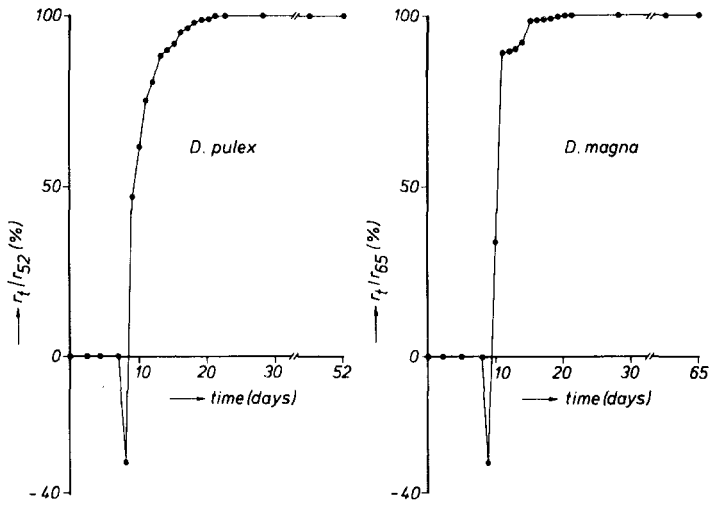

Fig. 4. Temporal changes in the intrinsic rate of natural increase (r_m) for *D. pulex* (blank control data of Daniels and Allen (1981) and *D. magna*. Data are expressed as percentage of r_m calculated for the entire life table.

The overriding importance of early reproduction in terms of population growth and competition was clearly demonstrated by Birch (1948), Lewontin (1965), and Daniels and Allen (1981). Daniels and Allen (1981) found an almost perfect correlation ($r=0.99$) between r_m values calculated for a 3-week exposure and values based on entire-life experiments with *D. pulex*. Their control data were used to calculate r_m for 0-1, and 0-2 days, and for successive intervals. These results are shown in Fig. 4. A study of the progress of r_m in time for *D. magna* was carried out in our laboratory. This experiment was replicated ten times using 10 daphnids per jar containing 500 ml Lake IJssel water. The daphnids were fed *C. pyrenoidosa* cells at a concentration of 5 x 10^8 cells/l. The test solutions were renewed daily. The mean r_m values and their standard errors for 0-14 and 0-21 days, and r_m, calculated for the entire life-table were 0.3054 ± 0.0082, 0.3311 ± 0.0047 and 0.3322 ± 0.0046, respectively. For *D. magna* this progress of r_m with time is also depicted in Fig. 4, showing a similar trend to that was found for *D. pulex*. The discontinuity of the line for *D. magna* is caused by the large and synchronous reproduction of daphnids at Days 11 and 15, m_x rated 22.4 and 29.3, respectively. In both experiments, r_m calculated for the first 21 days exceeded 99% of the r_m value calculated for the entire life-table, indicating the importance of early reproduction for population growth. For this reason it can be concluded that a 3-week life-table study will be appropriate to calculate the intrinsic rate of natural increase. The significance of the age at which reproduction occurs, is however often underestimated whereas the importance of total progeny is generally overemphasized. Therefore delay in reproduction, caused by pollutants, should be included in reproduction tests with daphnids. For these reasons, age-specific survival and fecundity rates derived from life-table experiments and subsequent calculation of r_m values, are considered to be a sound basis for the description of the exponential (not logistic) growth under toxicant stress. The growth itself is found by substituting the r_m values in Eq. 2.

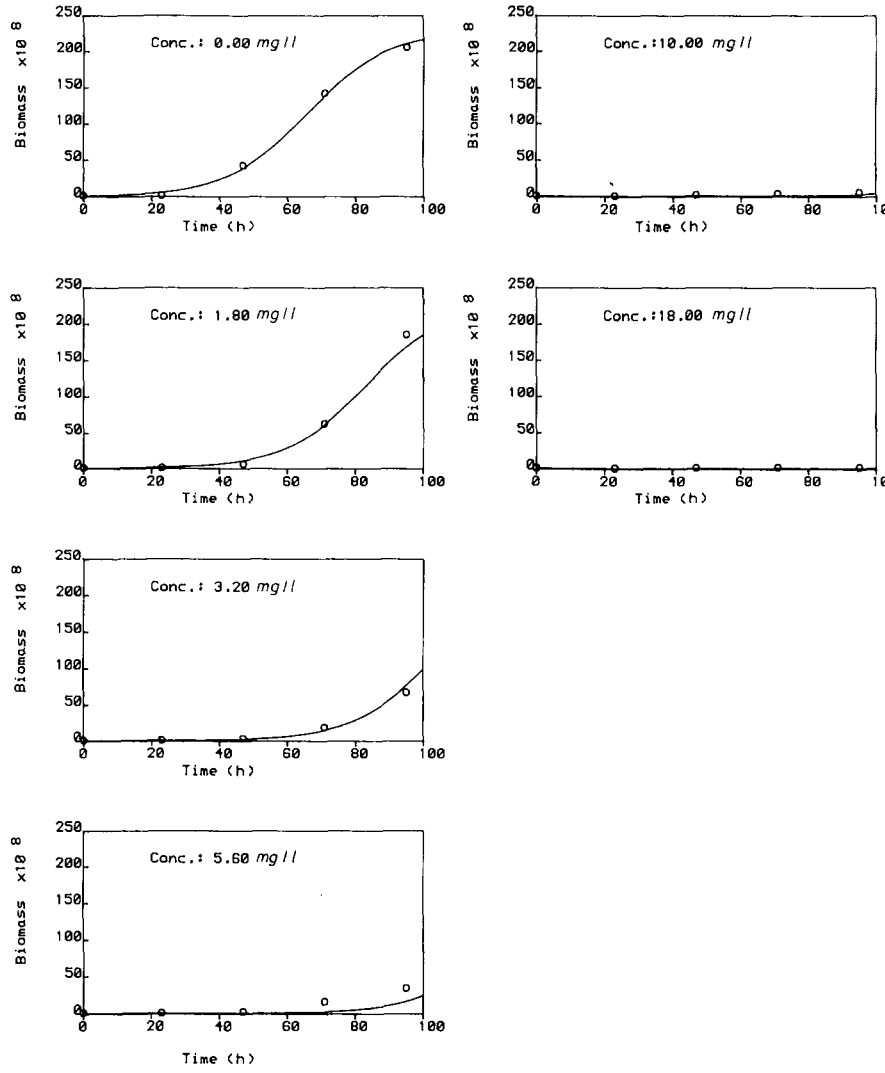

Fig. 5. The effects of cadmium on the growth of *Chlorella pyrenoidosa* populations. Circles represent the observed and lines the expected values based on model calculations.

This test procedure, however, may result in an underestimation of long-term toxicity. As most experiments start with unexposed animals, compounds showing a slow increase of toxicity with time will exhibit their effects only after one or more broods. As early reproduction contributes to r_m to a great extent (Fig. 4), these chronic effects on reproduction and longevity will generally not contribute to r_m at all, which is illustrated from our results. From Fig. 3 it can be derived that 1.0 µg/l Cd clearly delays and reduces reproduction after two broods. This accounts for the fact that its Σm_x is significantly different from the blank while r_m is not (Table II).

Geiger et al. (1980) also showed that 21-day reproduction data were moderately

predictive of long-term effects. It is likely that the cadmium concentration at which responses occur would drop if the tests were to be prolonged. At first sight these long-term (> 21 days) data on mortality and reproductive impairment seem to be unimportant from the point of view of population dynamics, but since these experiments were started with neonates from previously unexposed parents, neglecting exposure during an important part of the life cycle (oogenesis and early embryogenesis) and presumably toxicant transfer from parents to neonates, these tests do not really reflect the chronic effects of cadmium. The results of a test with F_1-generations from a third or fourth brood of preexposed animals would probably give a more realistic figure for chronic toxicity. The second objection is the fact that in static tests the food concentration is generally unrealistically high (Buikema et al., 1980). This aspect will be discussed below.

As a control for the effects of cadmium on *C. pyrenoidosa* an algal toxicity test was performed according to NEN 6506 (1980). The results are shown in Fig. 5. Cadmium affected the inoculum, which is shown by the extension of the lag time. The EC_{50} and EC_{10} for this criterion were 1.3 and 0.66 mg/l, respectively, thus excluding any possibility of cadmium affecting daphnids by reducing algal food levels.

Unfortunately this was the only chronic experiment within a period of 15 months in which blank survival and reproduction of daphnids reared and tested on a synthetic medium were acceptable. Blok[1] noticed that daphnids kept on synthetic NPR 6503 medium showed infectious diseases in the brood pouch. The discussion he brought about led to the conclusion that many aquatic toxicologists, performing chronic tests with daphnids in synthetic media, had these same problems. Most of the time these difficulties disappeared when, e.g., tap water, surface water or ground water were used, suggesting that one or more essential biological and/or chemical factors are present or lacking in synthetic media. After making considerable efforts to improve this medium, we finally came to the same conclusion as Winner (1976): 'Given the quality of distilled water available to most laboratories, reconstituted waters are likely to create more problems in acute and chronic toxicity studies than they solve'. Therefore in subsequent tests Lake IJssel water was used.

TABLE III. Results of a semistatic life-table experiment with *D. magna* in cadmium-contaminated Lake IJssel water (Experiment B).

Nominal Cd concentration (μg/l)	Survival after 21 days (%)	$\Sigma m_x \pm$ SE	$r_m \pm$ SE	Length ± SE (mm)
0	96	51.0 ± 3.7	0.291 ± 0.005	4.29 ± 0.13
0.32	98	49.4 ± 1.4	0.295 ± 0.006	4.19 ± 0.09[a]
1.0	100	50.7 ± 3.1	0.301 ± 0.011	4.19 ± 0.08
3.2	98	50.7 ± 3.5	0.294 ± 0.008	4.15 ± 0.09
10	90	46.8 ± 3.8[a]	0.249 ± 0.015[a]	3.98 ± 0.23
32	0	2.2 ± 2.3	-0.050 ± 0.057	—

[a]Lowest rejected concentration tested (α = 0.01).

[1] Blok, J., AKZO Research Laboratories, Arnhem, The Netherlands. Personal communication.

Experiment B

The results of the second experiment are summarized in Table III. The 14- and 21-day LC_{50} of cadmium for *D. magna* was 24 and 14 μg/l, respectively. The 'nontoxic concentration' for r_m was 3.2 μg/l. In this test the number of lifeless neonates and lost eggs increased again with elevated cadmium levels. No delay in reproduction occurred, although reproduction was completely inhibited after 14 days at 32 μg/l.

The difference in toxicity between test A (reconstituted water) and test B (Lake IJssel water) may be explained by differences in the speciation of cadmium. Van De Meent et al. (1981) have shown that only a small fraction of cadmium in Lake IJssel is adsorbed by large (> 60 μm) suspended particles. In experiment B, 50-μm filtered Lake IJssel water was used. It can be assumed that the concentration of cadmium-binding sites in this water is in excess of its 'background' concentration. Therefore, up to a certain level, the bioavailability of experimentally added cadmium is likely to be reduced through sorption onto suspended particles, which may explain why in experiment B, as opposed to experiment A, biological responses occur at higher (total) cadmium levels. In fact, from these differences one may even conclude that the introduction of standardized reconstituted test media is accompanied by a reduction in chemical and physical realism and hampers the extrapolation of laboratory experiments to the field situation.

The reduction in length caused by cadmium is remarkable. Although length appeared to be the most sensitive parameter in this test, as it also was in tests with hydrocarbons (Geiger et al., 1980), the ecological significance of these small differences is unclear.

Experiment C

Experiment C was carried out to study the effects of cadmium on populations. To a certain extent this experimental design offers the possibility of studying effects on suc-

TABLE IV. Results of an intermittent-flow experiment with *D. magna* populations in cadmium-contaminated Lake IJssel water (Experiment C).

Cd concentration (μg/l)				Yield			
					Measured		
Nominal	Measured[a]	Measured[b]	r_m[c]	Calculated ($t=\infty$)	$t=16$ days	$t=19$ days	$t=21$ days
0	0.089±0.011	0.076±0.012	0.39	1235	1291	1112	876
0.32	0.363±0.038	0.300±0.014	0.37	788	727	594	555
1.0	1.05 ±0.17	0.88 ±0.11	0.35	680	614	417	340
3.2	3.25 ±0.32	2.48 ±0.46	0.35	565	576	531	522
10.0	9.74 ±1.52	7.94 ±1.56	0.33	465	631	620	589
32.0	32.3 ±3.7	34.5 ±7.1	0.25	355	162	152	144

[a]Total ± SE.
[b]'Dissolved' ± SE.
[c]Calculated by means of linear regression for the exponential growth phase (t=0-9 days).

cessive generations. For practical reasons it was not possible to increase the low renewal rate. The results are shown in Figs. 6 and 7 and Table IV. The mean coefficient of variation of the four replicates was low, only 17% with lowest and highest values of 4 and 44%, respectively. Three major conclusions may be drawn from this experiment:

Small populations of *D. magna* composed of cohorts of different ages and having a stable age distribution show an almost perfect logistic growth (Fig. 6).

Cadmium significantly reduces the carrying capacity ('yield'), i.e., the upper numerical limit for *D. magna* (Figs. 6 and 7).

A 'nontoxic' concentration for cadmium toxicity cannot be established (Fig. 7).

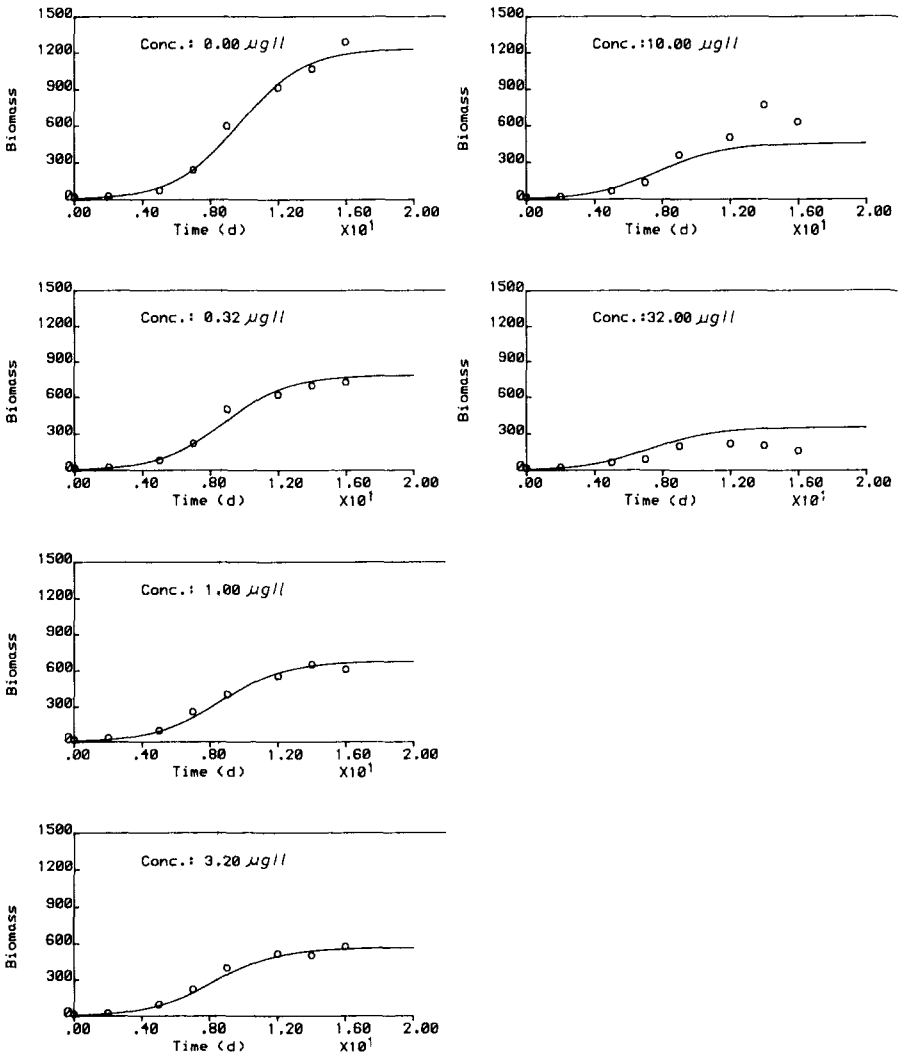

Fig. 6. The effects of cadmium on the growth of *Daphnia magna* populations. Circles respresent the observed and lines the expected values based on model calculations.

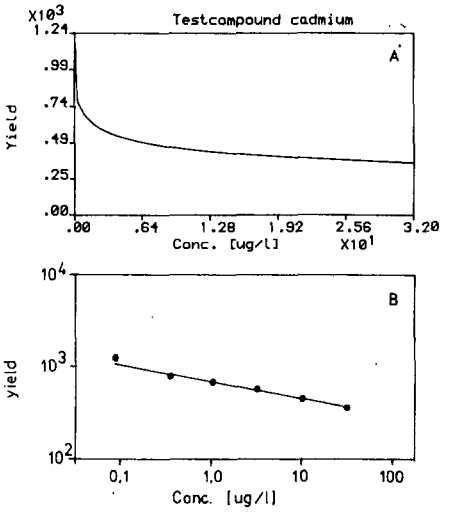

Fig. 7. The effects of cadmium on the carrying capacity (yield) for *Daphnia magna* populations. (A) Model values versus nominal concentrations (linear scale). (B) Model values versus measured concentrations (log scale). [Cd] as total cadmium. Log Y = 2.85 - 0.20 log [Cd]; r= -0.99.

These results are in good agreement with the work of Marshall (1978a). Marshall (1978a) showed cadmium to reduce the carrying capacity for *Daphnia galeata mendotae* populations. He showed that a threshold for this parameter could not be established either and cited Woodwell (1975) to indicate that thresholds do not exist for many types of chronic stress. Marshall (1978a) showed *D. galeata mendota* populations to have no constant upper numerical limit. Their numbers fluctuated after reaching a certain level. To some extent this is also shown for *D. magna* (Fig. 6 and Table IV), but our experiment was terminated after 21 days. At Days 19 and 21 the 'yield' dropped. These points were excluded from the calculations. The numerical oscillations, as found by Marshall (1978a), and the decrease in carrying capacity for *D. magna* in our test may be ascribed to the absence of an instantaneous reaction to changes in population density, i.e., time lags are likely to occur, which cause *Daphnia* populations to 'overshoot' and then 'undershoot' the equilibrium density in a repetitive way. A change in the mean size of daphnids may also account for the fact that no constant upper numerical limit is achieved. At the end of the exponential phase a shift occurs to a population with a zero growth rate, which will lead to a change in the stable age structure, i.e., size structure of the population. As a result, the proportion of large-size daphnids and therefore resource competition will increase (Eq. 3) thus explaining the decrease in carrying capacity for *D. magna* in a numerical sense. In fact, population growth, measured as numerical changes in time may only be appropriate, as no great changes in population size structure occur.

The difference in the results of this test and experiment B can possibly be explained by the fact that successive generations were exposed and/or crowding resulted in a competition for food. Resource depletion, which most certainly will lead to an increase in the filtering rate of daphnids, i.e., a decrease in foraging efficiency (Kersting, 1978), will

possibly have a negative effect on survival and reproductive output. It was shown that the chlorophyll *a* concentration in the controls decreased in the course of this type of experiment. After about 10 days, many young daphnids died, possibly from starvation, both in the exposed and unexposed test vessels. At 32 μg/l reproduction was almost completely inhibited. Since in static experiments, including our own, daphnids are fed *ad libitum*, no competition for food is likely to occur. This is unlike the natural situation because even in eutrophic environments competition for food is very likely to occur (Vijverberg, 1976; Buikema et al., 1980). Toxicant adsorption by > 0.45-μm particles ('food') was not found to play an important role in our experiment (Table IV).

As *Cladocera* constitute an important link in the food web (Vijverberg and Richter, 1982) and differences in sensitivity of species belonging to this suborder are not expected to be large, this reduction in carrying capacity, is a serious ecotoxicological problem. A cadmium-induced inhibition of reproduction will eventually lead to senescence of a population (Eq. 3). As age is positively correlated with the size of daphnids and positive size-selective fish predation probably will take place (Vijverberg and Richter, 1982; Marshall, 1978a), cadmium will interact synergistically with fish predation. A reduction in carrying capacity combined with an increased chance of being predated may seriously alter the functional structure of freshwater ecosystems. As no 'nontoxic concentration' for cadmium can be established, it may be stated that, for practical as well as legislative reasons its concentration should never exceed the measured 'background' of 0.1 μg/l. Hence, this figure is also proposed as a freshwater quality criterion for this heavy metal. Although many biological factors were excluded from this experiment, it is believed that the introduction of the concepts of population dynamics in *Daphnia* reproduction tests is a realistic step towards ecotoxicology.

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Chapter 4

The use of cohorts and populations in chronic toxicity studies with Daphnia magna. II. A bromide example

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Abstract

Two chronic toxicity experiments were conducted with *Daphnia magna*. In a semi-static experiment with cohorts, the no effect level for bromide in respect of the intrinsic rate of natural increase (derived from age-specific survival and fecundity) was 10 mg/l. A second test was started with small populations in an intermittent-flow system. These populations had a stable age distribution, were composed of cohorts of different ages, and showed an almost perfect logistic growth. Model calculations showed that bromide reduced the upper numerical limit (carrying capacity). It also increased the time-lag required to attain the maximum reproduction rate. For the first parameter, a no-effect-level of 14 mg/l was calculated. For the latter a threshold could not be detected. The EC_{50} and EC_{10} were 27 and 18 mg/l, respectively. Additional experiments showed that individual growth of *D. magna* in time could also be described by a logistic equation. The age structure of the populations changed when food became limiting. This was parallelled by a reduction of the mean brood size. In conclusion it is stated that the results of the toxicity studies with populations support Halbach's view (1984), that population dynamics can be used like a 'magnifying glass' to detect small sublethal ecotoxicological effects of environmental pollutants.

Chapter 4

The use of cohorts and populations in chronic toxicity studies with Daphnia magna. II. A bromide example

Introduction

Most of the literature dealing with acute and chronic effects of chemicals on aquatic ecosystems is concerned with effects on a relatively few species of fish and invertebrates. The vast majority of tests on invertebrates have dealt with only one species, *Daphnia magna*. One of the reasons *Daphnia* species have been used extensively in toxicity testing programs is their demonstrated sensitivity to a broad spectrum of toxicants. Their short life-cycle and the good correlation of 21-day chronic tests with chronic fish toxicity data, made these tests an attractive alternative to long-term fish toxicity studies (Maki, 1979). As the principal objective of aquatic toxicology is to determine concentrations of pollutants which, when released into the environment, will not impact natural populations and community integrity (Gentile et al., 1982), parameters should be studied which cover these aspects. Single-species tests have a place in evaluating these effects, provided that relevant parameters are studied.

Analyses of the effects on longevity and reproductive rate can be based on the results of demographic life history data. Integrated by life-table calculations, mortality and natality data of cohorts provide information on the intrinsic rate of natural increase (r_m), an ecologically more relevant parameter than the total number of young produced in a fixed time-interval (Van Leeuwen et al., 1985). This parameter can be calculated from the formula of Lotka (1913):

$$\sum_{x=0}^{\infty} l_x m_x e^{-r_m x} = 1. \qquad (1)$$

where l_x is the proportion of individuals surviving to age x, m_x is the age-specific fecundity (number of females produced per surviving female at age x), and x is time expressed in days.

Experiments with populations may provide a good alternative to life-table studies. Population toxicity studies are started with small exponentially growing populations. Slight reductions in vitality, such as a decrease in lifespan or offspring production which can hardly be detected at the level of the individual are then summed over many individuals in the next level of integration, population dynamics, thus facilitating detection (Halbach, 1983, 1984). The underlying assumption for projecting future growth with either the exponential or logistic equation, however, is that the population has a stable-age structure. At low population densities, growth will proceed exponentially and the stable-age structure can easily be calculated from:

$$c_x = [l_x e^{-r_m x}][\sum l_x e^{-r_m x}]^{-1}. \quad (2)$$

where c_x is the proportion of the total population in the xth age class. Derivations of these equations can be found in Roughgarden (1979) and Pielou (1977).

In routine toxicity testing, population growth is only studied in experiments with algae. The objective of this study is to extend this approach to the experiments with daphnids, and to describe several aspects of population dynamics. Bromide was chosen as the test compound because it is reported to inhibit reproduction specifically (Canton et al., 1983), and can easily be detected spectrophotometrically.

Materials and methods

Experimental procedures

Experiments with *D. magna* were carried out in a constant temperature room at 20 ± 0.5° C with a 12 h photoperiod. The test medium used was 50 μm filtered, UV-sterilized Lake IJssel water with a pH of 8.1 and a hardness of approximately 225 mg/l (as $CaCO_3$). Sodium bromide (chemical purity 99.6%) was obtained from Baker Chemicals B.V. (Deventer, The Netherlands).

The life-table experiments were started with newborn (< 24 h) daphnids from a laboratory stock culture. The tests were conducted in 800 ml test vessels to which 500 ml of test solution was added. Daphnids were fed daily with $3x10^8$ cells/l of the unicellular green alga *Chlorella pyrenoidosa*. The pooled neonates were randomly distributed into cohorts of ten animals each in 8 toxicant concentrations and a control. To enable statistical treatment of the data, the experiments were replicated five times. The number of surviving females and the number of neonates produced were recorded daily, with new neonates discarded from the test vessels after counting. After three weeks, the experiments were terminated and the carapace length of the daphnids was determined from the anterior margin of the head to the base of the caudal spine using an ocular micrometer.

Population dynamics of *D. magna* under different levels of bromide stress were studied in an intermittent-flow system, equiped with electric valves. Water was aerated before algal cells and toxicant solutions were added. The water flow through the 20-l test vessels was 667 ml/h. The *C. pyrenoidosa* concentration was $3x10^8$ cells/l. The test vessels consisted of an outer and inner container. The 16-l inner container was divided into four compartments, each holding one population. The test was initiated with exponentially growing populations of 20 daphnids composed of cohorts of different ages. The stable-age structure was calculated from equation 2, with $r_m = 0.3$ and $l_x = 1.0$. The total number of daphnids in each test compartment (biomass) was counted at regular intervals. Concentrations of bromide were determined spectrophotometrically at 590 nm with phenol red as indicator (APHA, 1980). The results of both experiments were based on actual concentrations.

Additional intermittent-flow experiments were carried out to study several aspects of the population dynamics in further detail, i.e. the development of the age structure, reproductive ratio (the proportion of adult females with broods), and mean brood size (the number of eggs per reproductive female) in time. These measurements were performed at regular intervals on the total number of daphnids in one compartment. The reproductive ratio was determined by classing as adults all females larger than the smallest reproductive female actually observed in the populations (Hebert, 1978). The determinations of the age structure were based on length measurements which were transformed to age, using the logistic equation for growth. The development of length in time was determined in experiments with cohorts under semistatic conditions. These experiments were all performed at *Chlorella* densities of $3x10^8$ cells/l.

Calculations and statistics

Results of the semistatic tests were summarized in life-table form. The intrinsic rate of natural increase was calculated for each replicate by successive approximation from equation 1. As r_m calculated after 21 days is indistinguishible from r_m estimated for the entire life-span, due to the great importance of early reproduction (Van Leeuwen et al., 1985), the calculations were based on 21-day experiments. Finally, means and standard errors were calculated.

Differences in mean survival, r_m and carapace length, between treatments and control were tested using the procedure described by Williams (1971, 1972). In case of the survival data, the arc sin square root transformation was applied. Differences were considered significant at $\alpha < 0.01$.

The effects of bromide on the population dynamics of *D. magna* were calculated by means of a parametric model developed by Kooyman et al. (1983). In this model, it is assumed that population growth is a logistic function of time and that survival probability, reproduction rate or yield (carrying capacity) decreases according to a logistic function of the concentration of the test substance. In formula:

$$N(t,c) = EY\{E + (Y-E)\exp(-R_c t)\}^{-1} \quad (3)$$

where $N(t,c)$ is the mean number of daphnids per compartment at time t and bromide concentration c, E is the inoculum, i.e. mean number of daphnids per compartment at time $t=0$, Y is the yield, i.e. the mean number of daphnids per compartment obtained in the culture and R_c is the growth rate at bromide concentration c. The three types of effects can occur singly or in combination. The logistic equation was also applied to the growth of *D. magna* in time. The experimental data were fitted to:

$$L(t) = L_m L_o\{L_o + (L_m - L_o)\exp(-\alpha t)\}^{-1} \quad (4)$$

where $L(t)$, L_m and L_o is length at time t, ∞ and 0, respectively and α is the growth rate.

The parameters were quantified by means of non-linear regression. Calculations and curve plottings were performed with a Sperry Univac 1100/84 computer.

Total biomass estimations were based on length measurements and weight was subsequently calculated using the regression equation given by Dumont (1975, Fig. 1):

$$W = (1.5 \times 10^8) L^{2.84} \quad (5)$$

where L is length in μm and W is dry weight in μg. For the weight of eggs and embryos 8 μg was substituted (Dumont, 1975, Table III).

Results

Life-table studies

Impairment of reproduction was clearly related to exposure concentration and significantly inhibited at 32 mg/l (Table I). Bromide did not delay the onset of

TABLE I. Demographic information for *Daphnia magna* from life-table experiments at various concentrations of bromide.

Conc. (mg/l)	$r_m \pm$ SE (day^{-1})	Survivorship (%)	Mean length ± SE (mm)
0	0.298 ± 0.006	100	4.09 ± 0.08
3.2	0.288 ± 0.008	98	4.09 ± 0.08
10	0.283 ± 0.011	100	4.03 ± 0.11
32	0.037 ± 0.042[a]	92	4.05 ± 0.10
100	-	100	4.02 ± 0.07[a]
320	-	100	4.01 ± 0.07
1,000	-	100	3.98 ± 0.08
3,200	-	100	3.93 ± 0.13
10,000	-	58	3.58 ± 0.16

[a] lowest concentration significantly different ($\alpha < 0.01$) from the control.

reproduction nor the brood frequency, but disturbed embryonic development as many aborted eggs were found on the bottom of the test vessels. At 100 mg/l, reproduction was completely inhibited and only aborted eggs were found. Survivorship was unaffected at this concentration, and not reduced until 10 g/l (Fig. 1). It may therefore be concluded that bromide specifically inhibits the reproduction process. This inhibition, however, was reversible. Daphnids from this experiment which had previously been exposed to bromide concentrations up to 32 mg/l released broods within three days upon transferring to uncontaminated water. At higher levels of pre-exposure, this brood was skipped and a first brood appeared within 7 days. Growth was significantly inhibited at 100 mg/l (Table I).

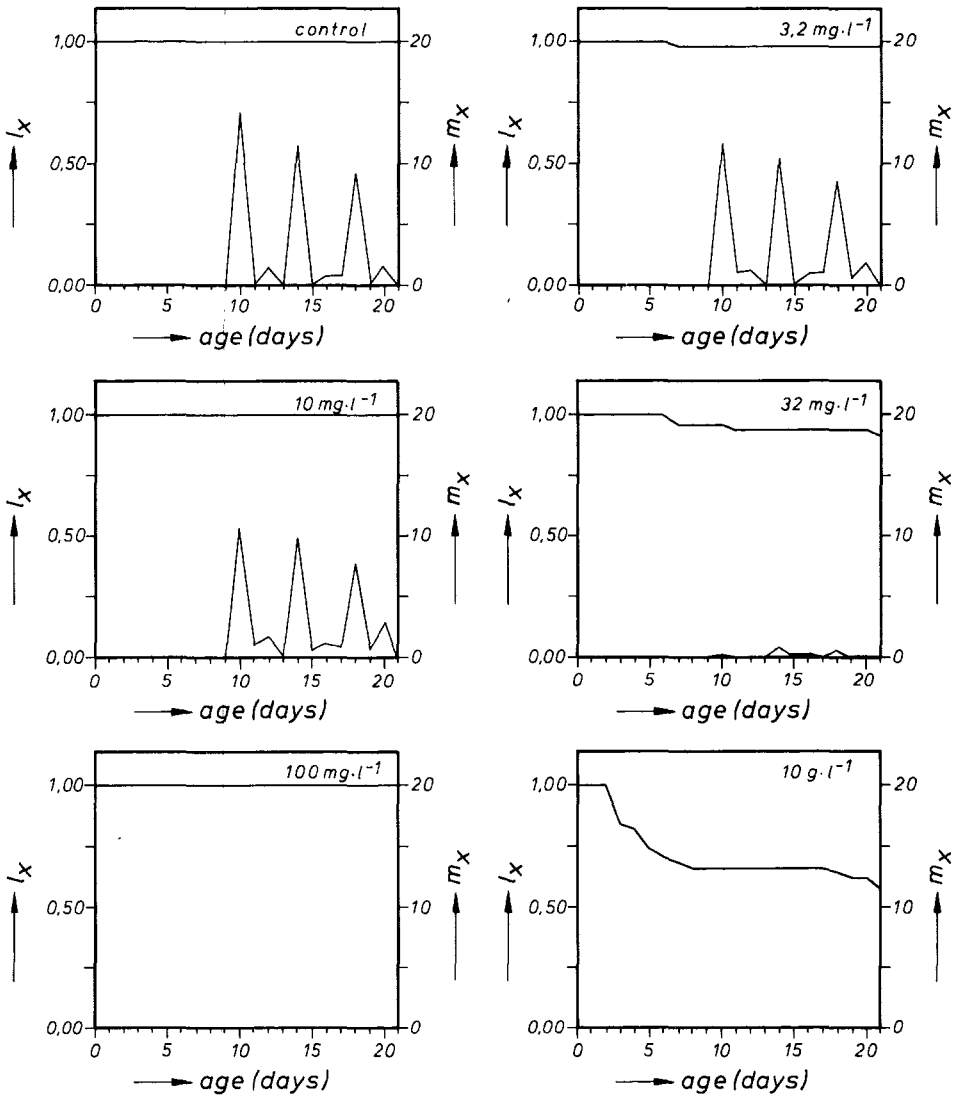

Fig. 1. Life-table of *Daphnia magna* at various concentrations of bromide: survivorship curve (thick line) and fertility curve (thin line).

Population toxicity studies

The population toxicity experiment showed that small populations of *D. magna* composed of cohorts of different ages and having a stable age structure grew in an almost ideal logistic fashion (Fig. 2). This was true provided that experimental data obtained after 15 days were excluded from the calculations. After the population had attained its maximum at day 13, the mean yield dropped. At concentrations of 0, 4.5, 8.6, 16.3, 27.9,

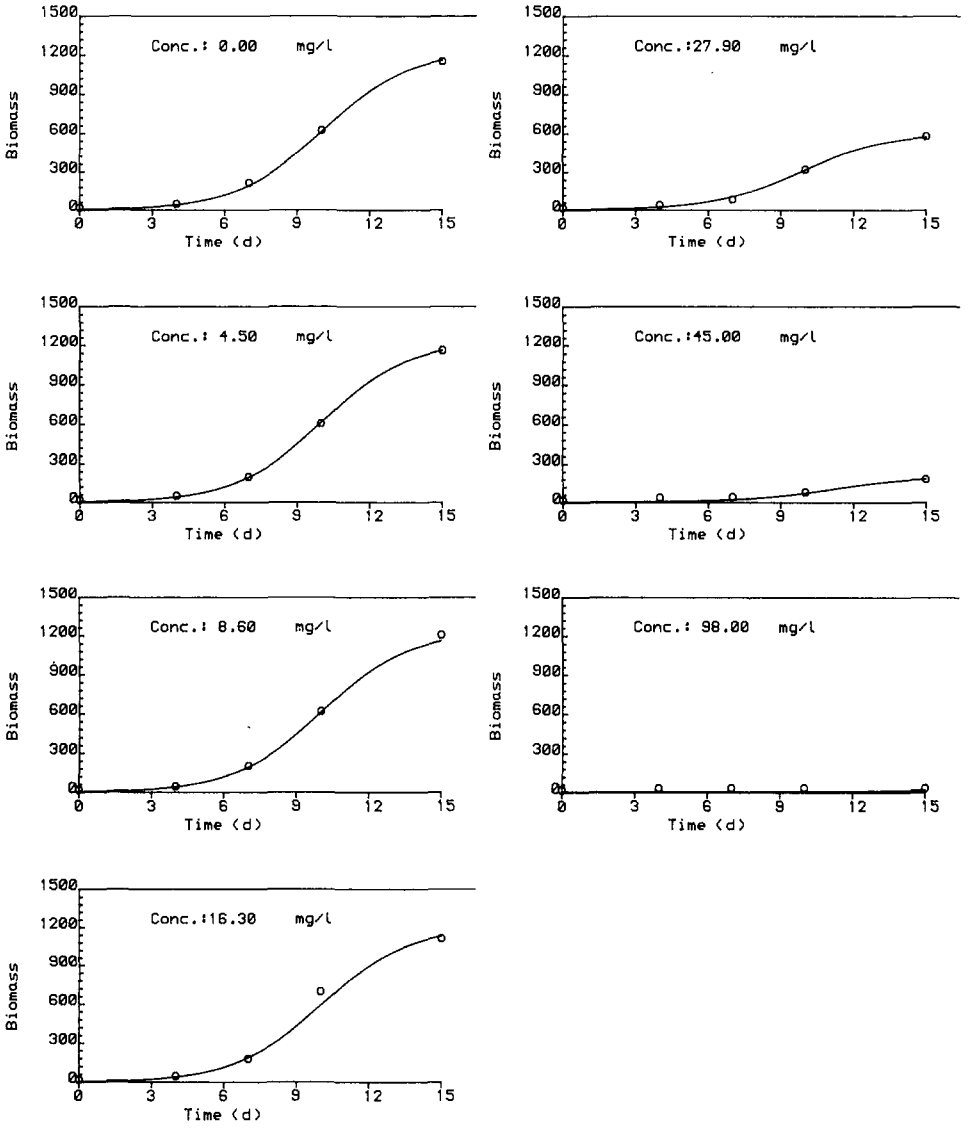

Fig. 2. The effects of bromide on the growth of *Daphnia magna* populations. Circles represent the observed and lines the expected values based on model calculations.

45.0 and 98.0 mg/l the yield at t=18 and, in parentheses, at t=21 days was 862 (673), 1117 (821), 1236 (986), 748 (582), 463 (430), 185 (188) and 37 (36), respectively.

The ultimate factor limiting population growth and size clearly was the amount of food available for consumption. During the experiments, the chlorophyll *a* concentration decreased. This was also shown by a color phase shift from '*Chlorella*-green' to 'Chablis-yellow'. The depletion of food was parallelled by an increasing number of neonates which adheared to the water surface and died, likely from starvation. Resource

depletion, neonatal mortality, and drops in the yield were less dramatic at high levels of bromide exposure.

Bromide clearly delayed population growth. This was shown by the extension of the lag phase, i.e. the time required to attain the maximum reproduction rate. For this effect on the inoculum, a threshold could not be detected. The EC_{50} and EC_{10} were 27 and 18 mg/l, respectively. Bromide also reduced the yield, i.e. the upper numerical limit for *D. magna*. The no effect level for this parameter was 14.3 mg/l. The results of the parametric analysis are given in Table II.

TABLE II. Results of a parametric analysis of population growth experiments with *Daphnia magna* under various levels of bromide stress (mg/l).

Model Parameter	Symbol	Estimate	Variance
Inoculum in blank	E_b	5.3	3.2
Inoculum gradient	E_g	5.3	3.0
ln EC_{50} for inoculum	E_e	3.3	0.008
Blank reproduction rate	R_b	0.55	0.0014
Yield in blank	Y_b	1240	1010
Yield gradient	Y_g	1.9	0.3
ln EC_{50} for yield	Y_e	2.6	0.047
No effect level	γ	14.3	6.8
Model variance	σ^2		534

Additional experiments

The relation between growth, expressed as linear growth, and time is given in Fig. 3.

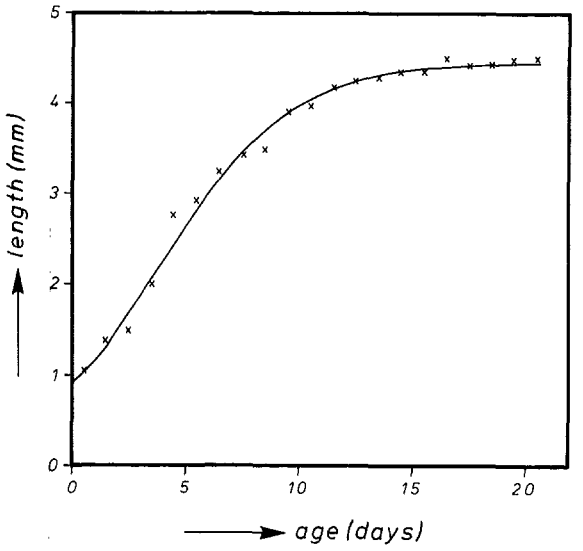

Fig. 3. Length development of *Daphnia magna* as a function of time t. The fitted curves have the form $L(t)=L_m L_o\{L_o + (L_m-L_o) \exp(-\alpha t)\}^{-1}$, where L_m = 4.47 mm, L_o = 0.905 mm and α = 0.348 d^{-1}.

In *D. magna*, the growth rate seems to be exponential in the pre-adult instars. There is no appreciable growth beyond this stage in the reproductive phase, i.e. the active growth levels off, indicating that the animal reaches maximum growth at this stage.

The experimental data fit the logistic equation quite well. The standard error of estimate was 0.11. This logistic equation was used to reconstruct changes in the age structure of *D. magna* populations in time. At increasing population densities (in a numerical sense), a shift occurred towards a population with a large proportion of adults (Figs. 4 and 5). The greatest changes in age structure occurred after the population had attained its numerical maximum. As food levels declined, the proportion of poorly reproducing adults increased. This response to food limitation was shown primarily by a decrease in the mean brood size, as opposed to the reproductive ratio, which remained approximately constant, although it dropped after 23 days (Table III and Fig. 5). The carapace length of the smallest reproductive female detected, necessary for the calculation of the reproductive ratio was 2.49 mm.

TABLE III. Demographic information for *Daphnia magna* from an intermittent-flow experiment with populations.

Time (days)	Mean number per compartment	Biomass (mg)	Reproductive ratio	Mean brood size
0	20	1.98	0.59	9.9
8	286	16.03	0.65	18.8
13	533	31.24	0.67	7.5
20	424	32.36	0.57	3.5
23	365	41.74	0.78	2.5
27	246	24.05	0.37	3.2

In the exponential phase of population growth, total biomass rose in proportion to the total number of daphnids. Subsequently, this relation became disturbed. The obvious conclusion therefore is that population counts are good estimates for total biomass, provided that no great changes in age structure occur.

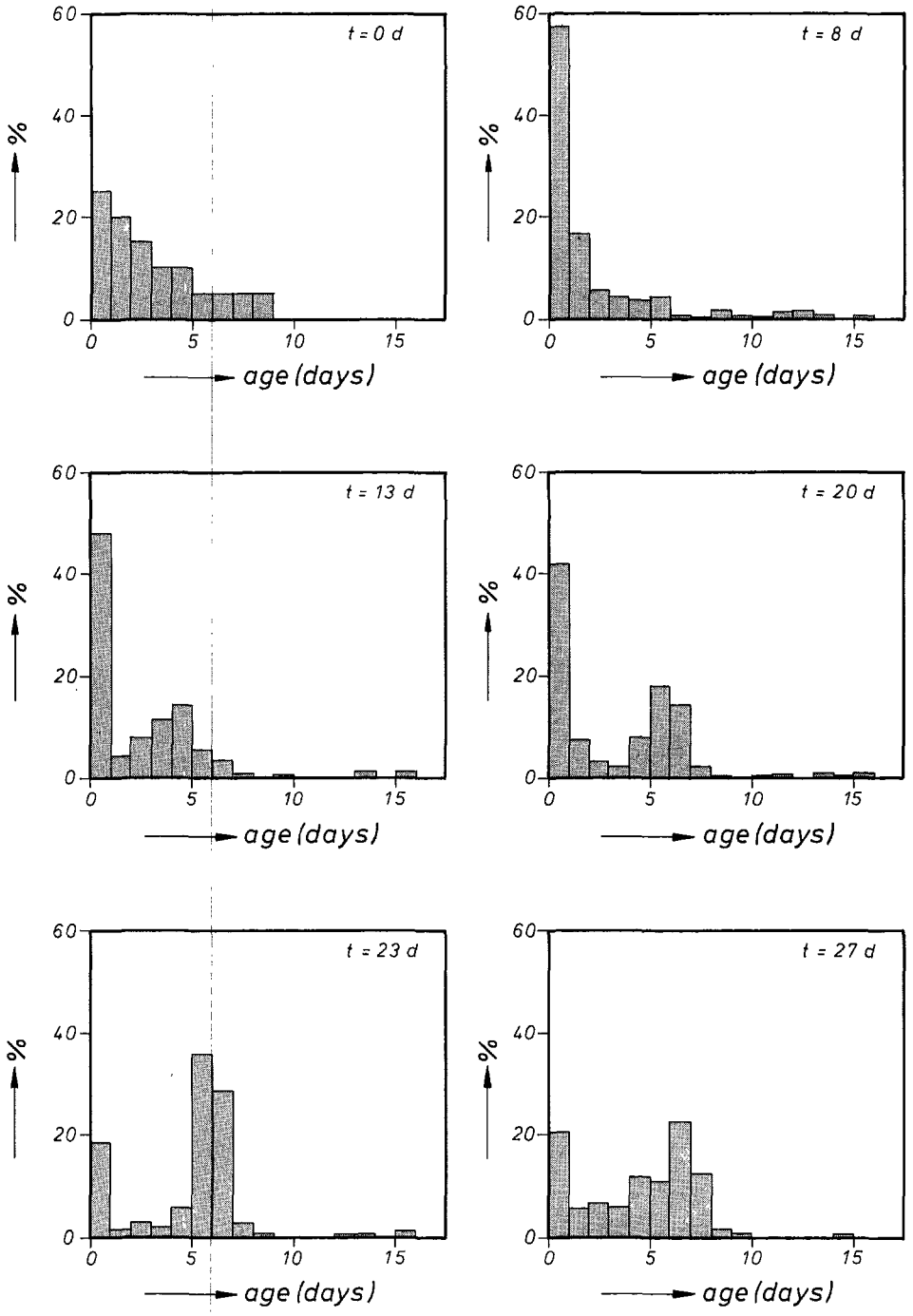

Fig. 4. Changes in the age structure of a *Daphnia magna* population in time.

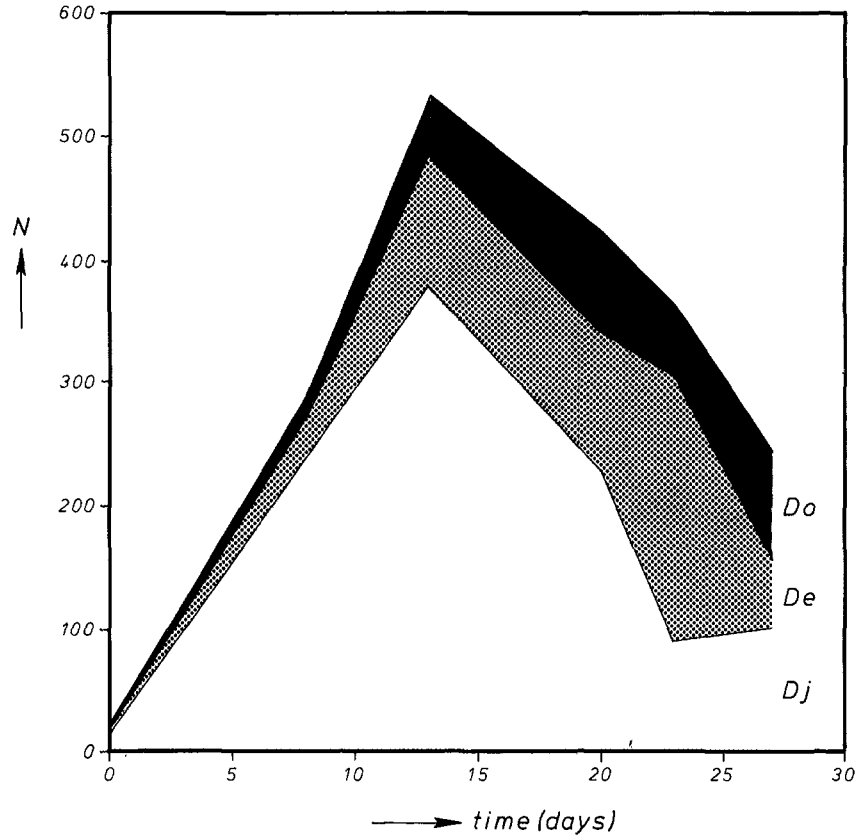

Fig. 5. Growth of a *Daphnia magna* population in time. De= egg-carrying females, Do= adults without eggs or embryos, Dj= adolescents and juveniles.

Discussion

Logistic equations provide an excellent fit of the available data for both population and individual growth (Figs. 2 and 3). Population growth, however, was far from logistic when the tests were continued after the population had attained its numerical maximum (Fig. 5). This may be ascribed to the absence of an instantaneous reaction to changes in population density, i.e. time-lags are likely to occur, which cause daphnid populations to 'overshoot' and then 'undershoot' the equilibrium density in a repetitive way (cf. Marshall, 1978). Changes in the age structure may also account for the absence of constant upper numerical limits (Fig. 4). It should be noted that the age structure determinations are estimations, as actual *Chlorella* densities in the population growth experiments diminished, whereas the equation for individual growth (Fig. 3) was derived from experiments with cohorts under an approximately constant food density.

It was striking that total biomass still increased after the daphnid population had reached its numerical maximum. This may be explained by the buildup of energy

reserves in the form of lipid droplets (Tessier & Goulden, 1982) in the preceeding food-unlimited phase, i.e. the production of daphnids with a high calorific value. It may also be explained by a decrease in nutritional requirements at older ages. When growth and reproduction cease, energy is spent on maintenance only. Maintenance, or respiration, i.e. oxygen consumption on a unit weight basis, is smaller in older animals (Richman, 1958).

Bromide inhibited reproduction. In the population toxicity study, this is shown by an increased time-lag prior to attainment of the maximum reproduction rate (Fig. 2). The EC_{10} for this parameter was 18 mg/l, whereas the lowest concentration which significantly reduced r_m in the life-table study was 32 mg/l. The results are consistent with those reported by Canton et al. (1983).

The major difference between life-table and population toxicity tests is, that in the latter type of experiment the population will meet the inevitable situation of food limitation. In contrast, life-table studies are conducted at a constant food density. The amount of food generally applied is unrealistically high (Buikema et al., 1980), and clearly above the threshold food density (cf. Kersting & Van Der Leeuw-Leegwater, 1976). Thus, effects of chemicals on the threshold food density for daphnids cannot be detected in life-table studies. As pointed out by Kooyman and Metz (1983), the effects of chemicals on populations are strongly dependent on food availability. A shortage of food is likely to greatly increase chemical stress. This possibly offers an explanation for the bromide-induced reduction of the yield in the experiment with populations. By which this effect was caused, i.e., a slight reduction of the filtration rate, digestion rate or changes in basal metabolism, remains unsolved.

Yet, this result supports Halbach's view (1984), that population dynamics can be used like a 'magnifying glass' to detect small sublethal effects of environmental pollutants.

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Chapter 5

Aquatic toxicological aspects of dithiocarbamates and related compounds. Effects on survival, reproduction and growth of Daphnia magna

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Abstract

Life-table studies on *Daphnia magna* were carried out with various dialkyldithiocarbamates (DDCs), ethylenebisdithiocarbamates (BDCs) and major transformation products. DDCs were generally more toxic than BDCs. Survival, reproduction and growth were clearly reduced at levels of approximately 10 and 80 μg/l, respectively. Quantitative structure-activity relationships were calculated for a limited number of the compounds involved. The calculations revealed that the *n*-octanol/water partition coefficient (P_{oct}) explained nearly all variation in toxicity. Over the broad range of lipophilicities (10^7), toxicity could best be described by a sigmoid function of P_{oct}. The application of DDCs as heavy metal scavengers in waste water treatment is questionable against the background of the concentrations needed, since these compounds are generally more toxic than the metals.

Chapter 5

Aquatic toxicological aspects of dithiocarbamates and related compounds. Effects on survival, reproduction and growth of Daphnia magna

Introduction

Previous studies in this laboratory have demonstrated that dithiocarbamates (DCs) were toxic to highly toxic to bacteria, green algae, crustaceans and fish, after short-term exposure. It was concluded that DCs were cytotoxic and should be regarded as broad-spectrum biocides (Van Leeuwen et al., 1985a). The study reported on in the present paper was undertaken to examine the effects of prolonged exposure to ethylenebisdithiocarbamates (BDCs) and dialkyldithiocarbamates (DDCs) on survival, fecundity and growth of *Daphnia magna*. As DCs are unstable in the aquatic environment, a number of major transformation products were tested as well. In order to describe relationships between biological activity and hydrophobicity, quantitative structure-activity relationships (QSARs) were calculated for a limited number of the compounds involved.

Materials and methods

Test compounds

The chemicals used are listed in Table I. For structural formulas see Van Leeuwen et al. (1985a).

Toxicity tests

The experiments were carried out with *D. magna* in a constant temperature room at 20 ± 0.5°C with a 12 h photoperiod. The tests were conducted in 800-ml vessels to which 500 ml test solution was added. The test medium used was 50-μm filtered and UV-sterilized Lake IJssel water, with a pH of 8.1 and a hardness of approximately 225 mg/l (as $CaCO_3$). Stock solutions were prepared in this water. In several instances acetone or dimethylsulphoxide were used as solvents for the test compounds. The test solutions were renewed three times a week and were prepared fresh at each renewal. Oxygen concentration and pH were measured at regular intervals. The ratio of concentrations was 1.8; actual concentrations were not verified during the experiments.

The experiments were started with newborn (< 24 h) daphnids from a laboratory stock culture. These were fed daily with $3x10^8$ cells/l of the unicellular green alga

TABLE I. Test compounds.

Compound	Abbreviation	Supplier[a]	[illegible] (≥%)
Disodium ethylenebisdithiocarbamate	nabam	Lamers & Indemans	99
Manganese ethylenebisdithiocarbamate	maneb	ICN	90
Zinc ethylenebisdithiocarbamate	zineb	ICN	95
5,6-dihydro-3H-imidazo (2,1-c)-1,2,4-dithiazole-3-thion	DIDT	TNO	98
Ethylenethiourea	ETU	EGA	99
Ethyleneurea	EU	EGA	97
Sodium dimethyldithiocarbamate	NaDMDC	Fluka	97
Zinc dimethyldithiocarbamate	ZnDMDC	ICN	95
Ferric dimethyldithiocarbamate	ferbam	ICN	95
Tetramethylthiuramdisulfide	thiram	ICN	98
Tetramethylthiurammonosulfide	TMTM	ICN	95
Tetramethylthiourea	TMTU	Riedel de Haen	97
Tetramethylurea	TMU	Riedel de Haen	98
Sodium diethyldithiocarbamate	NaDEDC	Fluka	99
Zinc diethyldithiocarbamate	ZnDEDC	ICN	90
Tetraethylthiuramdisulfide	disulfiram	Fluka	97
Tetra-*n*-propylthiuramdisulfide	T(*n*)PTD	TNO	95
Tetra-isopropylthiuramdisulfide	T(i)PTD	OCS	98

[a]The chemicals were obtained from Lamers & Indemans B.V. ('s-Hertogenbosch, The Netherlands), Organic Chemicals Service (Vlissingen, The Netherlands), Baker Chemicals B.V. (Deventer, The Netherlands), TNO, Institute for Applied Chemistry (Utrecht, The Netherlands), Fluka AG (Buchs, Switzerland), EGA (Steinheim, F.R.G.), Riedel de Haen (Hannover, F.R.G.) and ICN Pharmaceuticals Inc. (Montreal, Canada).

Chlorella pyrenoidosa. The pooled neonates were randomly distributed into cohorts of ten animals each, in 5-8 toxicant concentrations, a solvent control and a blank control. To allow statistical treatment of the data the experiments were replicated five times. The number of surviving females and the number of neonates produced were recorded daily, the new neonates being removed from the test vessels after counting. At the end of the experiments carapace length of the daphnids was determined from the anterior margin of the head to the base of the caudal spine using an ocular micrometer. When daphnids appeared to be males, these animals were excluded both from fecundity, survival and growth analysis.

Calculations and statistics

Results of the chronic tests were summarized in life-table form. The intrinsic rate of natural increase, r_m, was calculated for each cohort of ten animals by successive approximation from the equation:

$$\sum_{x=0}^{\infty} l_x m_x e^{-r_m x} = 1.$$

where l_x is the proportion of individuals surviving to age x, m_x is the age-specific fecundity (number of females produced per surviving female at age x), and x is days. A derivation of this equation can be found in e.g. Roughgarden (1979) and Pielou (1977). As r_m calculated after 21 days is indistinguishable from r_m estimated for the entire life-span, due to the great importance of early reproduction (Van Leeuwen et al., 1985b), all calculations were based on 21-day experiments. Finally, means and standard errors were calculated.

Differences in mean survival, r_m and carapace length, between treatments and blank control, were tested using the procedure described by Williams (1971, 1972), after verification of differences between blank control and solvent control (Student's *t*-test; Sokal and Rohlf, 1981). Prior to applying the Williams' test, the data were tested for homogeneity of variances using the Bartlett test (Sokal and Rohlf, 1981). Normality was verified by means of the Shapiro-Wilk test, or graphically, by plots of the ordered data (x_i) against the normal order statistic $\xi\,(i/n)$ (Pearson and Hartley, 1972). It should be noted that in case of the survival data we used the arcsin square root transformation of the proportion of survivors in each test vessel. The lowest concentration which was significantly different from the control was denoted as LRCT (lowest rejected concentration tested) according to Skalski (1981).

Differences were considered significant at $\alpha < 0.01$. The LC_{50} values and their 95% confidence limits were determined according to Kooyman (1981). Relationships between toxicity and the partition between *n*-octanol and water (P_{oct}) were calculated with a computer program, based on the method of least squares.

Results

Survival

The results of the life-table experiments with DDCs and BDCs and related compounds are shown in Tables II and III. Effects of DCs on survival occurred at concentrations below the ppm-level. DDCs were generally more toxic than BDCs. Complexing with metals had little influence on the toxicity. The organic solvents used did not significantly alter the responses at the concentrations tested. The slopes of the concentration-response curves were generally steep; the LC_{50} values for all compounds studied, differed less than a factor 2 from their LRCT, provided that the same criterion was tested for. The LRCTs for survival were therefore omitted from Tables II and III.

The survival pattern of daphnids was clearly related to exposure concentration, and a gradual decline of the LC_{50} with time was observed for most compounds. This is illustrated in Figs. 1 and 2. The response of daphnids to EU, ETU, TMU and thiram was rather acute. Their incipient LC_{50} was reached within a few days. On some occasions the moulting of daphnids was disturbed and carapaces were deformed. When the new cuticle was in the process of formation, the old cuticle was still attached to the animal resulting in broken antennae and thoracic appendages. This clearly hindered filtering, and subsequently the animals died likely as a consequence of starvation.

TABLE II. Results of 21-day life-table and growth experiments with *D. magna* exposed to DDCs and related compounds.

Compound	LC_{50} and 95% C.L. (μg/l)		LRCT (μg/l)[a] r_m	carapace length
NaDMDC	14	(13-15)	14	>8.0
Ziram	11	(10-12)	10	⩽1.8
Ferbam	15	(13-16)	18	>10
Thiram	8	(7-9)	10	1.8
TMTM	164	(149-182)	320	100
TMTU	$7.5x10^4$	($6.8\text{-}8.2x10^4$)	5600	⩽3200
TMU[b]	$4.2x10^5$	($3.2\text{-}5.6x10^5$)	–	1.10^5
NaDEDC	30	(27-33)	24	14
ZnDEDC	14	(13-15)	10	⩽3.2
Disulfiram	12	(11-14)	18	10
T(*n*)PTD	13	(11-15)	5.6	>10
T(i)PTD	9	(8-10)	18	5.6

[a] Lowest rejected concentration tested ($\alpha < 0.01$).
[b] The experiment was terminated after 14 days.

TABLE III. Results of 21-day life-table and growth experiments with *D. magna* exposed to BDCs and related compounds.

Compound	LC_{50} and 95% C.L. (μg/l)		LRCT (μg/l)[a] r_m	carapace length
Nabam	80	(70-90)	56	32
Maneb	111	(94-126)	100	>56
Zineb	89	(78-102)	18	>56
DIDT	73	(67-81)	56	56
ETU	$1.8x10^4$	($1.0\text{-}3.2x10^4$)	⩽1000	$1x10^4$
EU[b]	$3.2x10^6$	($1.8\text{-}5.6x10^6$)	–	$1.8x10^5$

[a] Lowest rejected concentration tested ($\alpha < 0.01$).
[b] The experiment was terminated after 14 days.

Population growth

Generally, the lower rate of population increase caused by certain concentrations of the test compounds, was due to both increased mortality and a fewer clutches per female (Tables II and III). The latter was also due to reduced parental survival (Fig. 1 and Table IV). TMTU (Table V), zineb (Tabel VI) and ETU formed exceptions. For these compounds inhibition of population growth was observed at levels below those which caused a significant reduction in survival. The general pattern of toxicity is exemplified by disulfiram (Fig. 1). At 5.6 μg/l no significant depression in survival and fecundity was observed. At 10 μg/l, a first sign of disulfiram-induced toxicity was illustrated by reduced survival and fecundity. At 18 μg/l, survival was clearly reduced and reproductive output inhibited both by a delay in the onset of reproduction and by

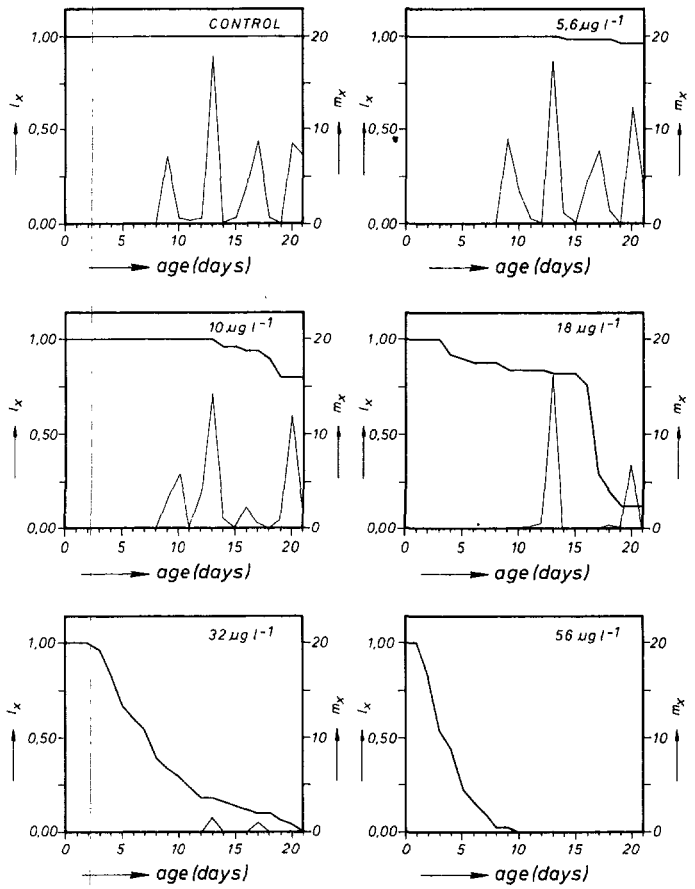

Fig. 1. Life-table data of *D. magna* at various concentrations of disulfiram: survival curve (thick line) and fertility curve (thin line).

fewer clutches per female. The effects increased in a concentration-dependent manner. The LRCT for r_m was 18 µg/l, but at this concentration survival was also clearly reduced (Table IV).

TABLE IV. Demographic information for *D. magna* from life-table experiments at various concentrations of disulfiram.

Concentration (µg/l)	Proportion surviving to day 21	Mean r_m ± SE (day^{-1})	Mean carapace length ± SE (mm)
0	1.00	0.302 ± 0.012	4.15 ± 0.16
3.2	0.96	0.320 ± 0.009	4.21 ± 0.16
5.6	0.96	0.317 ± 0.022	4.14 ± 0.15
10	0.80	0.296 ± 0.014	4.00 ± 0.19[a]
18	0.12	0.204 ± 0.016[a]	–
32	0.00	–	–

[a] Lowest rejected concentration tested ($\alpha < 0.01$).

TABLE V. Demographic information for *D. magna* from life-table experiments at various concentrations of TMTU.

Concentration (mg/l)	Proportion surviving to day 21	Mean $r_m \pm$ SE (day^{-1})	Mean carapace length ± SE (mm)
0	1.00	0.362 ± 0.004	4.13 ± 0.15
3.2	0.98	0.363 ± 0.009	3.92 ± 0.12[a]
5.6	1.00	0.313 ± 0.006[a]	3.75 ± 0.13
10	0.98	0.302 ± 0.012	3.73 ± 0.13
18	0.94	0.304 ± 0.007	3.66 ± 0.14
32	0.98	0.318 ± 0.002	3.60 ± 0.13
56	0.92	0.268 ± 0.009	3.11 ± 0.21
100	0.18	–	–
180	0.00	–	–

[a] Lowest rejected concentration tested ($\alpha < 0.01$).

TABLE VI. Demographic information for *D. magna* from life-table experiments at various concentrations of zineb.

Concentration (μg/l)	Proportion surviving to day 21	Mean $r_m \pm$ SE (day^{-1})	Mean carapace length ± SE (mm)
0	1.00	0.354 ± 0.006	4.04 ± 0.11
10	1.00	0.345 ± 0.005	4.02 ± 0.11
18	1.00	0.335 ± 0.007[a]	4.04 ± 0.13
32	0.96	0.339 ± 0.011	4.12 ± 0.17
56	0.62	0.299 ± 0.018	4.33 ± 0.26
100	0.30	0.197 ± 0.022	–
180	0.34	0.083 ± 0.033	–
320	0.04	–	–
560	0.00	–	–

[a] Lowest rejected concentration tested ($\alpha < 0.01$).

Carapace length

A significant reduction in mean carapace length of 21-day old daphnids was generally detected at levels comparable with their LC_{50} values (Table II and III). For ziram, ZnDEDC, thiram and TMTU, however, these effects occurred at levels clearly below their respective LC_{50}. For ziram, ZnDEDC and TMTU (Table V), carapace length was a monotonically decreasing function of concentration. No threshold level could be detected as the lowest concentration tested was the LRCT. The lowest concentration of thiram tested (1 μg/l), did not significantly differ from the control. Concentrations

which affected survival with more than 50%, were excluded from the statistical analysis as size-selective mortality might occur. The length data of zineb-treated *Daphnia* (Table VI) are apparently an example of such a selection.

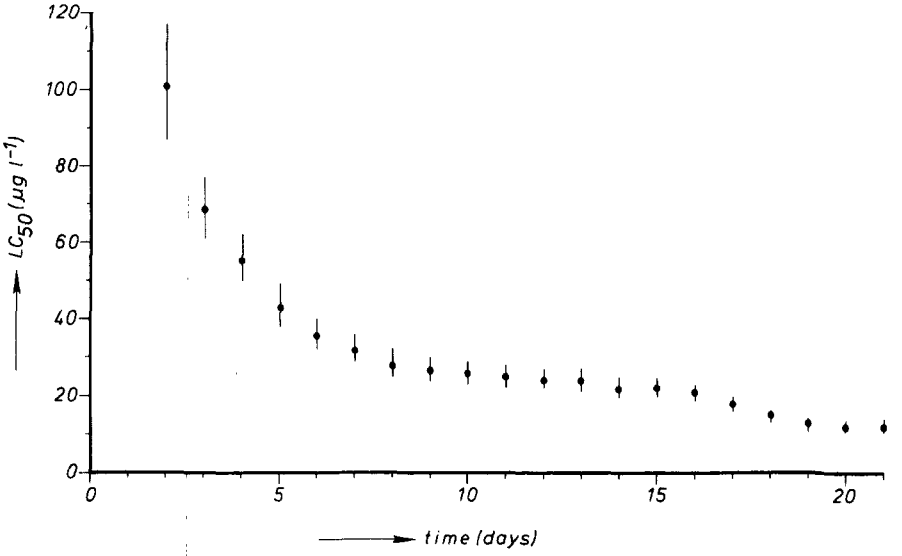

Fig. 2. Temporal changes in the LC_{50} for *D. magna* exposed to disulfiram.

QSAR studies

The log P_{oct} values of the compounds selected for the QSAR studies are shown in Table VII. Metal derivatives were excluded from these calculations. The biological parameter used in the QSAR calculations was the LC_{50}, determined at different exposure times. No calculations were performed on the basis of the other toxicological

TABLE VII. Log P_{oct} values of the compounds used in QSAR calculations[a].

No.	Compound	Log P_{oct}
1	T(*n*)PTD	6.06
2	T(i)PTD	6.06[b]
3	Disulfiram	4.00
4	Thiram	1.82
5	DIDT	1.62
6	TMTM	1.17
7	TMTU	0.49
8	TMU	0.20
9	ETU	-0.67
10	EU	-0.96

[a] From Van Leeuwen et al. (1985a).
[b] Calculated from the experimental value of thiram and f(CH_2) from Rekker (1977).

criteria studied as they were closely linked to mortality. The calculations revealed that the *n*-octanol/water partition coefficient explained nearly all variation in toxicity (Table VIII). Over the broad range of lipophilicities (10^7), toxicity was a sigmoid function of log P_{oct} (Fig. 3). ETU appeared to be an outlier and was excluded from all calculations. The sigmoid QSAR remained undisturbed when LC_{50} values of longer

TABLE VIII. QSARS for *D. magna* and the compounds shown in Table VII[a].

QSAR equation	$\log 1/C = ab\{a+(b-a)\exp(-k \log P_{oct})\}^{-1}+c$					
C (μmol/l)	*a*	*b*	*c*	*k*	*n*	*s*
2-day LC_{50}	0.238	5.13	-4.83	3.39	9	0.077
4-day LC_{50}	0.492	5.55	-4.69	2.98	9	0.398
7-day LC_{50}	0.632	5.83	-4.70	2.72	9	0.454
14-day LC_{50}	0.572	5.90	-4.59	2.83	9	0.353
21-day LC_{50}[b]	0.679	6.11	-4.64	2.69	9	0.338

[a] n= number of data points; s = standard error of the estimate.
[b] For EU and TMU the 14-day LC_{50} values were substituted.

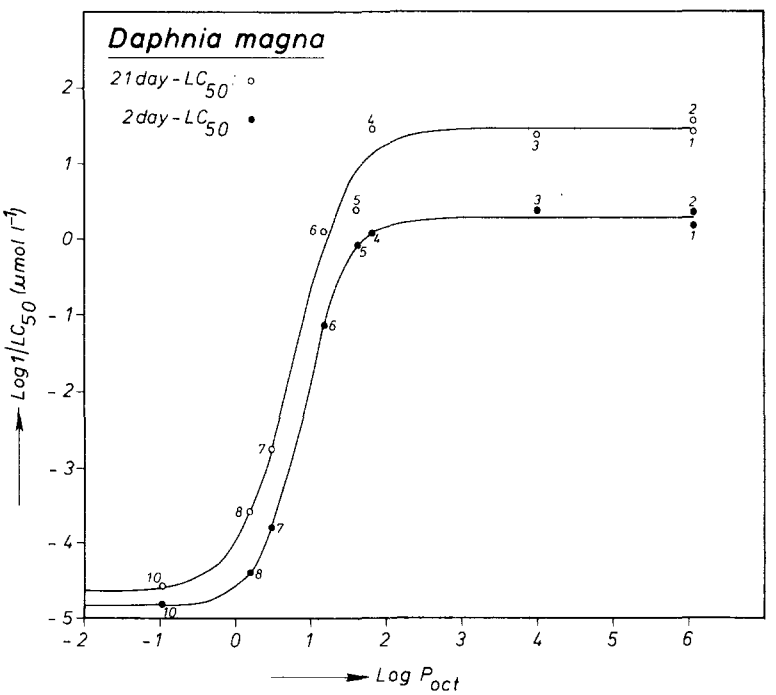

Fig. 3. QSARs for DCs and related compounds. Circles represent the observed and lines the expected values based on the equations presented in Table VIII. Numbers correspond to those of Table VII.

test times were used instead of 2-day LC_{50} values. Differences in toxicity between the hydrophilic and lipophilic compounds, however, increased with exposure time (Fig. 3). This was due mainly to the relatively large increase of toxicity of the lipophilic compounds (parameter *b* in Table VIII), whereas the toxicity of the hydrophilic compounds remained approximately constant (parameter *c* and *k* from Table VIII).

Discussion

Life-table tests

DCs were very toxic to *D. magna*. Growth and reproduction were not specifically inhibited since effects on these properties were generally detected at levels comparable to their LC_{50}. This non-specific mode of action can be explained by their cytotoxic nature (Van Leeuwen et al., 1985a), BDCs being less toxic than DDCs.

Heavy metal complexation may explain the incidental problems encountered in moulting as at least two metallo-enzymes (phenol oxidase and glutamate dehydrogenase) are involved in this process (cf. Scheer, 1966; Lockwood, 1968; Hochachka and Somero, 1973). Owens (1954) has demonstrated the *in vitro* inhibition of phenol oxidase by several DCs and thiourea derivatives.

QSAR studies

Quantitative structure-activity relationships are increasingly used in aquatic toxicology (Könemann, 1981; Govers et al., 1984; Hermens et al., 1984.). In this study the derivation of such relationships and their quantitative aspects were applied to a series of pesticides and some of their degradation products. The relationship between lipophilicity and toxicity of these compounds was non-linear. Deviations from linearity were observed both at low and high log P_{oct}. The deviations from linearity in the hydrophilic range may be related to diffusion through aqueous membrane pores (Seydel and Schaper, 1982), whereas deviations from linearity at high P_{oct} may be related to reduced solubility and limited diffusion, i.e. reduced accessibility to the site of action.

Deviations from linearity in the hydrophobic range are not uncommon and generally described by parabolic equations. For many biological systems and non-specific neutral compounds the ideal log P_{oct} is about 2 (Hansch, 1973). The same holds true for DCs and related compounds. No indication was found, however, of any decrease in toxicity in response to increasing lipophilicity above this value (Fig. 3). This is in line with the theoretical considerations of Dearden and Townend (1976). They pointed out that biological activity not necessarily falls off again with increasing lipophilicity but depends on the test-time and probably also on the test species, route of administration and on metabolism.

Acknowledgments

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Chapter 6

Differences in susceptibility of early life stages of rainbow trout (Salmo gairdneri) to environmental pollutants

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Abstract

To compare the susceptibility of early life stages (ELS) of rainbow trout (*Salmo gairdneri*), acute toxicity studies were carried out with cadmium, maneb, pentachlorophenol, 1,2,4,5-tetrachlorobenzene, parathion and dieldrin. The tests were performed with fertilized eggs before and after water hardening, early eye point eggs, late eye point eggs, sac fry and early fry. In all cases the early fry stage appeared the most critical period. Additionally, short-term static bioaccumulation experiments were performed with [^{14}C]dieldrin. A kinetic model was developed for the static accumulation studies. The bioconcentration factor increased during embryonic development, reached a maximum at the sac fry stage and fell off again at the early fry stage. The uptake rate constant clearly increased once the egg membrane had disappeared and reached its maximum at the early fry stage. The clearance rate constant sharply increased at the early fry stage. Dieldrin was recovered almost completely from the yolk, which acted as a temporary 'toxicant sink'. Changes in the susceptibility of ELS of *S. gairdneri* to pollutants are explained by metabolic modifications during embryolarval development and only partly by differences in the accessibility of the toxicants.

In conclusion it is stated that in view of the lack of fish toxicity data, short-term exposure of early fry is a practical approximation in establishing toxicant concentrations acceptable for fish. For compounds having a high bioaccumulative potential the exposure period should also include the sac fry stage.

Chapter 6

Differences in susceptibility of early life stages of rainbow trout (Salmo gairdneri) to environmental pollutants

Introduction

For a full evaluation of the aquatic toxicity of chemicals, life cycle tests have been proposed by Mount and Stephan (1967). Effects of pollutants on the reproduction process are implicitly studied in these tests. Embryolarval tests are generally started with artificially fertilized eggs of fish reared in uncontaminated water and therefore, effects of chemicals on gametogenesis cannot be detected. As a rule embryolarval tests yield results which are consistent with those obtained from life cycle tests and at the same time enable the test period to be appreciably shortened (McKim, 1977). Macek and Sleight (1977) give details of the conditions such tests must meet.

The critical stages to be distinguished during embryogenesis from a physiological and toxicological point of view are perivitelline fluid formation, which takes place immediately following fertilization, hatching of the eggs, and the resorption of the yolk as the sac fry develop into free-swimming juveniles. Differences in sensitivity between early life stages (ELS) of fish may possibly be traced in part to differences in levels of accumulation. Before a substance can exercise its harmful effects it has to reach the 'site of action'. Access to this 'site of action' depends on the barriers to be overcome. In the case of fish, these include the gills, the skin and the intestine. It may be assumed that amongst others major physiological changes take place in the course of the development of the embryo. The hatching process whereby the protective egg membrane disappears and the gills of the sac fry begin to develop functionally may alter the path of exposure and therefore the sensitivity to environmental pollutants. The development of a functional digestive system including detoxification mechanisms during ontogenesis, may also alter the toxicological response.

Although embryolarval tests as compared to life cycle studies reduce the time to produce information on the toxicity of chemicals, they remain laborious. A further reduction of the exposure time, i.e. short-cut methods, are needed because fish toxicity data are still very scarce. In order to detect susceptible stages during the ontogenesis of fish simultaneous static accumulation experiments with [^{14}C]dieldrin and acute toxicity tests have been undertaken with different ELS of rainbow trout (*Salmo gairdneri*) together with fat content and fresh-weight determinations. The sensitivity of fingerlings was not studied as several studies showed that body size is inversely related to the toxic response (Anderson and Weber, 1975; Tsai and Chang, 1981).

Materials and methods

Experimental animals

Rainbow trout (*Salmo gairdneri*) were obtained from a fish hatchery at Vaassen (Gelderland). Eggs and milt were collected in the laboratory by stripping the ripe spawners. Fertilization took place according to the dry method described by Huisman (1975). The fertilized eggs were directly used in the experiments or put into glass aquaria having a capacity of 15 l. The aquaria were filled with 10 l standard water (in accordance with NPR 6507 (1980); pH 7.2; hardness 50 mg/l as $CaCO_3$). This water was moderately aerated and renewed once a week. The aquaria were placed in a constant temperature room of 10 ± 1°C. During embryogenesis this chamber was kept dark. Unfertilized eggs and dead ELS were removed and after resorption of the yolk, the fry were fed on a commercial fish food (Trouvit, Trouw & Co. N.V., The Netherlands).

Chemicals

The chemicals used in the toxicity experiments were cadmium chloride, manganese ethylenebisdithiocarbamate (maneb), pentachlorophenol (PCP), parathion-ethyl, 1,2,4,5-tetrachlorobenzene (TeCB) and dieldrin with a chemical purity equal to or exceeding 99, 90, 97, 99, 97 and 99%, respectively. Maneb was obtained from ICN Pharmaceuticals Inc. (Montreal, Canada), TeCB from Fluka AG (Buchs, Switzerland), $CdCl_2$ and PCP from Merck (Darmstadt, F.R.G.) and dieldrin and parathion-ethyl from Riedel de Haen (Hannover, F.R.G.). Stock solutions of dieldrin, PCP and TeCB were made in acetone. Parathion-ethyl was dissolved in DMSO and maneb and $CdCl_2$ in distilled water. The stock solutions of maneb, dieldrin and parathion were freshly prepared for each test. The other stock solutions were prepared once only and kept in the dark at 10°C. [^{14}C]dieldrin (specific activity 0.222 μCi/μg), used in the accumulation experiments was obtained from Amersham Radiochemical Centre (England). The stock solution was prepared in acetone and had an activity of 0.1 μCi/μl.

Experimental set-up

The investigation consisted of six separate experiments in which toxicity and accumulation tests as well as weight and fat content determinations were performed simultaneously with different ELS. The first series of experiments was started with eggs directly after fertilization. The other series of experiments were performed with water-hardened eggs, early eyed eggs, late eyed eggs, sac fry and early fry. At the start of the experiments the ELS were, respectively, 0, 1, 14, 28, 42 and 77 days old. The fresh weight and fat content determinations, however, were not carried out with freshly fertilized eggs and water-hardened eggs. Instead, only 3-h-old eggs were used.

Toxicity experiments

Toxicity experiments were carried out in the dark in a constant temperature room at 10 ± 1°C. The tests were performed in duplicate, using 10 eggs or fry per litre. The test solutions were renewed each day. At the egg stage, the eggs were placed in a small Petri dish on the bottom of the test vessel and at each renewal the Petri dish was transferred into a fresh solution. Sac fry and early juveniles were transferred with a net. The criterion applied to establish death was the appearance of a non-transparant (white) egg membrane at the egg stage, the abolishment of heartbeat for the older embryos and sac fry, and the abolishment of respiratory movements for the juveniles.

The LC_{50} values and their 95% confidence intervals were either calculated according to Litchfield and Wilcoxon (1949) or determined graphically on log probability paper. In the case of the latter, the concentrations resulting in 0 and 100% mortality respectively were taken as a 99% confidence interval (Stephan, 1977).

Accumulation experiments

Accumulation studies were carried out with ELS in the dark at 10 ± 1°C. The experiments with freshly fertilized eggs were performed in 1.5 l test vessels to which 1 l well-aerated standard water was added. The other experiments were carried out in 10 l aquaria filled with 4 l standard water. The [^{14}C]dieldrin solution was added below the water surface. The test solution was then thoroughly stirred for 1 h.

Afterwards a Petri dish containing 100-120 eggs or fry was placed in the aquarium. These experiments were static, i.e., the test solutions were not renewed. These solutions were not aerated during the test either. Samples were taken at regular intervals. In each case 10 eggs or fry were taken out of the test solution and rinsed with standard water. After removal of adhering water by blotting on filter paper the eggs or fry were distributed over combustion cups, weighed, and incinerated in a sample oxidizer (Packard, Tri Carb model B 306). CO_2 was trapped in Carbo-sorb and Perma-Fluor V. Radioactivity of the test solutions and eggs or fry was measured with a liquid scintillation counter (LKB/Wallac Rackbeta 1215). All measurements were carried out in five replicates. Additional semi-static experiments (i.e., test solutions were renewed at regular intervals) were carried out to study accumulation just before and after hatching of the eggs, elimination, as well as to localize dieldrin in the various tissues. Eggs and larvae were dissected under a stereomicroscope and tissues analyzed according to the methods described above.

Model Calculations

Static accumulation experiments differ from continuous flow experiments in the sense that the concentration of the test substance in water gradually declines. Therefore two mass balance equations are needed to describe the kinetics for static tests: one for the concentration in the fish (eq. 1) and one for the concentration in the water (eq. 2):

$$\frac{d}{dt} C_f(t) = k_1 C_w(t) - k_2 C_f(t) \tag{1}$$

$$\frac{d}{dt} C_w(t) = -\{\lambda + k_1 \frac{m_o}{V} N(t)\} C_w(t) + k_2 \frac{m_o}{V} N(t) C_f(t) \tag{2}$$

$C_f(t=0) = 0$, $C_w(t=0) = C_w^o$, the initial concentration in the water.

The following symbols were used:

		dimensions
t	: time	h
$C_f(t)$	: concentration of test substance in one organism at time t	µg/kg
$C_w(t)$	: concentration of test substance in the water at time t	µg/l
m_o	: weight of one organism	kg
V	: water volume	l
k_1	: uptake rate constant	l/kg/h
k_2	: clearance rate constant	1/h
λ	: loss rate constant	1/h
$N(t)$	: number of organisms at time t	--

It is assumed that uptake and release of the test substance are proportional to its concentration in water (C_w) and organism (C_f), respectively. Processes like volatilization and adsorption to the walls of the test vessels are taken into account by introduction of a loss term, which is assumed to be proportional to C_w. The volume (V) and the weight of the organism (m_o) are considered to be constant during the experiment. $N(t)$ changes with time because of sampling and $C_f(t)$ is regarded as the population mean.

The constants k_1, k_2 and λ are estimated by minimizing a least-squares criterion defined by:

$$J(k_1, k_2, \lambda) = \sum_{i=1}^{M} \varepsilon_f^2(t_i) + \sum_{i=1}^{M} \varepsilon_w^2(t_i) \tag{3}$$

where:

$$\varepsilon_f(t_i) = \{C_f(t_i) - \bar{C}_f(t_i)\} \frac{N(t_i) m_o}{V} \tag{4}$$

and:

$$\varepsilon_w(t_i) = C_w(t_i) - \bar{C}_w(t_i). \tag{5}$$

In these equations $\bar{C}_f(t_i)$ and $\bar{C}_w(t_i)$ are sample means of the measured concentrations both at time t_i, $i = 1...M$(the total number of sampling times). It is assumed that sample means are good approximations of population means, i.e., the influence of biological variability and variations due to sampling are neglected. For the numerical minimiza-

tion of J with respect to k_1, k_2 and λ, the IMSL-routine ZXMIN was used, which is based on a quasi-Newton method. The time-dependent bioconcentration factor (BCF; dimension: l/kg) was calculated according to its definition ($BCF = C_f/C_w$). The BCF under equilibrium conditions (notation: BCF_∞), where it is assumed that N is constant, can be derived from eqs. 1 and 2:

$$BCF_\infty = \frac{k_1 \frac{m_0 N}{V} + \lambda - k_2 + \sqrt{(k_1 \frac{m_0 N}{V} + \lambda - k_2)^2 + 4k_1 k_2 \frac{m_0 N}{V}}}{2k_2 \frac{m_0 N}{V}} \quad (6)$$

If λ is zero, this equation reduces to the more familiar equation:

$$BCF_\infty = \frac{k_1}{k_2} \quad (7)$$

Eq. 7 was used for the calculation of BCF_∞ for the various ELS.

The eigenvalues of the static accumulation model are:

$$e_{1,2} = -\tfrac{1}{2}(\lambda + k_1 \frac{m_0 N}{V} + k_2) + \tfrac{1}{2}\sqrt{(\lambda + k_1 \frac{m_0 N}{V} + k_2)^2 - 4k_2\lambda} \quad (8)$$

If processes like volatilization and adsorption are neglected (λ is set to zero) this equation further reduces to:

$$e_1 = 0, \quad e_2 = -(k_1 \frac{m_0 N}{V} + k_2) \quad (9)$$

The rate of change is completely determined by the second eigenvalue. In accumulation experiments under continuous flow conditions, however, the only eigenvalue is $-k_2$. In other words equilibrium concentrations under static conditions are reached more rapidly than under continuous flow conditions.

Weight and fat content

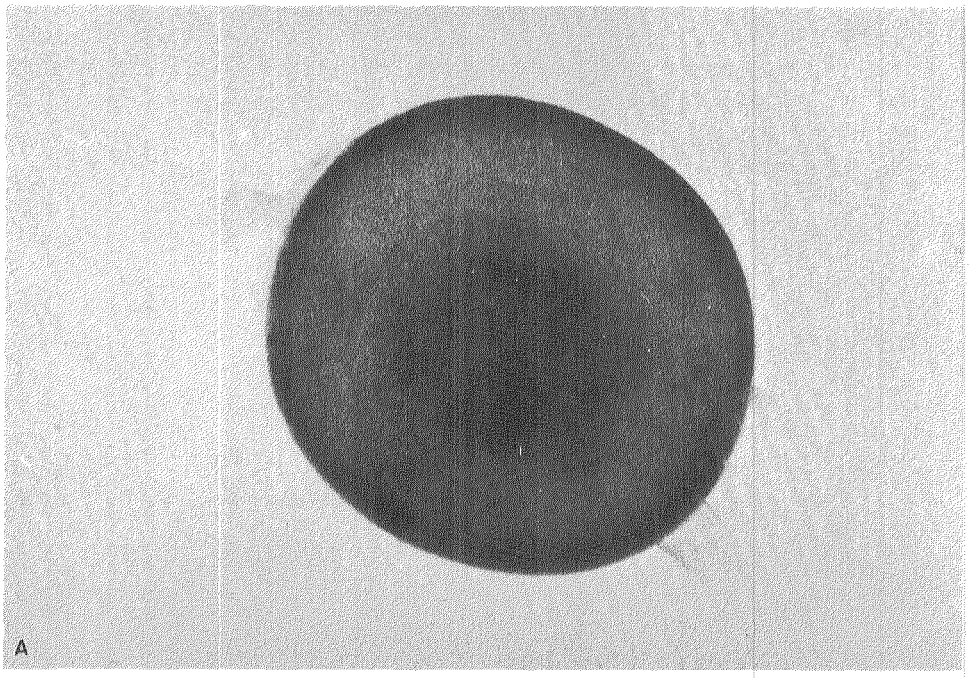
The weight of the ELS was determined using an analytical balance, the adhering water having been removed with filter paper. The fat content was determined gravimetrically after soxhlet extraction as described by Könemann and Van Leeuwen (1980). These determinations were made in replicates using ten and five samples for weight and fat content, respectively.

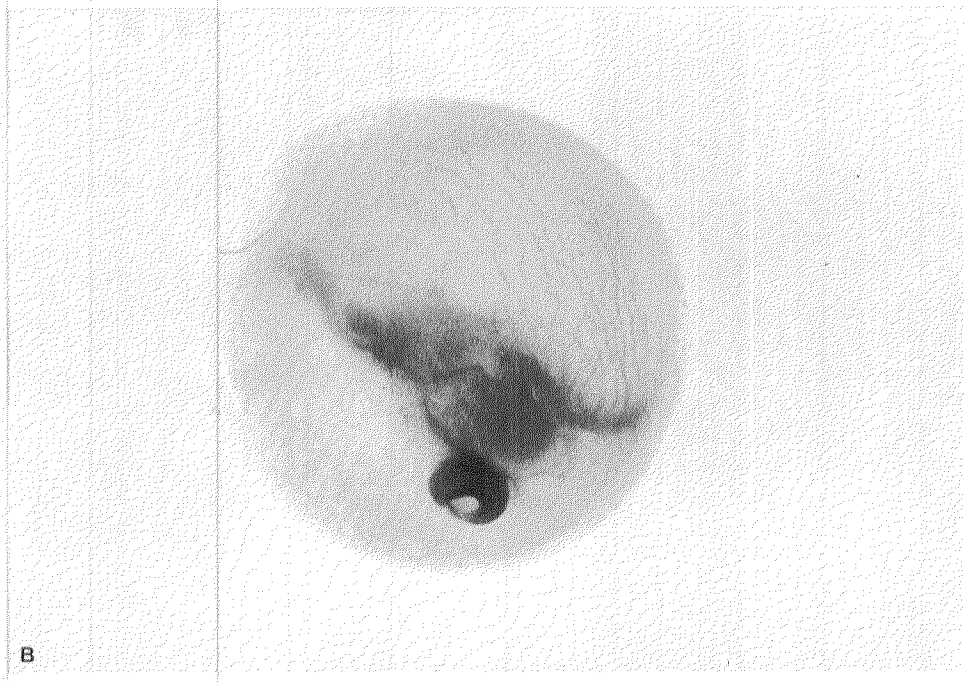
Results

Fig. 1 shows the physical appearance of the ELS of rainbow trout. The results of the toxicity experiments at various stages of development are shown in Table I. The susceptibility of the ELS clearly increased after hatching and reached its maximum at the early

fry stage for all compounds studied. Although in the experiments with dieldrin and TeCB, concentrations were used which were far greater than their aqueous solubility no mortality could be induced at these ELS up to and including the sac fry. In contrast, for the early fry very low LC_{50} values of 1.2 and 0.0032 mg/l were found for TeCB and dieldrin, respectively. The maximum concentration of parathion tested was 10 mg/l. At this concentration it induced partial mortality at the sac fry stage. The LC_{50} of parathion for early fry was 1.4 mg/l. Great differences in susceptibility between the various ELS were also noted for maneb, PCP and cadmium. For these compounds mortality was observed at earlier developmental phases. The freshly fertilized eggs were relatively insensitive to the chemicals tested. In experiments with sac fry and in particular with early fry, cadmium induced hyperactivity and mortality over a wide range of concentrations. LC_{50} values could only be determined after repeating these experiments for several times. The reported values should therefore be interpreted only as indicative.

Fig. 1. Early life stages of rainbow trout (*Salmo gairdneri*). (A) egg - 2 days, (B) late eyed egg - 25 days, (C) early sac fry - 35 days, (D) late sac fry - 55 days and (E) early fry - 85 days.





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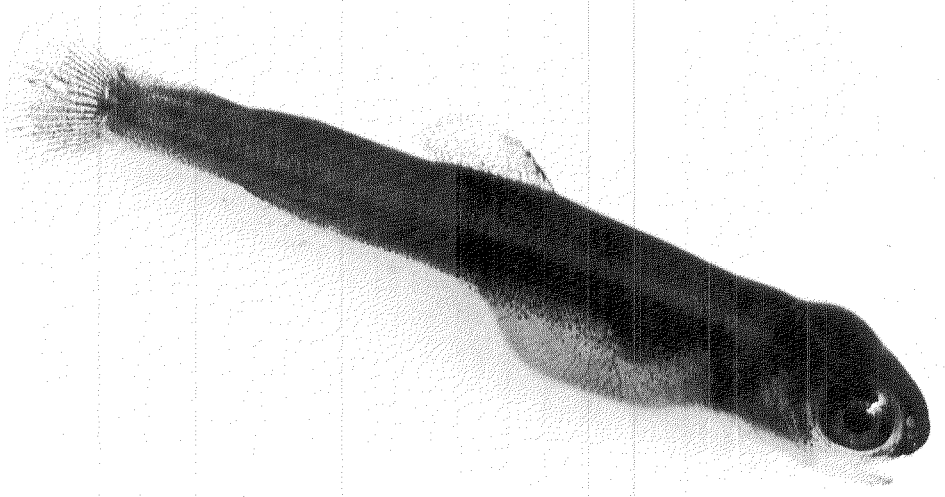

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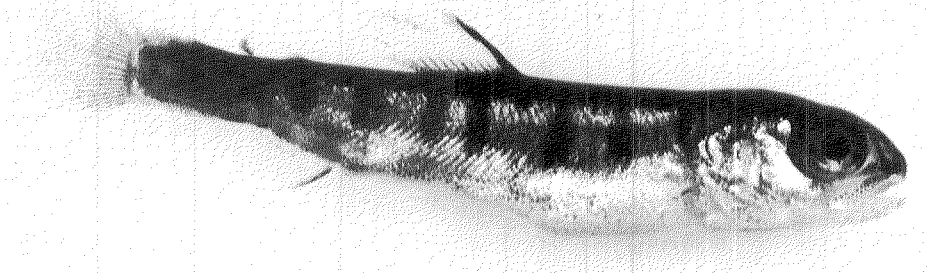


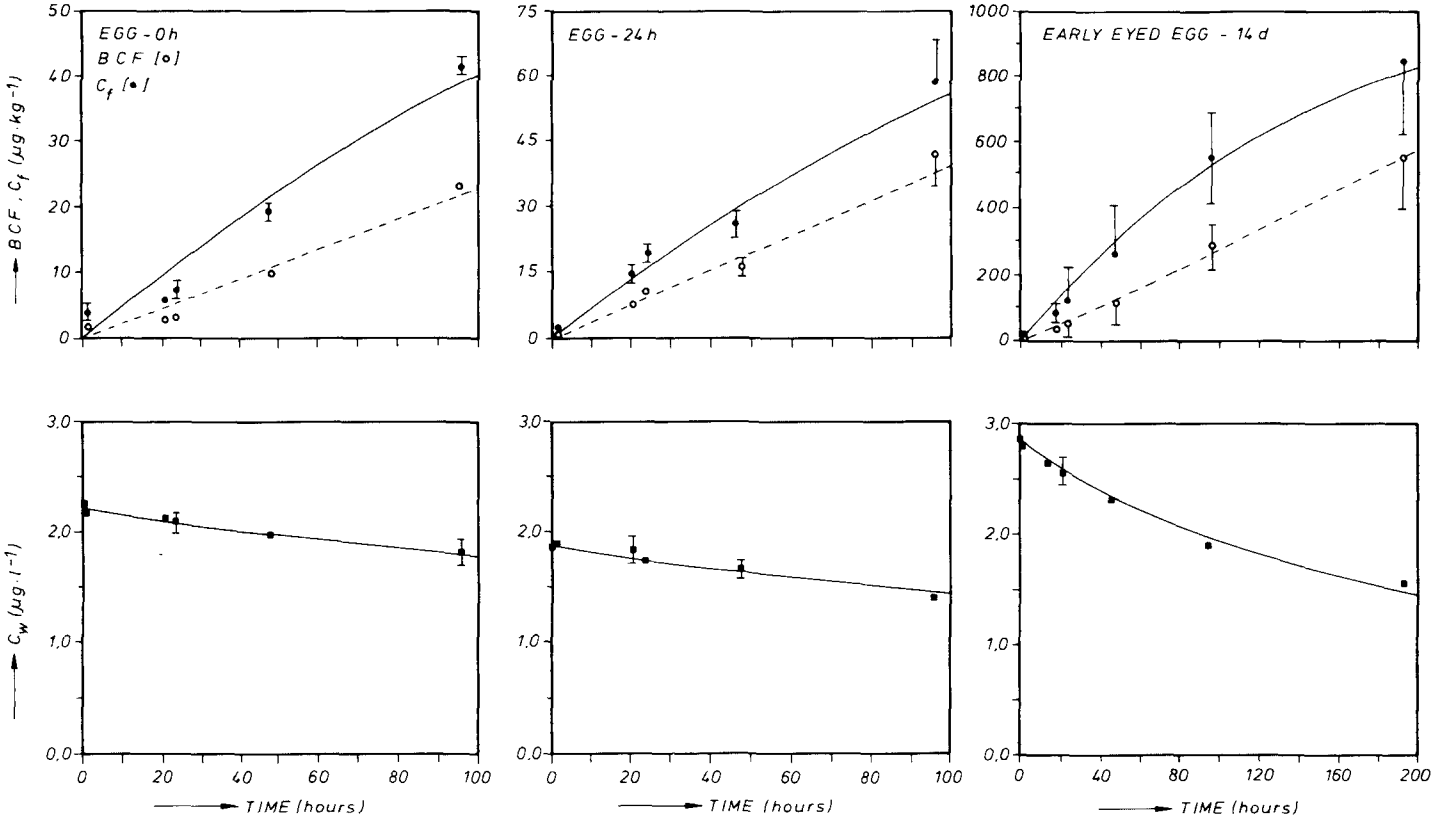
E

The results of the static accumulation experiments using [^{14}C]dieldrin are shown in Fig. 2 and Table II. During embryogenesis and larval development the uptake rate constant (k_1) clearly increased, in particular at the sac fry and early fry stage. The loss rate constant (λ) slightly fluctuated during embryogenesis but also increased after hatching of the eggs. The clearance rate constant (k_2) hardly varied during embryogenesis and larval development but sharply increased at the early fry stage. During embryolarval development the clearance half-life ($t1/2$) ranged from 15.2 to 22.2 days, whereas at the early fry stage a value of 0.2 days was attained. No equilibrium was reached between uptake and elimination within the eperimental period, except for early fry for which a 'steady-state' concentration was reached within 24 h (Fig. 2). This residue did not remain constant. After 24 h exposure it gradually decreased from (mean ± SE) 648 ± 107 to 519 ± 30 μg/kg at t=96 h, with a concomitant rise of C_w from 0.37 ± 0.02 to 0.40 ± 0.01 μg/l. These experimental data were excluded both from the calculations and Fig. 2. The 24-h and 96-h values for C_f and C_w were significantly different at $\alpha = 0.1$ and $\alpha = 0.01$ respectively (Student's t-test). Bioconcentration increased during development and

TABLE I. LC_{50} 96-h and confidence intervals (mg/l) of environmental contaminants at various stages of rainbow trout (*Salmo gairdneri*) ontogenesis.

Stage of development[a]	Test substance					
	Cd	Maneb	PCP	Parathion-ethyl	TeCB	Dieldrin
Egg-0 h	13[b] (10-18)	6.0[c] (4.7-7.6)	3.0[c] (2.3-3.8)	> 10	> 10	> 10
Egg-24 h	13[b] (10-18)	5.6[b] (3.2-10.0)	1.3[c] (1.0-1.5)	> 10	> 10	> 10
Early eyed egg-14 days	7.5[c] (5.4-10.4)	1.8[c] (1.4-2.4)	3.0[c] (2.5-3.6)	> 10	> 10	> 10
Late eyed egg-28 days	9.2[c] (6.4-13.2)	1.3[b] (1.0-1.8)	0.48[c] (0.35-0.66)	> 10	> 10	> 10
Sac fry -42 days	0.03[c] (0.01-0.06)	0.32[b] (0.18-0.56)	0.032[b] (0.010-0.056)	10[d]	> 10	> 10
Early fry -77 days	0.01[c] (0.007-0.015)	0.34[c] (0.27-0.43)	0.018[b] (0.010-0.032)	1.4[c] (1.2-1.6)	1.2[c] (1.0-1.5)	0.003[c] (0.002-0.006)

[a] The age of the ELS at the start of each experiment is given.
[b] Graphically interpolated value and, in parentheses, 99% confidence interval.
[c] Calculated LC_{50} and, in parentheses, 95% confidence interval.
[d] Partial mortality (30%) at the highest concentration tested.


EGG - 0h
BCF (o)
C_f (•)
EGG - 24h
EARLY EYED EGG - 14 d
BCF , C_f (µg·kg^{-1})
C_w (µg·l^{-1})
TIME (hours)
TIME (hours)
TIME (hours)

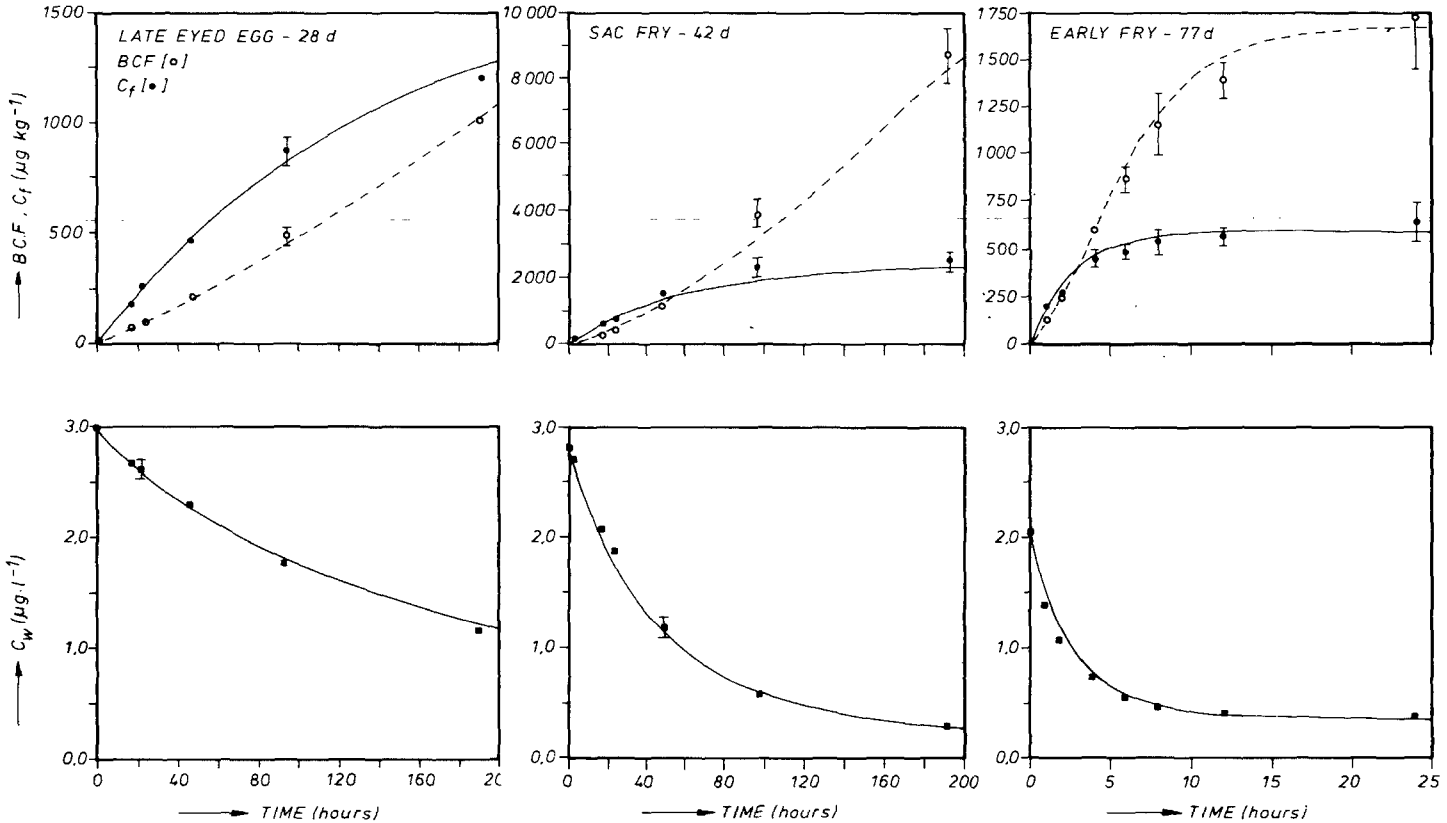


Fig. 2. Results of static accumulation experiments with [^{14}C]dieldrin in various developmental stages of rainbow trout (*Salmo gairdneri*). At each developmental stage the concentration in water (C_w), fish (C_f), as well as the bioconcentration factor (BCF = C_f/C_w) are shown. Solid circles, open circles and squares represent the observed and lines the expected values based on the model calculations. The age of the ELS at the start of each experiment is given. Bars represent standard errors.

TABLE II. Results of static accumulation experiments with [^{14}C]dieldrin in ELS of *Salmo gairdneri*.

Stage of development[a]	k_1 (h^{-1})	k_2 x 10^{-3} (h^{-1})	λ x 10^{-3} (h^{-1})	$t1/2$[b] (days)	BCF[c] (x 10^3)
Egg-o h	0.2	1.8	1.7	16.0	0.12
Egg-24 h	0.4	1.9	1.2	15.2	0.20
Early eyed egg-14 days	2.3	1.9	1.8	15.2	1.2
Late eyed egg-28 days	3.8	1.5	1.6	19.3	2.5
Sac fry-42 days	16	1.3	8.4	22.2	12
Early fry-77 days	140	94	18	0.3	1.5

[a] The age of ELS at the start of each experiment is given.
[b] Clearance half-life calculated from $t1/2 = \ln 2/k_2$.
[c] Calculated from eq. 7.

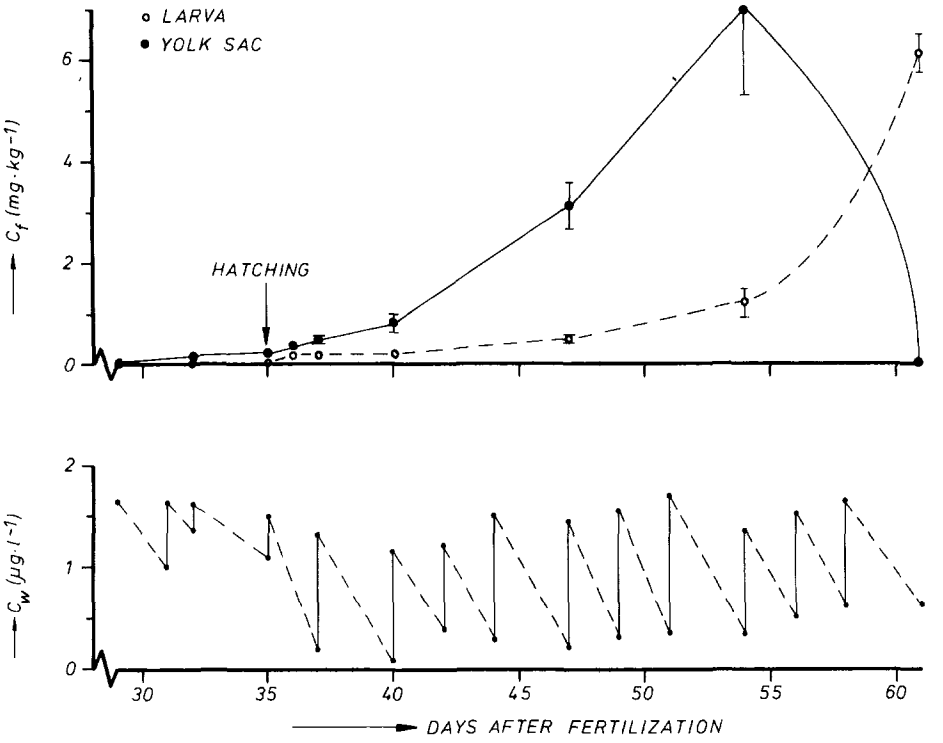


Fig. 3. Semi-static accumulation test with [^{14}C]dieldrin and rainbow trout (*Salmo gairdneri*) showing the concentration in fish (C_f) and water (C_w). The test was started with late eyed eggs and terminated after completion of the yolk resorption. Bars represent standard errors.

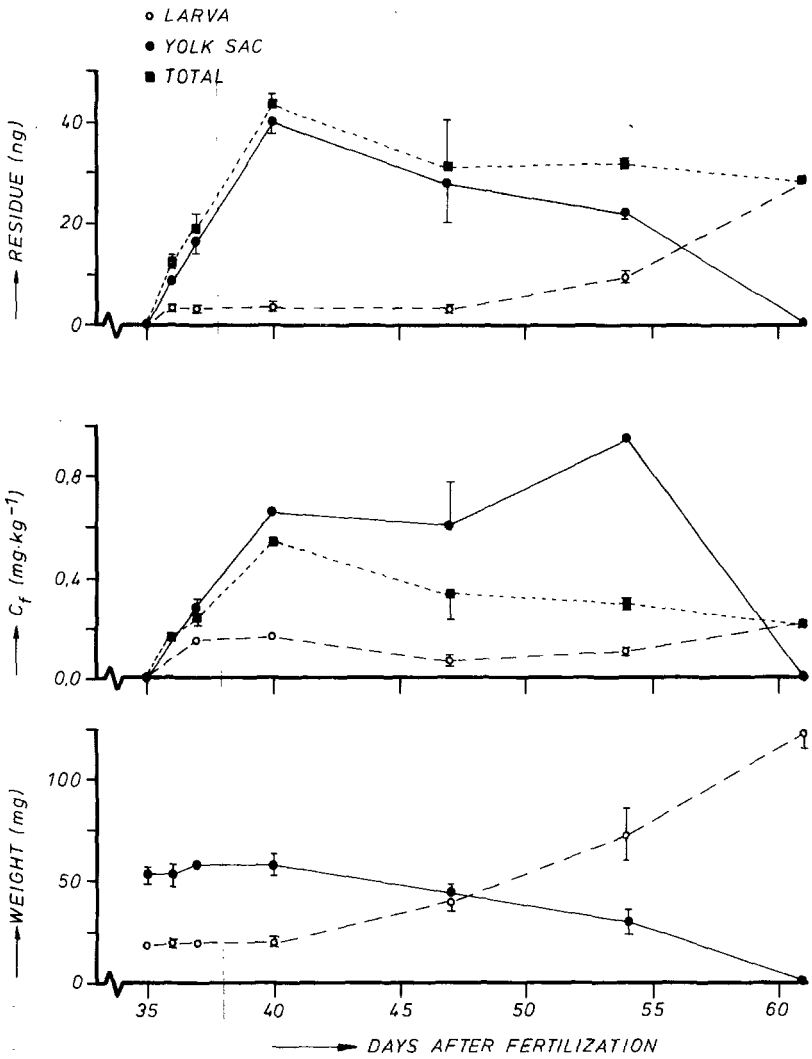


Fig. 4. Distribution and elimination of [^{14}C]dieldrin during the process of yolk resorption in rainbow trout (*Salmo gairdneri*). The test was started with sac fry, directly after hatching of the eggs and terminated after completion of the yolk resorption. After a 5-day exposure period the sac larvae were transferred to toxicant-free water. Bars represent standard errors.

reached a maximum at the sac fry stage. It decreased at the early fry stage as the clearance rate constant increased relatively sharply. Straightforward mass balance calculations showed the loss of radioactivity during the tests to be 10 - 20%.

The results of the semi-static accumulation and elimination studies are shown in Fig. 3 and Fig. 4. Increased accumulation once the egg membrane has disappeared (Fig. 3) is shown by the rise of C_f and by the deprivation of dieldrin in the test solution. During the sac fry stage a large residue was build op. This accumulation of dieldrin, however, is

TABLE III. Localization of [^{14}C]dieldrin in the 24-day-old eggs of rainbow trout (*Salmo gairdneri*) after 48-h exposure to 5.78 µg/l. Results are given as mean ± SE.

	Dieldrin content	
	ng/egg	% total
Chorion	0.05±0.03	0.8±0.3
Perivitelline fluid	0.33±0.18	5.3±3.0
Embryonic body	0.98±0.22	15.2±1.5
Yolk sac	5.02±0.63	78.7±3.3
Whole egg	6.38±0.82	100.0±0.0

underestimated because of the heavy loading of the test vessel and the semistatic procedure applied. The distribution of dieldrin between yolk and embryonic body during embryogenesis and the process of yolk resorption is also shown in Fig. 3. Results of a more precise localization of dieldrin in the egg are shown in Table III. The yolk acted like a temporary 'toxicant sink' (Fig. 3). This is also shown in Fig. 4; despite the relatively long elimination period, short-term accumulation of dieldrin during the beginning of the sac fry stage lead to an increased tissue concentration due to subsequent delivery from the yolk. The highest concentration of dieldrin in the larvae was reached towards the end of the sac fry stage when the yolk had been resorbed. Finally, when k is solved from $\ln C_f = -k_2 t + C$ ($C_W = 0$) and subsequently substituted in $t1/2 = \ln 2/k_2$, a clearance half-life of 16.6 ± 2.5 days is obtained for the sac fry which reasonably corresponds to those shown in Table II.

The mean fresh weight of the ELS appeared to be constant (40.4 - 43.7 mg) up to the early fry stage. Then it increased to (mean and 95% confidence limits) 63.4(61.4 - 65.2) mg due to the fact that the fry were fed. The mean fat content was constant up to the sac fry stage (2.79 - 3.07)% and decreased afterwards to 2.27(1.79 - 2.75)% for the sac fry and 1.84(1.72 - 1.96)% for the early fry, as a result of yolk resorption.

Discussion

Eggs were relatively resistant to the toxic effects of 6 chemicals as evidenced by high concentrations required to produce lethality. Susceptibility increased during embryonic development. Longwell (1977) postulated that pollutants may enter the egg with imbibed water during the process of perivitelline fluid formation after which the egg membrane affords a considerable protection against pollutants. In fact it is assumed that the membrane permeability decreases during early embryogenesis. In embryolarval tests with copper (Blaxter, 1977) teratogenicity only showed up when the experiments were started with eggs before water hardening. With toluene (Stoss and Haines, 1979), nickel (Blaylock and Frank, 1979) and under acid stress conditions (Lee and Gerking, 1980) a slight temporal increase in the susceptibility of eggs during early embryonic development was demonstrated. For maneb, pentachlorophenol, cadmium (Table I) and dibromochloromethane (Mattice et al., 1981) water hardening did not appear to create

great differences in toxicity. Although the partition of ^{14}C-activity between the chorion, perivitelline fluid and embryo was not studied during early embryogenesis, the results of the accumulation experiments did not show an increased rate of uptake during the first hours after fertilization.

Till (1978) reported that water hardening had no effect on ^{238}Pu uptake by embryos. Pu was uniformly distributed throughout the perivitelline fluid, embryo and yolk. Lead (Holcombe, 1976) was shown to penetrate the chorion only slightly and hardly to accumulate in embryos although the eggs were exposed before the termination of water hardening. In experiments with cobalt, Kunze et al. (1978) showed that ^{57}Co accumulated rapidly during the first 24 h, but almost all the accumulated cobalt was reversibly bound by mucopolysaccharide in the chorion. Preferential accumulation in the chorion was also found for cadmium (Von Westernhagen and Dethlefsen, 1975; Michibata, 1981) and zinc (Wedemeyer, 1968). Tetrachlorobiphenyl (Guiney et al., 1980), cadmium (Michibata, 1981), zinc and iron (Wedemeyer, 1968; Zeitoun et al., 1976) were shown to accumulate in embryos after water hardening. Therefore the chorion appears to 'protect' the embryo from the uptake of toxicants, not by completely preventing but by slowing down the intrusion. Differences in the susceptibility of early embryos must be ascribed to the ontogenetic stage at which experimental exposure is started, rather than to differences in chorion permeability. A distinction, however, should be made between the penetration of heavy metal ions and lipophilic compounds. The intrusion of heavy metal ions might be seriously hindered by complexation with SH-groups, which are abundantly present in the egg membrane (Blaxter, 1969). Most lipophilic compounds can easily penetrate membranes.

The early fry stage appeared to be the most susceptible stage for all compounds studied. This same conclusion can be drawn from a series of acute toxicity experiments with zinc sulphate (Skidmore, 1965), ammonia (Calamari et al., 1981), paradichlorobenzene (Galassi et al., 1982), nonyl phenol (Marchetti, 1965), fenitrothion (Klaverkamp et al., 1977) and toluene and naphtalene (Korn and Rice, 1981). Yet, on the basis of the dieldrin residue (Table II), the sac fry are expected to represent the most sensitive stage. This difference between sensitivity to toxic effects and total accumulation can be explained by the specific accumulation of dieldrin in the yolk tissue, with the yolk seen as acting like a passively functioning storage tissue, i.e. a fat and protein depot (Fig. 3). Aromatic hydrocarbons (Korn and Rice, 1981) and tetrachlorobiphenyl (Guiney et al., 1980) are also known to accumulate primarily in the yolk. Proteins, perhaps metallothionein in the yolk might play a crucial role in reducing free levels of heavy metals (Pierson, 1981).

It can be speculated that apart from the yolk acting as a temporary ('toxicant sink'), metabolic modifications also play an important role as to the differences in susceptibility of the ELS. This can be derived from the concentrations of dieldrin in the early fry and the larval body of the sac fry. Based on a 96-h LC_{50} for early fry of 3.1 μg/l and a BCF of 1700 (Fig. 2), a critical tissue concentration of approximately 5 mg/kg can be calculated. The concentration of dieldrin in the larval body of sac fry can be estimated by using the aqueous solubility of dieldrin (90 μg/l; Butyn and Koeman, 1977), the 96-h

BCF (Fig. 2) and the partition coefficient of dieldrin between yolk and larval body (Fig. 4). Thus, a value exceeding 36 mg/kg is obtained, but mortality was not observed. This can be explained by the development of liver enzymes which may alter the rate of biotransformation of xenobiotic compounds, resulting in either activation or deactivation (Van Genderen, 1980).

Biotransformation of dieldrin by fish is known. In a study of Sudershan and Khan (1981) with bluegill (*Lepomis macrochirus*), 8% pentachloroketone, 8% aldrin-trans-diol and 74% dieldrin respectively, were recovered from the 94% accumulated radioactivity together with other metabolites. Biotransformation of dieldrin, which results in decreased lipophilicity, may account for the fact that no constant equilibrium was found in our accumulation experiments with early fry. It may also account for the relatively high sensitivity of early fry as evidence has been presented that aldrin-trans-diol rather than dieldrin itself, is the neurotoxic compound (Akkermans et al., 1974). Bioactivation also seems to be applicable to parathion, maneb and TeCB (De Bruin, 1976), but possibly does not hold for PCP and cadmium. Until now, hardly any attention has been paid to biotransformation processes in E.L.S. of fish and therefore more research is needed before clear conclusions can be drawn.

In addition to biotransformation of toxicants, differences in body metabolism can be expected to be of paramount importance. Changes in the amount of energy required, depend upon body activity which is parallelled by corresponding changes in the rate of respiration. Hamelink and Spacie (1977) point out that fish must extract large volumes of water each day to meet their gas exchange needs. In early fry, as compared to sac fry, gill development is at an advanced stage. This is shown by the different uptake rate constants for [^{14}C]dieldrin. A change in metabolism can be expected both in consequence of alterations in activity, as the fry turn from the non-free-living to the free-living stage and differences in metabolic pathways, as the fry turn from an internal to an external food source, with a concomitant change in the pattern of enzyme activities.

In embryolarval toxicity studies, ELS are exposed during the entire period of embryonic development. This is of great importance as accumulation of toxicants followed by metabolism of triglycerides and proteins during the process of yolk resorption (Guiney et al., 1980; Zeitoun et al., 1977; Fig. 4) increases the concentration and possibly also the accessibility of pollutants and therefore toxicity. The highest residues of dieldrin (Fig. 3), methyl mercury (McKim et al., 1976), tetrachlorobiphenyl (Guiney et al., 1980), lead (Holcombe et al., 1976), paradichlorobenzene (Galassi et al., 1982), toluene, naphtalene, 2-methylnaphtalene (Korn and Rice, 1981), benzo(*a*)pyrene (Hannah et al., 1982) and ammonia (Calamari et al., 1981) have been detected at the end of the sac fry stage. This yolk absorption process is believed to be a major factor in the redistribution of chemicals causing death among juveniles of rainbow trout in tests with tri-*n*-butyltinchloride (Seinen et al., 1981) and may also explain the 'delayed toxicity' of TCDD to pike (*Esox lucius*) after short-term exposure during the egg stage (Helder, 1980).

In conclusion it may be stated that in view of the lack of fish toxicity data, these and other reported results show that short-term exposure of early fry is a practical approxi-

mation in establishing toxicant concentrations acceptable for fish. For compounds having a high bioaccumulative potential the exposure period should also include the sac fry stage. When teratogenesis has to be studied, the exposure period should include the early embryonic stages as well.

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Chapter 7

Aquatic toxicological aspects of dithiocarbamates and related compounds. Embryolarval studies with rainbow trout (Salmo gairdneri)

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Abstract

Early life stages of *Salmo gairdneri*, from the fertilized egg to the early fry stage, were continuously exposed to various dialkyldithiocarbamates (DDCs), ethylenebisdithiocarbamates (BDCs) and a number of degradation products. Both DDCs and BDCs induced embryotoxicity, but DDCs were generally found to be more toxic than BDCs. For some DDCs adverse effects, below ppb-level were observed. All compounds, including carbon disulfide, were shown to have teratogenic properties. The most striking effects were severe spinal and vertebral abnormalities, mostly associated with retarded yolk sac resorption. These dysmorphogenetic effects compare favourably with those observed in amphibians, birds and mammals. Quantitative structure-activity relationships (QSARs) revealed that toxicity could be described by a sigmoid function of the *n*-octanol/water partition coefficient. Chronic toxicity for *Daphnia magna* and embryolarval toxicity for *S. gairdneri* were significantly correlated ($r=0.93$; $\alpha < 0.01$). The same held true for embryotoxicity in White Leghorn chicken eggs and eggs of *S. gairdneri* ($r=0.97$; $\alpha < 0.01$). As tentative water quality standards, concentrations of 1.0 and 0.1 μg/l for BDCs and DDCs, respectively, are proposed.

Chapter 7

Aquatic toxicological aspects of dithiocarbamates and related compounds. Embryolarval studies with rainbow trout (Salmo gairdneri)

Introduction

Dithiocarbamates (DCs) are widely used in industry, agriculture and medicine (Thorn and Ludwig, 1962). Their chemistry is very complicated as they are subject to manifold degradation, which begins in production and continues during storage and application. Nevertheless, the only official analytical method both for ethylenebisdithiocarbamates (BDCs) and dialkyldithiocarbamates (DDCs) is nonspecific and based on the colorimetric determination of a yellow complex formed by reaction of evolved CS_2 with a reagent after acid hydrolysis of the sample (Horowitz, 1975).

Until recently DCs were regarded as relatively harmless, given their generally low mammalian toxicity and their biodegradability; besides, they have been used continuously for over 40 years. However, the temporary ADIs (acceptable daily intakes) for man have been lowered on the basis of reported teratogenic and genotoxic effects, as well as their capability for being nitrosated *in vitro* and *in vivo* (Vettorazzi, 1979). So far the embryotoxic and teratogenic potential of some fungicide preparations, both pure compounds and several degradation products, have been investigated in tests with amphibians, birds and mammals (Fishbein, 1977 and Tabacova et al., 1978), but not with fish. Therefore, following previous studies on the effects of DCs and related compounds on aquatic life (cf. Van Leeuwen et al., 1985a,b), the present study reports on their effects on the embryolarval development of rainbow trout (*Salmo gairdneri*). The histopathological and mechanistic aspects of teratogenicity will be dealt with in a following paper.

Materials and methods

Test compounds

The chemicals used are listed in Table I. Structural formulas of some are given in Fig. 1.

Toxicity tests

The experiments with *S. gairdneri* were carried out in a constant temperature room at

TABLE I. Test compounds.

No	Compound	Abbreviation	Supplier[a]	Purity (≥%)
1	disodium ethylenebisdithiocarbamate	nabam	Lamers & Indemans	99
2	manganese ethylenebisdithiocarbamate	maneb	ICN	90
3	zinc ethylenebisdithiocarbamate	zineb	ICN	95
4	polymeric ethylenethiuramdisulfide	PETD	Pennwalt	96
5	5,6-dihydro-3H-imidazo(2,1-c)-1,2,4-dithiazole-3-thion	DIDT	TNO	98
6	ethylenethiourea	ETU	EGA	99
7	ethyleneurea	EU	EGA	97
8	sodium dimethyldithiocarbamate	NaDMDC	Fluka	97
9	zinc dimethyldithiocarbamate	ZnDMDC	ICN	95
10	ferric dimethyldithiocarbamate	ferbam	ICN	95
11	tetramethylthiuramdisulfide	thiram	ICN	98
12	tetramethylthiurammonosulfide	TMTM	ICN	95
13	tetramethylthiourea	TMTU	Riedel de Haen	97
14	tetramethylurea	TMU	Riedel de Haen	98
15	tetraethylthiuramdisulfide	disulfiram	Fluka	97
16	tetra-*n*-propylthiuramdisulfide	T(*n*)PTD	TNO	95
17	tetra-isopropylthiuramdisulfide	T(i)PTD	OCS	98
18	carbon disulfide	CS_2	Baker	99

[a] The chemicals were obtained from Lamers & Indemans B.V. ('s-Hertogenbosch, The Netherlands), Organic Chemicals Service (Vlissingen, The Netherlands), Baker Chemicals B.V. (Deventer, The Netherlands), Pennwalt Holland B.V. (Rotterdam, The Netherland), TNO, Institute for Applied Chemistry (Utrecht, The Netherlands), Fluka AG (Buchs, Switzerland), EGA (Steinheim, F.R.G.), Riedel de Haen (Hannover, F.R.G.) and ICN Pharmaceuticals Inc. (Montreal, Canada).

10 ± 1°C. The tests were conducted in 15 l all-glass tanks to which 10 l test solution were added. The test medium used was reconstituted water with a hardness of 50 mg/l (as $CaCO_3$) and a pH of 7.7 ± 0.2, and was prepared according to Alabaster and Abram (1965). Stock solutions were prepared in this water. In several instances acetone or dimethylsulphoxide were used as solvents for the test compounds. The concentration of these carriers never exceeded 100 μg/l. The test solutions were renewed three times a week and continuously aerated. To minimize compound decomposition, the stock solutions were prepared fresh at each renewal. The pH of the test solutions was measured at regular intervals; the actual concentrations of the test compounds were not verified during the experiments.

The embryolarval tests with trout were conducted in duplicate, and initiated with freshly, artificially spawned eggs obtained from a fish hatchery at Vaassen (Gelderland). Within 3 h after fertilization, egg samples (size 100) were introduced to the aquaria. Eggs were placed in a Petri dish (diameter 25 cm) on the bottom of the tanks. The embryolarval stages were exposed continuously for 60 days to 5-7 toxicant concentrations, a solvent control and a blank control. During embryogenesis the rooms were kept as dark as possible. After hatching of eggs, a photoperiod of 12 h light, 12 h dark, was im-

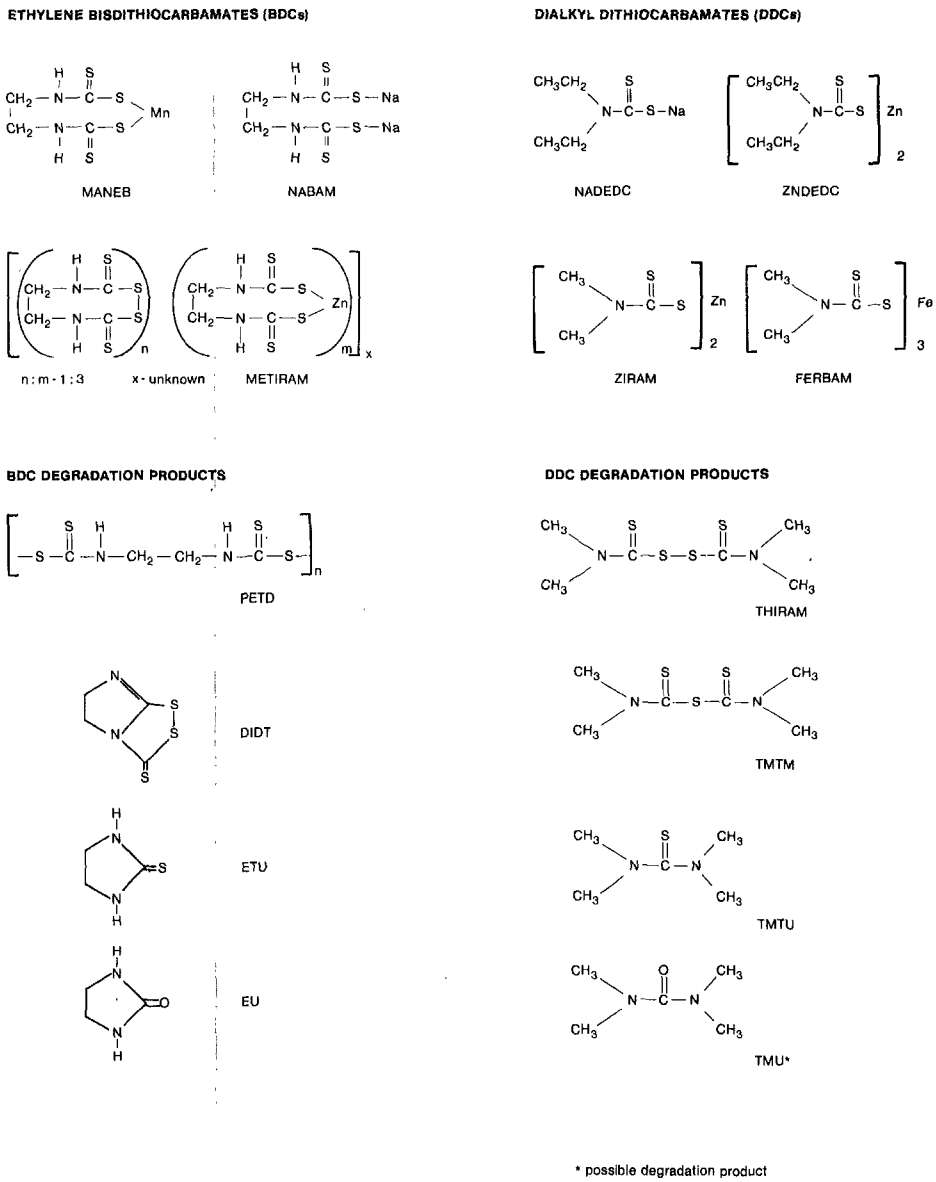

Fig. 1. Structural formulas of dithiocarbamates and several degradation products.

posed. The tanks were regularly inspected for dead specimens, which were removed; unfertilized eggs were removed from the tanks after 28 days. The animals were not fed during the tests. The experiments were terminated after 60 days. Surviving fish were anesthetized in buffered tricaine methane sulphonate (MS 222, Sandoz, Basel), and malformed and macroscopically normal fish were separated. A few juveniles per concentration (5-10) were removed for histopathological examination. The determination

of wet weight (all fish) and length (macroscopically normal fish) was performed only at the end of the tests.

In order to detect teratogenic properties of CS_2, short-term toxicity tests were performed in 1 l glass-stoppered erlenmeyers. Embryolarval stages of trout, of respectively 0, 7, 10 and 14 days old, reared at 10°C, were exposed to 100 mg CS_2/l from 0.25, 0.5, 1, 2 to 48 h. Afterwards, the eggs were transferred to reconstituted water. The experiments were terminated after hatching of eggs.

Calculations and statistics

Unfertilized eggs were excluded from the calculations. Survival, length, weight and teratogenicity data from both series were combined before conducting statistical analyses. The LC_{50} and 95% confidence limits were determined according to Kooyman (1981). The same procedure was applied to the calculation of the EC_{50}, after combining frequencies of embryonic mortality and abnormal development observed in juveniles. Differences in mean survival and normal development of the experimental concentrations were tested against the blank control by means of a χ^2 test (Sokal and Rohlf, 1981). Differences in mean length and weight between treatments and control were tested using the procedures described by Williams (1971, 1972), after verifying the differences between blank and solvent control (Student's *t*-test; Sokal and Rohlf, 1981). Concentrations affecting survival with more than 50% were excluded from the statistical analyses as size-selective mortality might occur. Prior to applying the Williams' test, the data were tested for homogeneity of variances using the Bartlett test (Sokal and Rohlf, 1981). Normality was verified graphically, by plotting the ordered data (x_i against the normal order statistic ξ (i/n) (Pearson and Hartley, 1972). The lowest concentration, which was significantly different from the control, was denoted as LRCT (lowest rejected concentration tested) according to Skalski (1981). Differences were considered significant at $\alpha < 0.01$. Relationships between toxicity, and the partition coefficient between *n*-octanol and water (P_{oct}), were calculated with a computer program based on the method of least squares.

Results

Early life stage toxicity tests.

The early life stage toxicity tests with *S. gairdneri* were conducted over a period of four years. The results are summarized in Tables II and III. Examples of more detailed actual test responses are shown in Tables IV and V. Both DDCs and BDCs displayed high embryotoxicity. DDCs were generally more toxic than BDCs; adverse effects were observed below ppb-level (Table IV). The degradation products PETD and DIDT showed higher toxicities than their parent compounds. Ethyleneurea proved nontoxic. In a 14-day static-renewal test with trout fry, lethality was observed at levels above 3.2

TABLE II. Results of 60-day early life stage toxicity tests with *S. gairdneri* exposed to DDCs and related compounds.

Compound	LC_{50} and 95% C.L. (μg/l)		EC_{50} and 95% C.L.[a] (μg/l)		LRCT (μg/l)[b]			
					mortality	total embryotoxicity[a]	length	weight
NaDMDC	6.4	(5.6-8.0)	1.0	(0.8-1.2)	1.4	$\leqslant$0.8	$\leqslant$0.8	1.4
Ziram	2.0	(1.8-2.1)	1.5	(1.4-1.5)	1.8	1.8	1.8	1.8
Ferbam	2.9	(2.7-3.2)	1.5	(1.3-1.6)	1.8	1.0	1.8	1.8
Thiram	1.1	(1.1-1.2)	0.64	(0.57-0.73)	1.0	$\leqslant$0.32	$\leqslant$0.32	0.56
TMTM	38	(33-43)	17	(14-20)	32	10	10	32
TMTU	8.8×10^4	($7.9-9.8\times10^4$)	7.2×10^4	($6.6-7.9\times10^4$)	1.0×10^5	1.0×10^5	1.0×10^5	1.0×10^5
TMU	3.0×10^5	($2.9-3.1\times10^5$)	2.4×10^5	($1.9-3.1\times10^5$)	3.2×10^4	3.2×10^4	3.2×10^5	3.2×10^5
Disulfiram	9.0	(8.0-10)	6.2	(5.8-6.8)	5.6	5.6	1.8	3.2
T(*n*)PTD	165	(132-206)	91	(79-105)	320	32	32	10
T(i)PTD	17	(15-18)	11	(10-12)	10	10	3.2	10

[a] Mortality and teratogenicity.
[b] Lowest rejected concentration tested ($\alpha<0.01$).

TABLE III. Results of 60-day early life stage toxicity tests with *S. gairdneri* exposed to BDCs and related compounds.

Compound	LC_{50} and 95% C.L. (μg/l)		EC_{50} and 95% C.L.[a] (μg/l)		LRCT (μg/l)[b]			
					mortality	total embryotoxicity[a]	length	weight
Nabam	2.1×10^3	($2.0-2.3\times10^3$)	1.8×10^3	(1.0-3.2)	3200	320	180	$\leqslant$100
Maneb	165	(150-181)	148	(136-162)	100	32	$\leqslant$18	$\leqslant$18
Zineb	211	(200-222)	188	(179-199)	180	100	$\leqslant$32	$\leqslant$32
PETD	24	(19-29)	11.7	(8.8-15.2)	10	10	32	320
DIDT	14	(12-16)	7.0	(5.7-8.6)	3.2	3.2	10	$\leqslant$0.32
ETU	1.8×10^6	($1.0-3.2\times10^6$)	1.0×10^6	($0.6-3.2\times10^6$)	3.2×10^6	1.0×10^5	1.0×10^5	3.2×10^6
EU[c]	1.0×10^7	($0.9-1.1\times10^7$)	–		–	–	–	–

[a] Mortality and teratogenicity.
[b] Lowest rejected concentration tested ($\alpha<0.01$).
[c] 14-day semistatic experiment with juveniles.

g/l. The LC_{50} was 10 g/l, and further testing was not considered to be relevant. The concentrations of the organic solvents used as carriers for some of the test compounds did not significantly alter the responses.

At 10°C the avarage hatching time for eggs of rainbow trout was 33 days. The survival pattern of the embryolarval stages was clearly related to exposure concentration

TABLE IV. Results of an embryolarval toxicity experiment with *S. gairdneri* and ferbam.

Concentration (μg/l)	Mortality (%)			Mortality and teratogenicity after 60 days (%)	Mean length and 95% C.L. (mm)	Main weight and 95% C.L. (mg)
	egg-stage	larval-juvenile stage	total after 60 days			
0	0.6	2.0	2.6	2.6	24.2 (24.0-24.4)	115.9 (112.2-119.6)
0.32	1.9	3.2	5.1	5.1	24.4 (24.2-24.6)	115.7 (112.2-119.2)
0.56	3.6	1.2	4.8	11.4	24.6 (24.4-24.8)	119.3 (115.8-122.8)
1.0	1.2	0	1.2	30.1[a]	24.1 (23.9-24.3)	112.5 (109.2-115.8)
1.8	20.2	9.2	29.4[a]	78.0	23.4[a](23.0-23.8)	97.6[a](93.9-101.3)
3.2	48.6	9.8	58.4	73.2	22.6 (22.1-23.1)	103.1 (96.9-109.3)

[a] Lowest rejected concentration tested ($\alpha < 0.01$).

TABLE V. Results of an embryolarval toxicity experiment with *S. gairdneri* and zineb.

Concentration (μg/l)	Mortality (%)			Mortality and teratogenicity after 60 days (%)	Mean length and 95% C.L. (mm)	Main weight and 95% C.L. (mg)
	egg-stage	larval-juvenile stage	total after 60 days			
0	2.8	0.6	3.4	4.0	24.4 (24.2-24.6)	131.9 (128.0-135.8)
32	5.7	1.7	7.4	8.0	23.5 (23.3-23.7)[a]	124.9 (121.3-128.5)[a]
56	6.4	0.7	7.1	10.7	22.8 (22.5-23.1)	118.5 (114.1-122.9)
100	5.7	0	5.7	20.0[a]	21.4 (21.0-21.8)	108.9 (104.8-113.0)
180	27.4	0.6	28.0[a]	44.1	21.8 (21.4-22.2)	119.1 (115.1-123.1)
320	94.5	0	94.5	98.0	– – –	– – –

[a] Lowest rejected concentration tested ($\alpha < 0.01$).

and time. All DCs and related compounds markedly affected embryogenesis. Mortality was highest during the egg stage, especially during late gastrulation (when somites began to form) and early organogenesis. For most compounds no appreciable mortality occurred at later stages of embryolarval development (Fig. 2), with the exception of the tests with TMTM, TMTU, TMU, T(i)PTD, maneb, PETD and DIDT (Fig. 3), which revealed substantial larval and juvenile mortality rates. Generally, the LC_{50} gradually declined with time, and the incipient LC_{50} was reached before hatching of the eggs (Fig. 4). Growth of early developmental stages of rainbow trout was not adversely affected by most of the compounds studied.

All DCs and related compounds were shown to have teratogenic properties. The most pronounced effects were severe spinal and vertebral abnormalities including lateral flexures (scoliosis), ventral curvatures (lordosis), dorsal curvatures (kyphosis) and irregular dwarfed structures of the trunk. The qualitative study of the teratogenic ef-

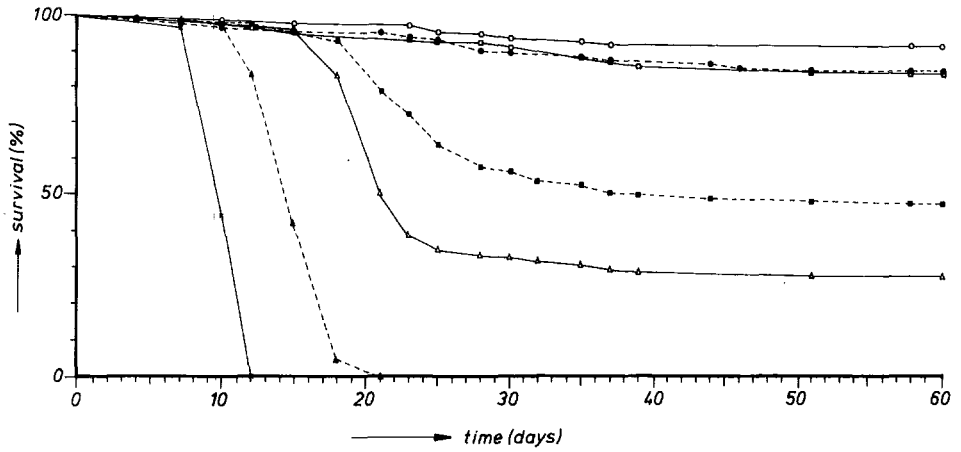

Fig. 2. Survival pattern of early life stages of *S. gairdneri* at various concentrations of thiram; ○,control; •,0.32 μg/l; □,0.56 μg/l; ■,1.0 μg/l; △,1.8 μg/l;▲,3.2 μg/l; and x,5.6 μg/l.

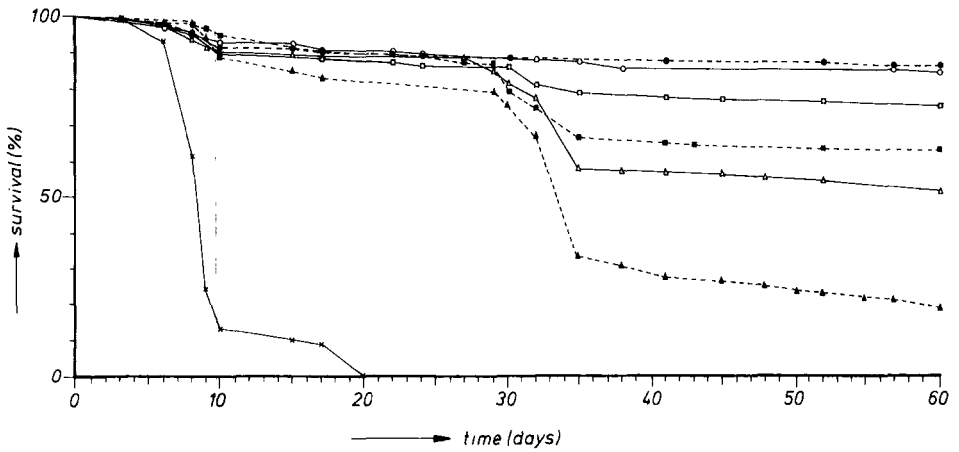

Fig. 3. Survival pattern of early life stages of S. *gairdneri* at various concentrations of DIDT; ○, control; •,0.32μg/l; □,1.0 μg/l;■,3.2 μg/l;Δ,10 μg/l;▲,32 μg/l; and x,100 μg/l.

fects of CS_2, revealed that only embryolethality was induced after short-term exposure of 3-h and 14-day-old developmental stages of *S. gairdneri*. Both mortality (3% and 5%) and skeletal deformations (13% and 21%) were observed after acute (7 h) exposure of 7-day and 10-day-old eggs, respectively. Mortality and teratogenic effects clearly increased with prolonged exposure. After a 24-h exposure period the proportion of malformed embryos was 33% and 32%, respectively. The graphically determined ET_{50} (the exposure time, which induced 50% effect, i.e. lethality and/or teratogenicity) for the four successive stages was 0.7, 14, 25 and 15 h, respectively.

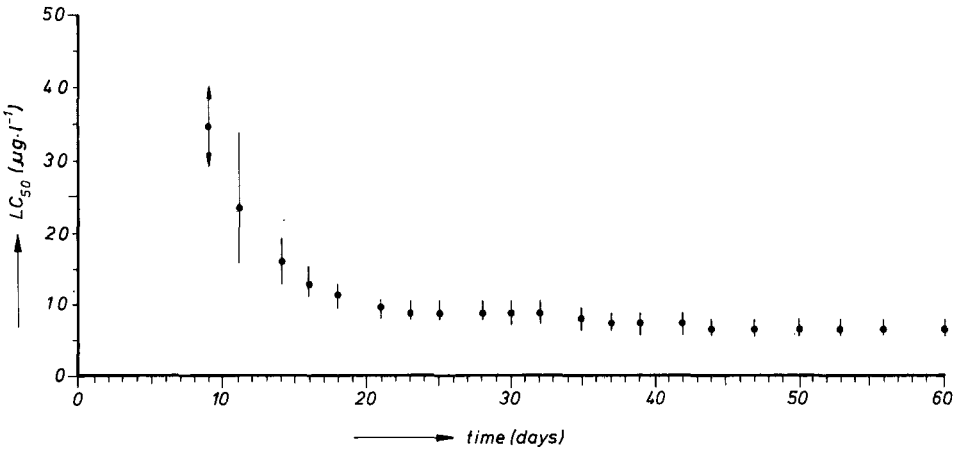

Fig. 4. Temporal changes in the LC_{50} for *S. gairdneri* exposed to NaDMDC.

Examples of congenital deformities are shown in Figs. 5-8. Severe skeletal anomalies were often associated with retarded yolk sac resorption, probably because normal movement was mechanically impossible or suppressed. Balance was clearly impaired and the fish were capable only of weak quivering motions, rested or tumbled about on the bottom of the aquaria. The frequency of malformations increased with exposure concentration, but decreased again at the highest concentrations tested, probably because these anomalies were too severe to survive (Tables IV and V). Locomotor behaviour of fish with normal morphology did not seem to be impaired.

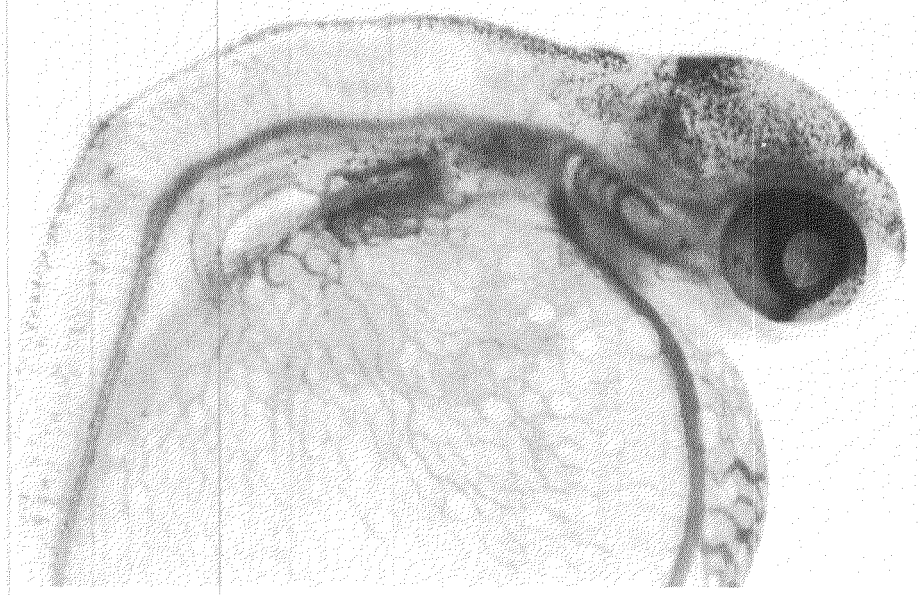

Fig. 5. Severely distorted and wavy notochord in an eleutheroembryo of *S. gairdneri* (30 days postfertilization) after treatment with maneb (320 μg/l).

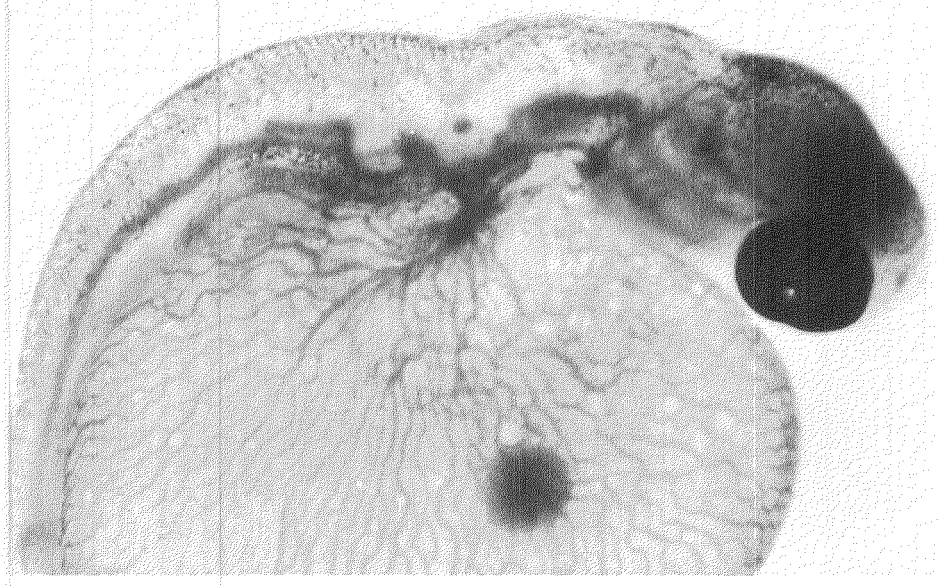

Fig. 6. NaDMDC-induced anomalous notochordal structure, distortion of the normal course of blood vessels, and hemorrhage in the yolk sac in a 30-day-old eleutheroembryo of *S. gairdneri*, resulting from treatment with 18 μg/l.

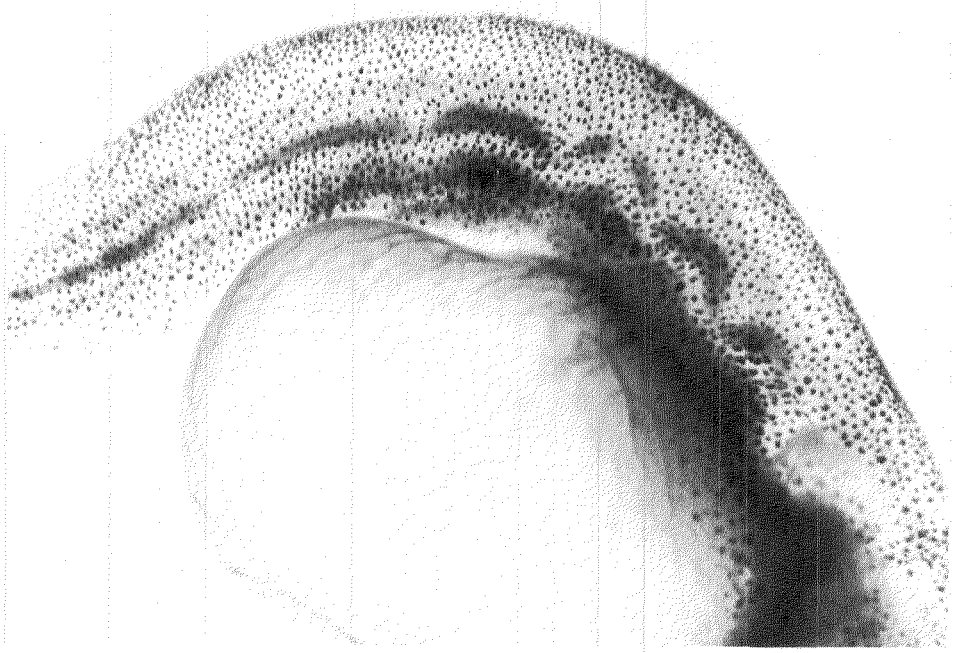

Fig. 7. Severe mechanical derangements in normal hemodynamics in a 46-day-old eleutheroembryo of *S. gairdneri* after treatment with thiram at a concentration of 1.0 μg/l.

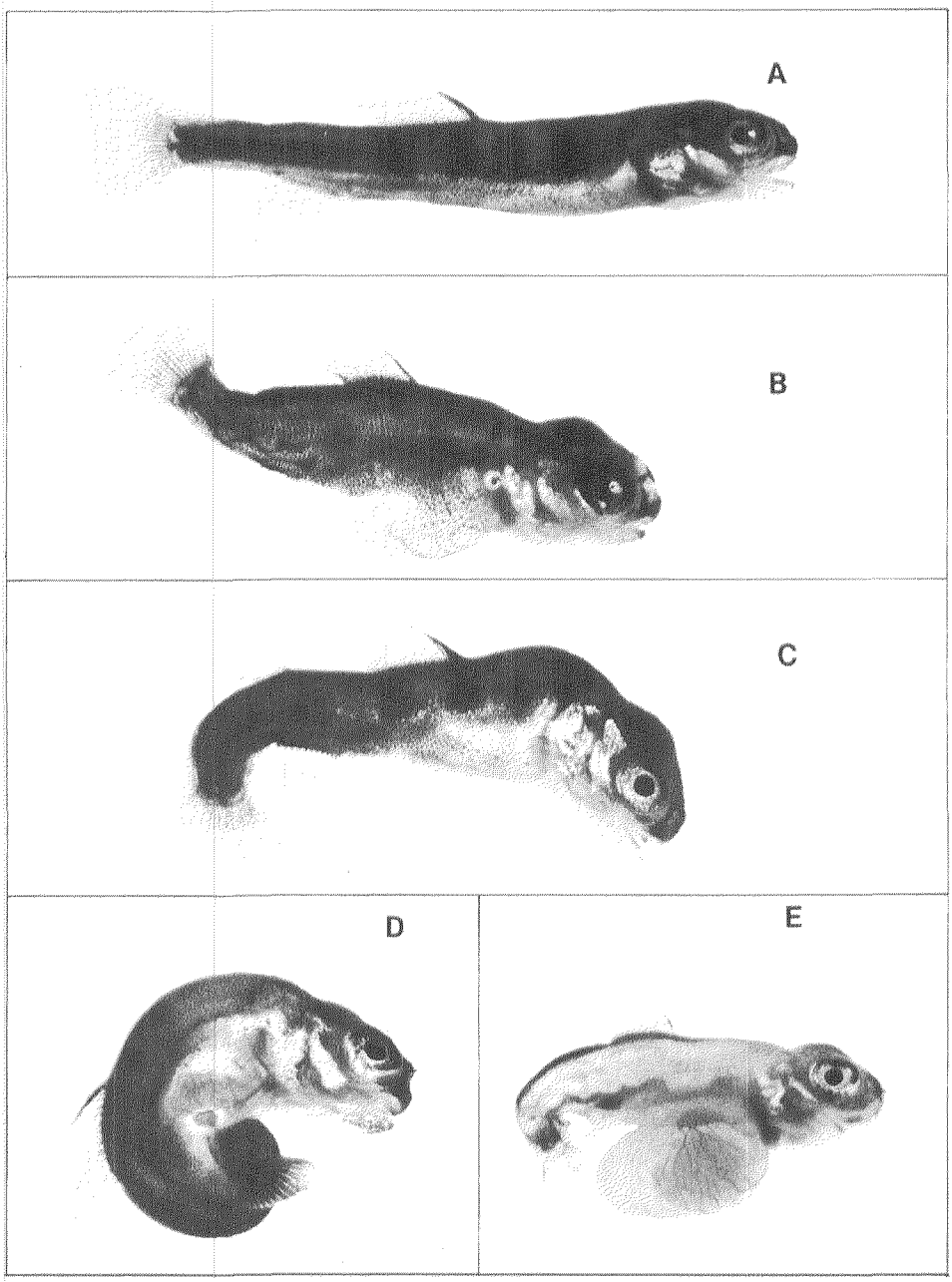

Fig. 8. Several examples of teratogenic effects in alevins of *S. gairdneri*, 27 days posthatching. (A) normal trout alevin (B) kyphosis of the vertebral column with irregular dwarfed trunk structure, reduced lower jaw, and retarded yolk sac resorption (NaDMDC 18 µg/l), (C) skeletal defects including a kyphotic caudal peduncle (PETD: 10 µg/l), (D) nabam-induced, inflexible curvature of the spinal column, resulting from treatment with 1.8 mg/l and (E) congenital defects including dwarfed kyphotic trunk, irregular and defective structure of notochord, operculum, caudal fin, and jaws. Yolk sac resorption and pigmentation of the skin are also severely retarded (DIDT: 32 µg/l).

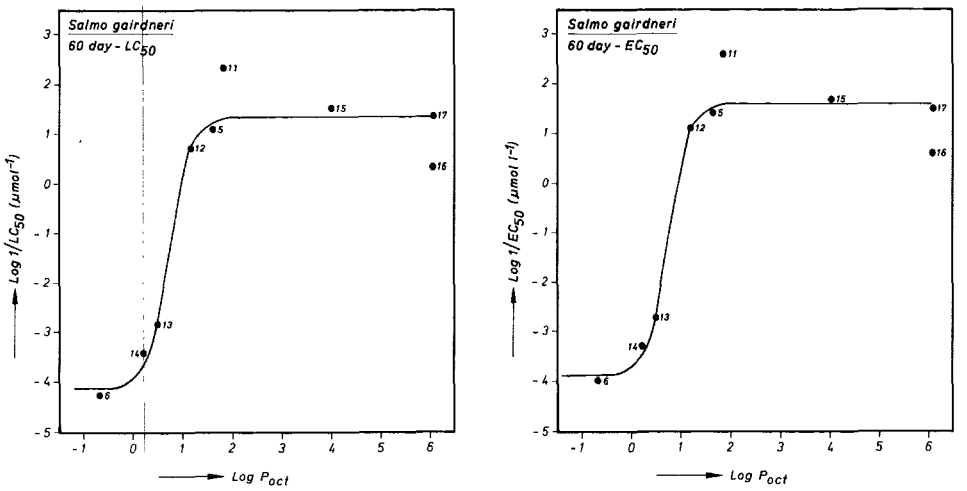

Fig. 9. QSARs for DCs and related compounds. Lines represent the expected values based on equations presented in Table VI. Numbers correspond to those of Table I.

QSAR studies

The log P_{oct} values of the compounds selected for the QSAR studies were drawn from Van Leeuwen et al. (1985b). Metal derivatives and polymeric ethylenethiuramdisulfide were excluded from the calculations. Both LC_{50} and EC_{50} values were used, but no calculations were done on the other toxicological criteria studied, as they are closely linked with these parameters. Toxicity could best be described by a sigmoid function of P_{oct} (Fig. 9), but a stepwise decrease in toxicity was observed with increasing lipophilicity in the high P_{oct} range, i.e., in the thiram, disulfiram and T(*n*)PTD sequence. Moreover, T(i)PTD was ten times more toxic than its *n*-propyl isomer, which accounted for the relatively poor quality of the QSARs (Table VI).

TABLE VI. QSARs for *S. gairdneri* and the compounds shown in Fig. 6[a].

QSAR equation[b] C (µmol/l)	$\log 1/C = ab\{a+(b-a)\exp(-k \log P_{oct})\}^{-1}+c$					
	a	*b*	*c*	*k*	*n*	*s*
60-day LC_{50}	0.189	5.46	-4.11	4.63	9	0.75
60-day EC_{50}	0.140	5.46	-3.88	5.00	9	0.73

[a] The P_{oct} values were drawn from Van Leeuwen et al. (1985[b]).
[b] n= sample size and s= SE of the estimate.

Comparison of toxicity data

The results of the embryolarval experiments with *S. gairdneri*, together with the test results on *Daphnia magna* (Van Leeuwen et al., 1985b) and chicken embryos

TABLE VII. Regression and correlation of *D. magna*, White Leghorn chicken embryos, and embryolarval toxicity of *S. gairdneri*.

Test organism	$\log(LC_{50}$ or $ED_{50}) = a \log(EC_{50}$ *S. gairdneri*$) + b$[a]				
	a	*b*	*n*	*r*	*s*
D. magna	1.13	-0.77	12	0.960	0.541
D. magna	1.18	-0.53	16	0.932	0.869
Chicken embryo	2.82	-3.09	5	0.967	0.854

[a] n = sample size, r = correlation coefficient and s = SE of the estimate.

(Korhonen et al., 1983), allow a comparison of toxicity. The data are presented in Table VII. The 21-day LC_{50} and 60-day EC_{50} values for *D. magna* and *S. gairdneri* respectively, were strongly correlated. Nabam, T(*n*)PTD and ETU were outliers. Including these outliers and substituting for EU a 14-day LC_{50} instead of the 60-day EC_{50}, did not affect the correlation which remained fairly good ($r=0.93$) and highly significant ($\alpha < 0.01$). The embryotoxic effects of DCs and related compounds in rainbow trout and chicken also showed a fairly good correlation ($r=0.97$).

Discussion

It is becoming increasingly apparent that, with regard to toxic effects of environmental trace contaminants, embryonic and early juvenile stages constitute the critical 'sensitive links' in the life cycles of fish (McKim, 1977; Van Leeuwen et al., 1985c). DCs were also found to be highly toxic to the ontogenetic stages of rainbow trout (Tables II and III). Embryotoxicity and teratogenicity were concentration-related and were found for all compounds studied. Generally, a wide concentration range in which there was an overlap between the responses for skeletal malformations and lethality was observed. The parallel concentration-response c.q. P_{oct}-L(E)C_{50} curves (Fig. 9 and Table VI) for embryolarval lethality and total embryotoxicity, suggest that embryos die because of severe dysmorphogenetic effects. The teratogenic effects in trout proved to be in agreement with those observed in mammals, birds and amphibians (Fishbein, 1977, Chernoff et al., 1979, Tabacova et al., 1978 and Korhonen et al., 1983). The results for DIDT (ethylenebisdiisothiocyanato sulfide) are conflicting. The absence of DIDT-induced teratogenicity in rats, however, may be explained by the relatively low dose at which maternal limb paralysis (the limiting factor in dosing) was observed (Chernoff et al., 1979). On the whole, growth appeared to be a poor indicator of sublethal stress. In many other early life stage toxicity tests, it was not shown to be a very sensitive property either (Woltering, 1984).

A comparison of the embryolarval tests with life-table experiments with *Daphnia magna* (Van Leeuwen et al., 1985b) shows that developmental stages of trout generally display higher levels of sensitivity to the toxic action of DCs and related compounds. The data are clearly correlated (Table VII). It may therefore be concluded that

reproduction tests with daphnids form an attractive alternative to long-term toxicity testing with trout. This is essentially the same conclusion as was drawn by Maki (1979).

The great difference in toxicity for nabam between *D. magna* and *S. gairdneri* may in part be explained by the catalytic effects of several trace metals on its conversion to DIDT (Ludwig and Thorn, 1958), as the tests with daphnids were carried out in lake water. Dimethyldithiocarbamic acid, being a 'weak' acid, would exist in the test solution in great part in the unionized lipophylic form (Thorn and Ludwig, 1962). In tests with *S. gairdneri* this compound was found to be highly toxic (Fig. 4).

The difference in toxicity between T(*n*)PTD and T(i)PTD (Table II) is striking. It may be explained by differences in the spatial arrangement of the alkyl groups, i.e. molecular size, which in the case of T(*n*)PTD, possibly hinders rapid penetration into cells. A comparable difference in fungitoxicity between sodium di-*n*-propyldithiocarbamate and sodium diisopropyldithiocarbamate has been demonstrated (cf. Owens, 1969), and may also be explained by differences in accessibility.

More research needs to be done into the usefulness of embryolarval experiments with fish, before any definitive conclusions can be drawn as to its applicability as a teratogenicity screening test. The parallelism with the results of chicken embryo tests was striking (Table VII). This may be explained, apart from similarities in ontogenesis, by the fact that in both tests, chemicals act directly upon the embryos without interference from maternal metabolism such as in mammals.

From these and foregoing studies, a first approximation of water quality standards may be derived. However, this is compounded by the fact that degradation products, and those of BDCs in particular (PETD and DIDT), exhibited higher levels of toxicity than the parent compounds. The formation of both PETD, DIDT and ETU in water has been reported on (Kaars Sypesteyn et al., 1977), but more insight into the routes and rates of degradation in aquatic environments is needed, before firm conclusions can be drawn. Moreover, it should be pointed out that in this and previous studies done at this laboratory, only gross aquatic toxicological parameters have been examined. With these limitations in mind, it is tentatively proposed that concentrations of BDCs and DDCs in surface water should never exceed 1.0 and 0.1 μg/l, respectively.

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Chapter 8

Aquatic toxicological aspects of dithiocarbamates and related compounds. Teratogenicity and histopathology in rainbow trout (Salmo gairdneri)

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Abstract

Exposure of rainbow trout (*Salmo gairdneri*) during embryolarval development revealed that dithiocarbamates (DCs) and various related compounds are potent teratogens. Malformations were almost exclusively confined to the notochord, which increased considerably both in length and diameter. As a result, the normal rod-like appearance of the notochord was completely disturbed: it was twisted and distorted in all possible directions. Ectopic osteogenesis, was observed in almost every affected notochord. Other effects, such as the disruption of the integrity of myomeres and myosepta and organ dislocations, were closely related to the notochordal anomalies. X-ray photographs of malformed fish reared to the alevin stage revealed compression and fusion of vertebrae and 'wavyness' of various skeletal elements.

Semichronic exposure of juvenile rainbow trout to various DCs induced a concentration-related decrease in hepatic glycogen storage. At high exposure levels proliferation of bile duct epithelial cells and necrosis of individual hepatocytes were observed. Ziram and thiram induced extensive hemorrhages in the brain as well as intraspinal extravasates of blood cells.

Cellular and molecular aspects of the morphological changes are discussed.

Chapter 8

Aquatic toxicological aspects of dithiocarbamates and related compounds. Teratogenicity and histopathology in rainbow trout (Salmo gairdneri)

Introduction

In the preceding study on embryolarval development of rainbow trout (*Salmo gairdneri*) it was shown that dithiocarbamates (DCs), both ethylenebisdithiocarbamates (BDCs) and dialkyldithiocarbamates (DDCs), are highly toxic. Embryogenesis was clearly affected and skeletal malformations were most conspicuous (Van Leeuwen et al., 1986a). Similar DC-induced congenital abnormalities have been observed in mammals (Fishbein, 1976; Larsson et al., 1976; Lee et al., 1978), in birds (Fishbein, 1976 and Korhonen et al., 1982a, 1983) and in amphibians (Fishbein, 1976; Ghate and Mulherkar, 1980; Ghate, 1983). Exoskeletal abnormalities in larvae of insects (*Culex fatigans*) and crustaceans (*Daphnia magna*) have also been reported (Gretillat, 1962; Van Leeuwen et al., 1985).

The teratogenic potential of DCs is not restricted to the parent compounds but has been demonstrated for a host of degradation products as well. Many of these studies also revealed the occurrence of defects in the central nervous system (hydrocephalus, encephalocele and exencephaly) as well as visceral and cardiovascular anomalies (Khera, 1973; Korhonen et al., 1982a,b; Teramoto et al., 1981; Von Kreybig et al., 1969; Van Leeuwen et al., 1986a). An interesting discovery was made by Tabacova et al. (1978), who showed that CS_2, a degradation product of both BDCs and DDCs, also impaired prenatal development in rats. The high incidence of malformations, mostly affecting brains and limbs, points to a great resemblance between the intoxication of CS_2 and DCs. In northern pike (*Esox lucius*) skeletal deformations have been induced also after hydrogen sulfide exposure (Adelman and Smith, 1976).

Apart from their teratogenic effects, DCs are known to disturb many other functions as well (Fishbein, 1976). The present report deals with their teratogenicity and histopathology.

Materials and methods

Test compounds

Structural formulas, abbreviations, suppliers and purity of the test compounds are given in Fig. 1 and Table I of the preceding study (Van Leeuwen et al., 1986a).

Embryolarval tests

Semistatic embryolarval toxicity experiments with rainbow trout (*S. gairdneri*) were carried out according to the methods reported in the previous study. After a 60-day exposure period most juveniles were anesthetized in buffered tricaine methane sulphonate (MS 222, Sandoz Basel). A few trout (5-10 per concentration) were fixed in Bouin Hollande (Romeis, 1976) for 24 h and stored in 70% ethanol. The fish were embedded in paraffin, and 5 μm-transverse sections taken serially at distances of 130 μm were stained with hematoxylin-phloxin-saffran (HPS). The histopathological studies were only conducted in animals exposed to maneb, thiram and disulfiram.

Remaining juveniles were transferred to reconstituted water in order to raise them for skeletal examination. The fry were fed on a commercial fish food (Trouvit, Trouw & Co. N.V., The Netherlands). After the fish had attained a length of approximately 7 cm, they were anesthetized and stored in 70% ethanol. X-ray photographs (voltage: 28 kV; exposure time: 1.3 sec.) were made with a Philips Mammadiagnost U at the Zuiderzee Hospital, Lelystad.

Toxicity tests with juveniles

Juvenile rainbow trout (average size approximately 5 cm) were exposed in a semistatic manner, to various concentrations of zineb, DIDT, ziram, thiram and disulfiram. The tests with zineb, ziram and disulfiram were carried out in municipal tap water (pH: 7.4 ± 0.1; hardness: 100 ± 5 mg/l as $CaCO_3$) and terminated after 30 days. At the end of the experimental period 15 whole fish (5 per concentration) were processed by routine histological techniques as described above. Serial transverse sections at a distance of 2 mm were taken and stained with hematoxylin and eosin. The compounds DIDT and thiram were tested in reconstituted water as described in the previous report. These tests were terminated after 21 days. The fish were anesthetized in MS 222 and subsequently dissected. Brain, heart, intestine, liver, kidney, muscle, thyroid and eyes were fixed in buffered formalin. After a 24-h fixation period, the tissues were processed in a series of alcohol-water dilutions, a mixture of alcohol and liquid plastic (Technovit 7100, Kulzer & Co. GmbH, Bad Homburg), and pure plastic with a hardener, respectively. The 1 μm-sections were stained with hematoxylin and eosin (HE).

Results

Embryolarval tests

DCs disturbed the normal development of rainbow trout embryos. Stunted growth and retardation of yolk resorption were also observed. Among the survivors a high incidence of notochordal abnormalities was observed. The notochords were abnormally

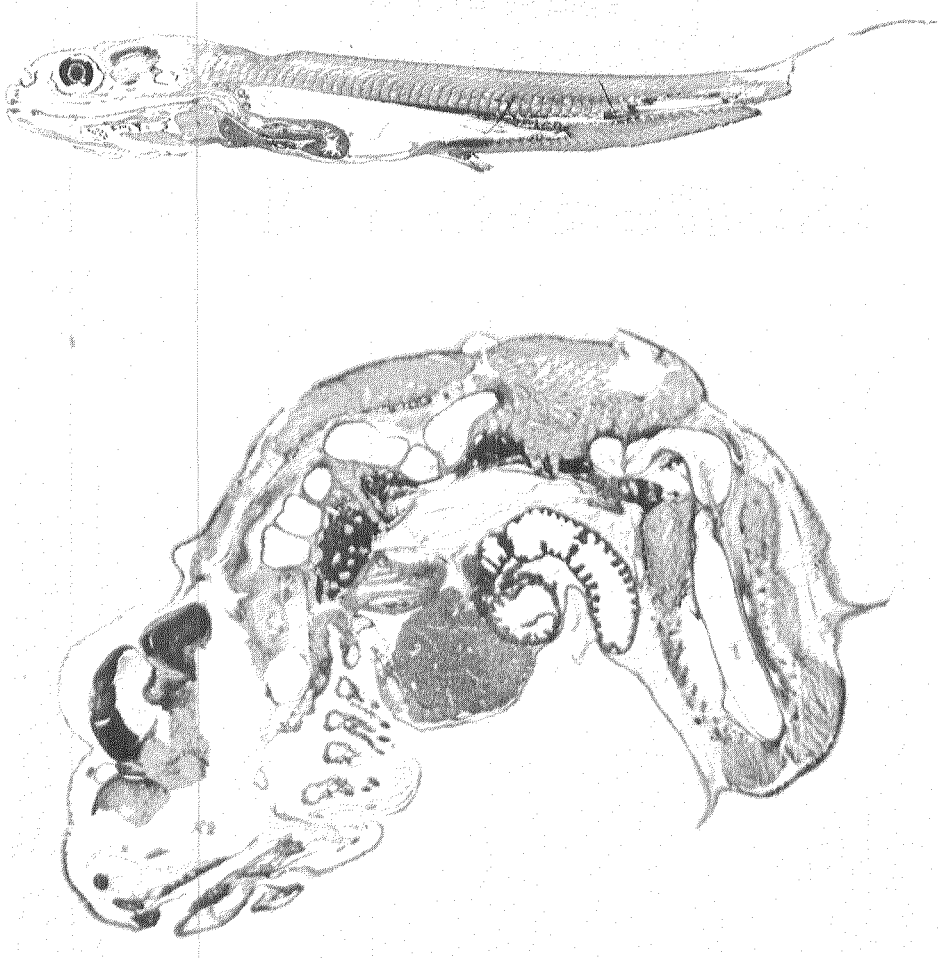

Fig. 1. Low power micrographs of sagittal sections of a control (top, HPS x 7.25) and a thiram-treated alevin of *S. gairdneri* after a 60-day embryolarval exposure period at a concentration of 0.56 µg/l. Note the extreme dilatation and torsion of the notochord (arrow), HPS x 15.

stretched, and had bends and kinks. In severe cases, one or more relatively large noduli of up to 2 mm in diameter were observed through the muscle and integumental layers.

Notochords of unexposed trout are circular in cross section and completely straight in sagittal section (Fig. 1). It is a cord-like structure of large cells virtually without nuclei. The notochordal cells are surrounded by the around cells (Hibiya, 1982). Further outward there is a fibrous secondary chorda sheath and a thin primary chorda sheath consisting of elastic fibres (the elastica externa). Here calcium salts are deposited and bone is formed. The entire chorda is surrounded by a layer of loose connective

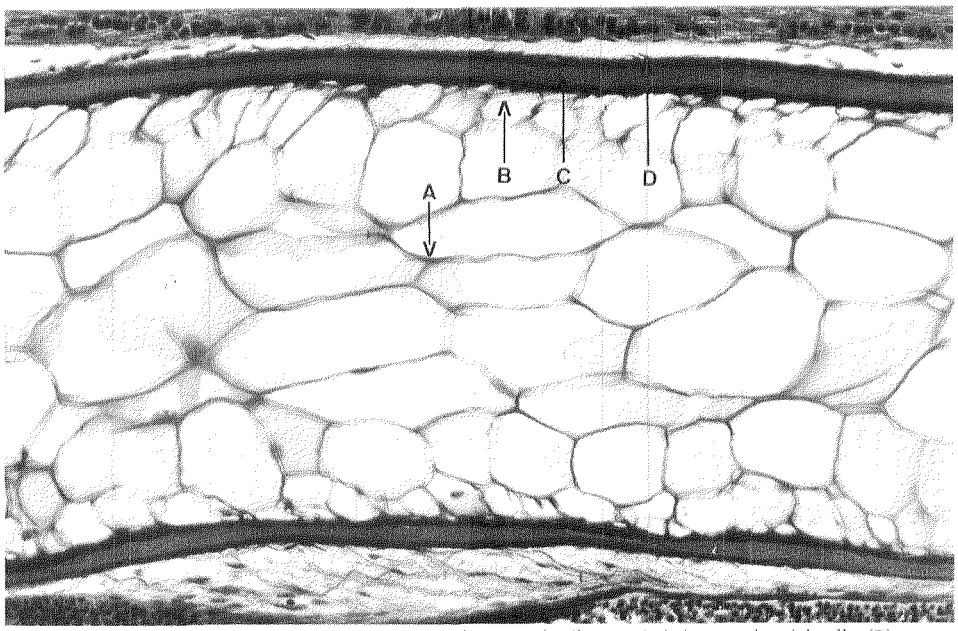

Fig. 2. Notochord of an alevin of *S. gairdneri* (60 days postfertilization). (A) Notochordal cells, (B) around cells, (C) secondary chorda sheath and (d) primary chorda sheath, HPS x 400.

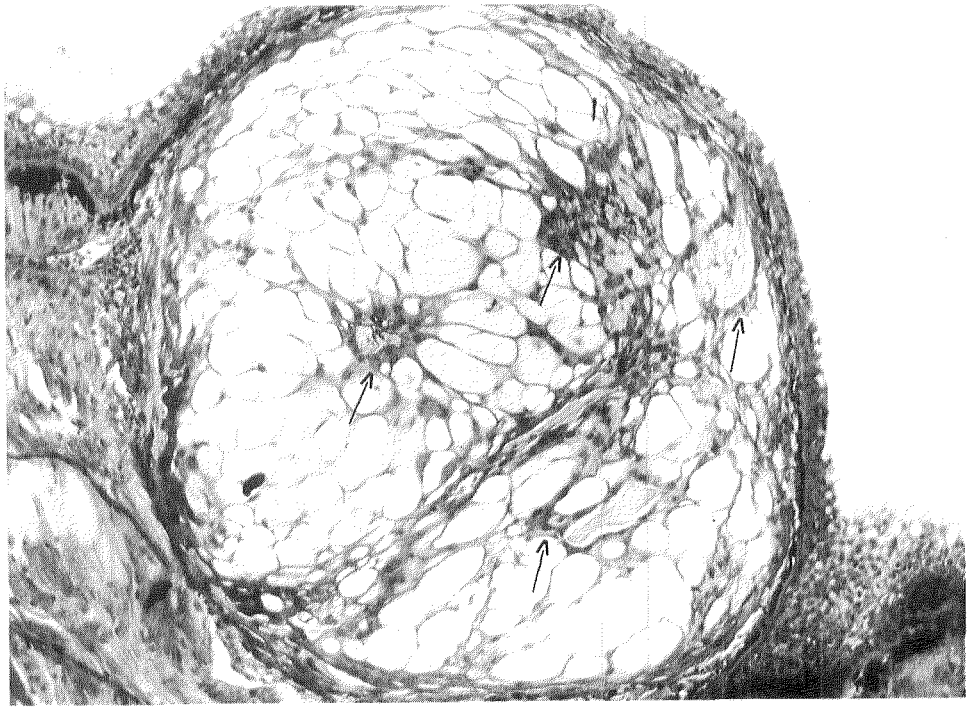

Fig. 3. Extreme dilatation of the notochord of *S. gairdneri*. The notochord is covered only by the integument. The fish were exposed during embryolarval development to maneb at a concentration of 80 μg/l. Note the irregular notochordal cells and the ectopic osteogenesis (arrows), HPS x 160.

tissue (Fig. 2). Sagittal sections of the exposed animals confirmed the notochordal anomalies. Both length and diameter had increased considerably. Hence, the normal rod-like shape of the notochord was severely disturbed. It was twisted and distorted in dorsal, ventral and lateral directions (Fig. 1). The number of notochordal cells had increased considerably. In between the notochordal cells nucleated cells, probably osteocytes, were frequently observed. In the vicinity of these cells irregular patches of newly formed bone were noticed (Fig. 3). This type of ectopic osteogenesis was observed in almost all affected notochords. Due to excessive dilatation and folding, the musculature was frequently dislocated, leaving the notochord covered only by the integument (Fig. 3).

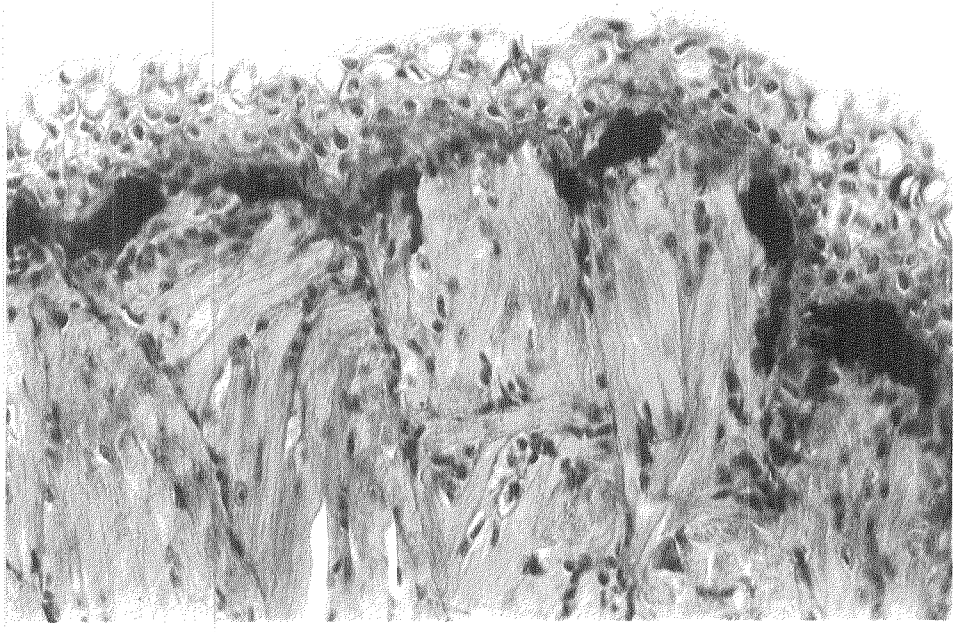

Fig. 4. Disorganization of muscle fibres in *S. gairdneri* after exposure to disulfiram (10 µg/l), HPS x 400.

In some cases, irregularities in the muscle tissue were observed. Whereas the muscle-fibers of a myomer normally run parallel, the test compounds induced a complete dissarray of fibers, apparently due to anomalous attachment of fibers to the collagenous myosepta (Fig. 4). The morphology of other organs and tissues was not affected, except that, apparently because of the extreme distortion of the notochord, some organs and tissues such as kidneys, muscles and neural tubes were severely dislocated.

Slightly malformed fish could be reared to the alevin stage in order to study the skeletal deformations, while badly deformed specimens, which were unable to swim

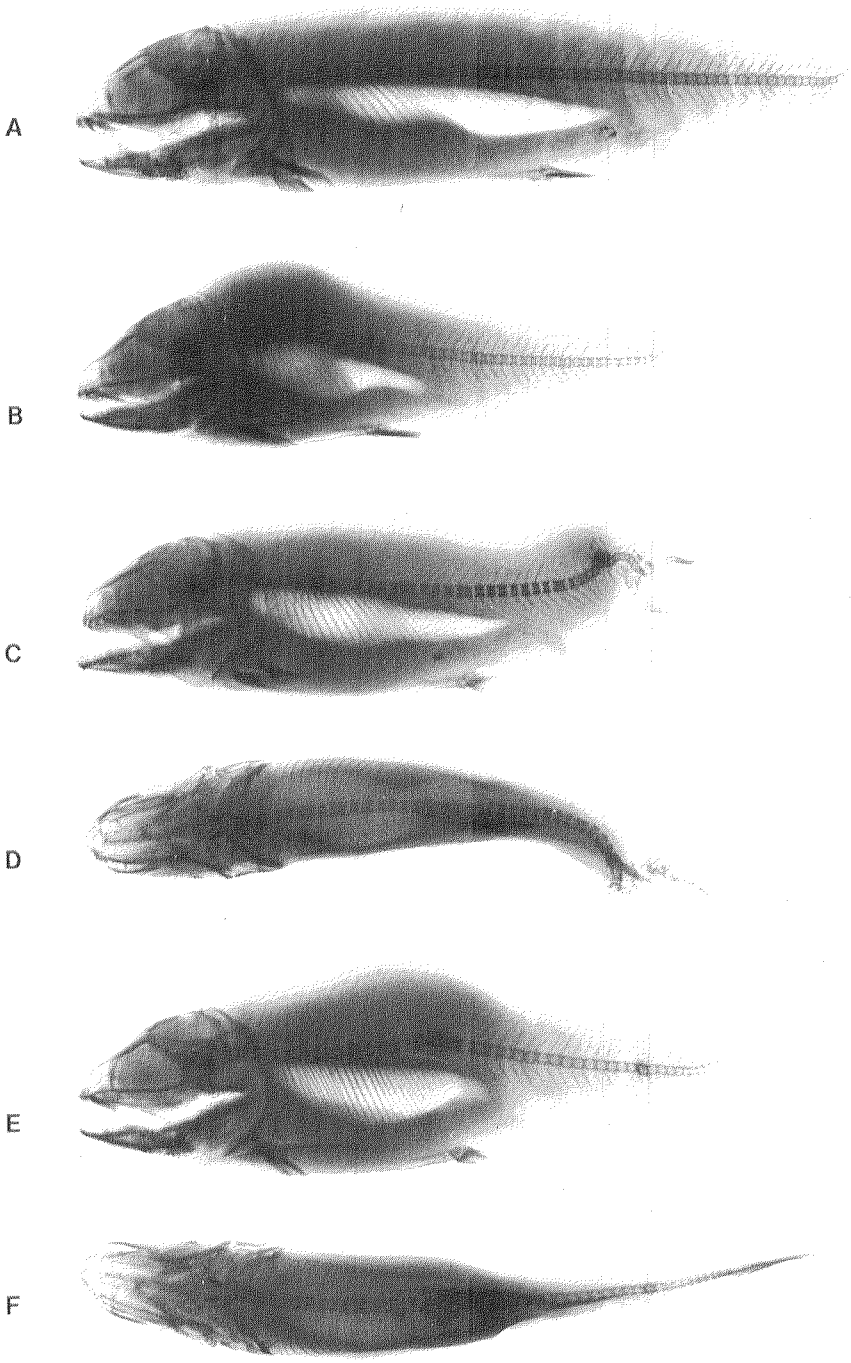

Fig. 5. X-ray photographs showing moderately malformed rainbow trout (*S. gairdneri*). After a 60-day embryolarval exposure period the fish were transferred to toxicant-free water. (A) control, (B) DIDT-3.2 μg/l, (C) ETU-1 g/l, (D) same fish dorsal view, (E) TMTM-10 μg/l and (F) same fish dorsal view. The actual size of the fish is 5-7 cm.

properly, eventually died of starvation. The results of the X-ray analysis are shown in Fig. 5. The deformities consisted of compressed, or fused, and dislocated vertebrae as well as 'wavy' ribs, spines and hemal arches. The fusion of vertebrae may account for the dwarfed trunk structure frequently observed in various trout.

Toxicity tests with juveniles

In the livers of normal *ad libitum* fed juvenile rainbow trout the hepatic cells are located among sinusoids forming cord-like and rosette-like structures. Within each double-rowed laminum there is a bile canaliculus. The hepatocytes contain fairly large quantities of glycogen in polygonal vacuoles, and lipids in much smaller droplets. Generally, in a liver section only one or two bile ducts with a single layer of low columnar epithelial cells can be seen (Fig. 6). All DCs tested induced a concentration-related loss of glycogen in the liver which was most prominent after exposure to ziram, thiram and DIDT. This was associated with reduced body weight gain. At concentrations of 5 μg/l ziram, 25 μg/l thiram and 50 μg/l DIDT, this depletion of glycogen was the only alteration found in the hepatic parenchymal cells. At higher concentrations, the loss of glycogen was complete, as a result of which the livers attained a very dense aspect. The nuclei had shifted to a basal position in the hepatocytes. At a concentration as high as 100 μg/l, thiram and DIDT induced a proliferation of bile duct epithelial cells, both with and without formation of new ductules. Occasionally, cell necrosis was observed, characterized by nuclear pycnosis and cytoplasmic eosinophilia of individual hepatocytes (Fig. 7). No histopathological changes were found in the thyroid follicles.

The DDCs ziram and thiram induced hemorrhages in the brains as well as in the spinal cords at a concentration of 5 μg/l. Large masses of erythrocytes were observed in the fourth and fifth ventricles and found diffusely within the nervous tissue, mainly of the cerebellum. Such extravasates were also found in the spinal cord, where they were located mainly in and around the central canal (Fig. 8). In the vicinity of these hemorrhages, occasionally foci of degenerating nerve cells were noticed. In juveniles exposed to DIDT, disulfiram and zineb at levels up to 100 μg/l, no hemorrhages were observed.

Discussion

The notochord seems to be the target organ in the DC-embryopathy in *S. gairdneri*. Dislocation of various organs and tissues is closely related with it. Similar DC-induced dysmorphogenetic effects have been observed in mammals, birds and amphibians (Fishbein, 1976; Korhonen et al., 1983; Ghate 1983).

Chelation of metal ions by DCs (Owens, 1969) might constitute the mechanism by which the teratologic effects are induced, as copper serves the catalytic function of the enzyme lysyl oxidase. This enzyme, a specific amine oxidase, catalyzes the oxidative deamination of peptidyl lysine and hydroxylysine residues in procollagen preparatory to the formation of covalent cross-links between the adjacent proteins (Opsahl et al., 1982). DCs have a pronounced affinity for copper ions (Thorn and Ludwig, 1962).

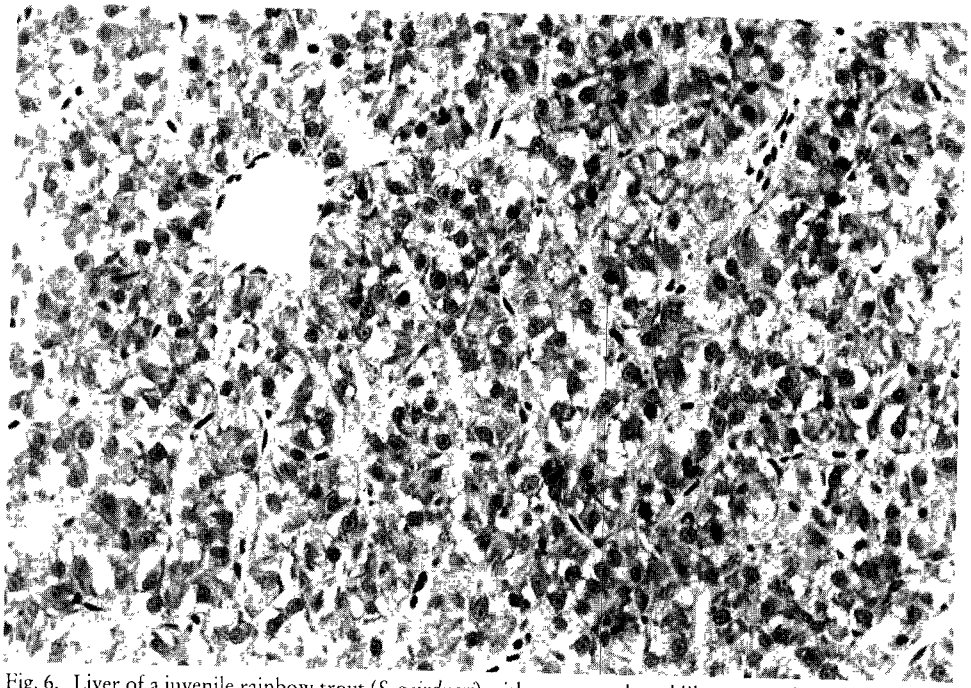

Fig. 6. Liver of a juvenile rainbow trout (*S. gairdneri*) with rosette and cord-like arrays of hepatocytes, loaded with glycogen, HE x 400.

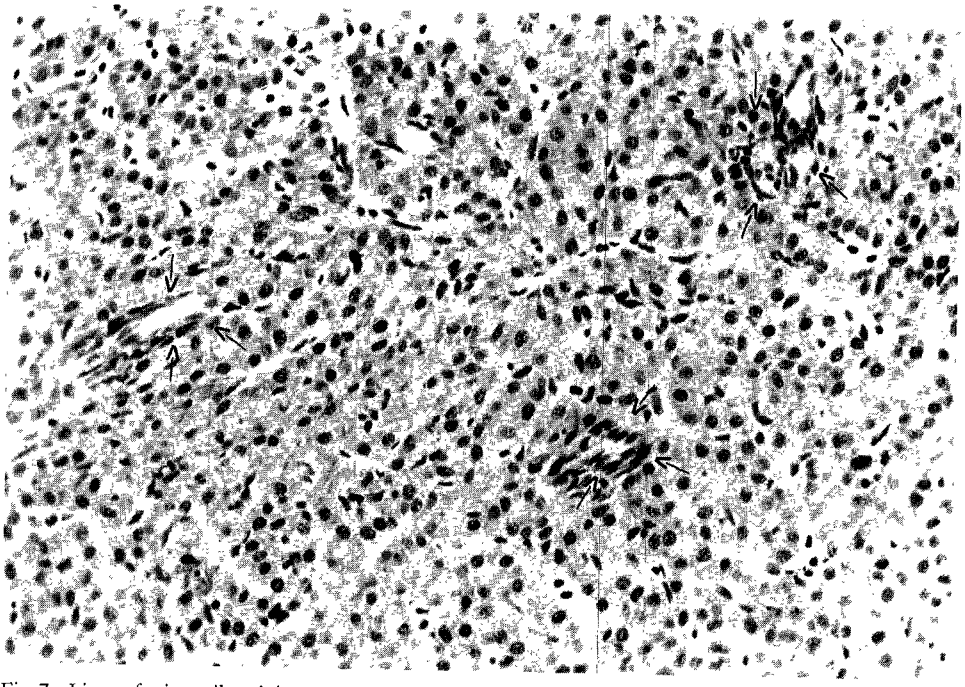

Fig. 7. Liver of a juvenile rainbow trout (*S. gairdneri*) after a 21-day exposure period to thiram (100 µg/l). Loss of glycogen and proliferation of bile ducts (arrows), HE x 400.

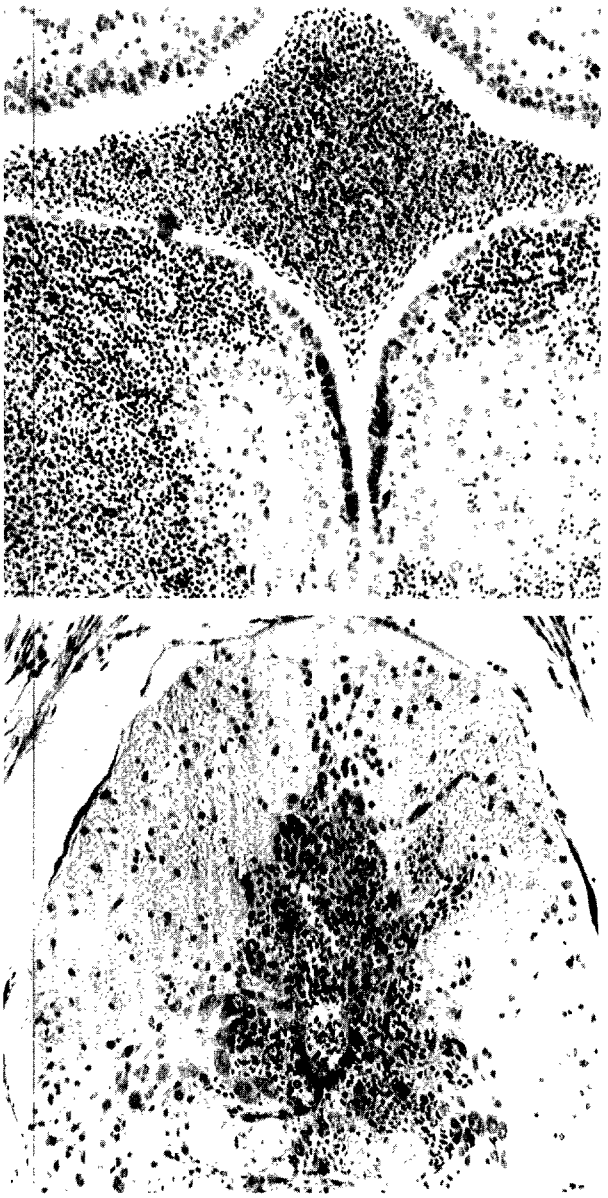

Fig. 8. Hemorrhages in juvenile rainbow trout (*S. gairdneri*) after exposure to thiram (5 µg/l) for 21 days. Top: brain tissue (HE x 256). Bottom: spinal cord (HE x 256).

They might reduce the intracellular availability of copper to lysyl oxidase, thus impairing the structural integrity of collagen. Evidence supportive of this mechanism has come from *in vitro* experiments with lysyl oxidase. Its activity is inhibited by diethyldithiocarbamate and can subsequently be restored by addition of copper sulfate (Shieh and Yasunobu, 1976). Further evidence has been presented by Prahlad et al.

(1974), who reported that nabam induced disorders in the fibrillar arrangement in the notochordal sheath of *Xenopus laevis* embryos. This nabam-mediated increase in the size of the notochord was ascribed to the loss of the limiting force exerted by the primary and secondary chorda sheath. The hypothesis that the notochordal defects might in fact be due to alterations in collagen metabolism is a likely explanation for our observations in rainbow trout. The skeletal malformations in the older fish are to be considered merely as a sequela to the extreme dilatation and distortions of the notochord.

The extensive hemorrhages observed in ziram and thiram-treated juveniles of *S. gairdneri* are obviously induced by the same mechanism, as lysyl oxidase initiates cross-linking of elastine as well. Low levels of lysyl oxidase activity induced by hypocupremia initiate pathological changes in elastic fibres rendering the vasculature more fragile (Harris et al., 1977). Vascular defects may entail a risk for hemorrhage and may account for the blood extravasates observed in our studies with *S. gairdneri*. Similar hemorrhagic changes have been observed in regenerating forelimbs of newt (*Triturus cristatus*) after treatment with maneb (Arias and Zavanella, 1979; Zavanella et al., 1984), and in studies with thiurams (Korhonen et al., 1982a), DDCs (Short et al., 1976), various BDCs (Fishbein, 1976; Larsson et al., 1976; Pacces Zaffaroni et al., 1978), carbon disulfide (Tabacova et al., 1978) and some urea and thiourea derivatives (Von Kreybig, 1969; Cross et al., 1972; Stula and Krauss, 1977). Since these compounds do not possess equally strong metal-binding properties (cf. Owens, 1969), other mechanisms are likely to be involved as well.

Hepatotoxicity is a well-known feature of both carbon disulfide and DC-intoxication (WHO, 1979; Fishbein, 1976; De Bruin, 1976). In our experiments with rainbow trout the only consistent effect, however, was a progressive depletion of glycogen in the hepatocytes. No fatty changes or serious degenerative processes were noted. The proliferation of bile duct epithelial cells is apparently related to the high-rated biliary excretion of these compounds or their metabolites. Evidence for this has been obtained from a recent study with ^{14}C-labelled zineb and ziram, in which high levels of ^{14}C-activity were found in liver and bile (Van Leeuwen et al., 1986b).

From this study it may be concluded that the main expression of DC-toxicity is teratogenicity. Histopathological evidence is given that this teratogenic action is confined to the notochord. Since these effects are induced at concentrations which are likely to be achieved in the aquatic environment, they may pose a serious hazard to the functioning of aquatic ecosystems.

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Chapter 9

Uptake, distribution and retention of zineb and ziram in rainbow trout (Salmo gairdneri)

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Abstract

Uptake, distribution and retention of ^{14}C-labelled zinc ethylenebisdithiocarbamate (zineb) and zinc dimethyldithiocarbamate (ziram) were studied in short-term experiments with rainbow trout (*Salmo gairdneri*). Upon exposure both compounds were rapidly disseminated through the tissues. Whole-body accumulation was low, with bioconcentration factors (BCFs) < 100. In both ziram and zineb-treated trout relatively high radioactivity levels were found in the liver, gall bladder and intestinal contents, which suggests a prominent role for hepatic biotransformation and biliary excretion. In ziram-treated trout eyes and skin also appeared to be major distribution sites. Tissue disposition of radioactivity differed for the two compounds, with ziram having the higher tissue-BCFs.

Whole-body elimination of zineb residues was rapid, with approximately 75% of radioactivity being eliminated within the first 4 days, and very little thereafter. In fish exposed to ziram 45% of the initial total ^{14}C-content in the body was retained by the end of the 16-day depuration period. Differences in the extent of elimination were most noteworthy for eyes, skin and kidney.

Whole-body autoradiography confirmed the results of the liquid scintillation countings. Radioactivity was localized in digestive tract, liver, bile, gills, thyroid follicles and in melanophores of the skin, in the choroid epithelium complex of the eyes, and in other melanin-containing tissues such as the kidneys. The observed differences in toxicokinetics between zineb and ziram may, in part, explain the differences in toxicity to fish between ethylenebisdithiocarbamates and dialkyldithiocarbamates.

Chapter 9

Uptake, distribution and retention of zineb and ziram in rainbow trout (Salmo gairdneri)

Introduction

Dithiocarbamates (DCs) are among the most important classes of fungicides currently used in agricultural practice. Because of their chelating properties DCs are also applied in industry and medicine (Thorn and Ludwig, 1962; Wilholm, 1982; Wing and Rayford, 1982). A good deal is known about the effects of DCs on warm-blooded animals (Fishbein, 1977), but data on their effects on cold-blooded animals, particularly fish, are scarce. Recently, we have reported on the harmful effects of various dialkyldithiocarbamates (DDCs) and ethylenebisdithiocarbamates (BDCs) on aquatic life; DCs were found to be toxic to bacteria, green algae, crustaceans and fish. DDC-induced embryotoxicity and teratogenicity in rainbow trout (*Salmo gairdneri*), at concentrations below ppb-level, were among the most conspicuous effects. On the whole, BDCs appeared to be less toxic than DDCs (Van Leeuwen et al., 1985a,b; 1986a,b).

The present report deals with the results of experiments designed to obtain information about the uptake, distribution and retention of two representatives of the two groups. This was done by exposing early juvenile rainbow trout to ^{14}C-zineb (zinc ethylenebisdithiocarbamate) and ^{14}C-ziram (zinc dimethyldithiocarbamate) in water, transferring them to toxicant-free water and analyzing whole fish and tissues for ^{14}C-content. In addition, autoradiographic studies and short-term accumulation tests with ETU (ethylenethiourea) and disulfiram (tetraethylthiuram disulfide) were conducted.

Materials and methods

Test animals and standard water

Rainbow trout (*S. gairdneri*) were obtained from Fijge Trout Farm at Vaassen (The Netherlands). Guppies (*Poecilia reticulata*) were obtained from our laboratory stock culture. Standard water for the experiments was prepared according to Alabaster and Abram (1965). For *S. gairdneri* the pH of the water was 8.0±0.1, hardness 50 mg/l (as $CaCO_3$); for *P. reticulata* these values were 8.1±0.2 and 260 mg/l, respectively. During acclimatization and elimination, trout were fed with Trouvit pellets (Trouw & Co. N.V., The Netherlands), guppies with TetraMin (Tetra Werke, F.R.G.).

Test compounds

[Ethylene-^{14}C] zineb (specific activity: 11.7 μCi/mg), [ethylene-^{14}C] ETU (specific activity: 214 μCi/mg; radiochemical purity 98%), [methyl-^{14}C] ziram (specific activity: 14.5 μCi/mg) and [ethyl-^{14}C] disulfiram (specific activity 34.5 μCi/mg; radiochemical purity >99%) were obtained from Amersham Radiochemical Centre (England). Dimethylsulphoxide (DMSO) was used as a solvent for ziram, zineb and disulfiram. ETU was dissolved in distilled water.

Accumulation studies

Whole-body accumulation studies with *S. gairdneri* and *P. reticulata* were performed according to a static procedure (Van Leeuwen et al., 1985c). The weight of the fish was 0.42±0.13 g and 70±22 mg (mean ±SE), respectively. The respective water temperatures were 10±1 and 20±1°C. A 12-h light-dark cycle was imposed upon both species. Other accumulation experiments with rainbow trout were performed in an approximately analogous manner. These fish, with weights of 3.4 ± 0.4 g, were exposed to sublethal concentrations of the test compounds in 25 l all-glass fish tanks, housed in a water bath. They were fasted for 48 h prior to and during the exposure. In order to keep the NH_3 concentration below 0.025 mg/l (Water quality criteria for European freshwater fish, 1973), the mass of fish in each tank never exceeded 4 g/l. The test solutions were aerated continuously and not renewed. Fish and water were sampled after 6, 24, 48 and 96 h of exposure.

Elimination studies

Following 96 h of exposure to experimentally contaminated water, rainbow trout (weight 7.2±1.7 g) were transferred to toxicant-free water and sampled after 0, 4, and 16 days to measure depuration of radioactivity. Radioactivity in water was measured at regular intervals; a concentration of 1% of the initial ^{14}C-activity during the accumulation period was taken as a maximum, above which water was renewed. During depuration fish were fed once every four days.

Collection of organs and tissues

Fish were anesthetized with $NaHCO_3$-buffered tricaine methane sulphonate (MS 222, Sandoz, Basel). After removal of adhering water by blotting on filter paper, the fry were weighed. The following organs and tissues were dissected: eyes, gills, stomach, intestine, liver, gall bladder, head kidney, trunk kidney, brains, heart and spleen. Samples were taken from the vertebral column and muscles. Blood samples were collected from the ventral aorta. Rest fractions were homogenized with an Ultra-turrax mixer. Samples were weighed on a microbalance with a precision of 10 μg.

Radiotracer techniques

In the whole-body accumulation experiments, fish were incinerated in a sample oxidizer (Packard, Tri Carb model B 306). CO_2 was trapped in Carbo-sorb and Perma-Fluor V (Packard) and radioactivity was measured by liquid scintillation counting (LSC; LKB/Wallac Rackbêta 1215). In the other experiments samples were solved in Lumasolve (Lumac; 1 ml per 100 mg tissue) and placed in a stove at 40°C for 24 h. Subsequently, scintillator 299 (Packard) was added at a maximum of 5 ml per vial. Before LSC, vials were stored for approximately 6 h in complete darkness. Data were corrected for chemical quenching and background radiation. Measurements were carried out in 3-5 replicates.

Transverse sections for autoradiography were cut with a whole-body microtome according to the method described by Curtis et al. (1981). Sections of 30, 40 and 50 μm thickness were freeze-dried at -20°C for approximately 18 h before being pressed against autoradiography films (Kodak X-OMAT AR). Films were exposed in a light-tight box for 26 days at -20°C and developed in Kodak-LX 24 for 5 minutes. Next they were rinsed in an acetic acid stop-bath for 30 seconds. Fixation took place in Kodak AI-4 Röntgenfix for 4 minutes.

Data analysis

LSC results (dpm, per kg fish and per l water) were converted to μg/kg and μg/l respectively, dividing them by the specific activity of each compound. The rate constants were estimated from a kinetic model for static bioaccumulation experiments (cf. Van Leeuwen et al., 1985c), which also comprised biotransformation processes. The following set of equations was used:

$$\frac{d}{dt} C_f(t) = k_1 C_w(t) - k_2 C_f(t) - k_3 C_f(t) \quad (1)$$

$$\frac{d}{dt} C_w(t) = -\frac{N(t)w}{V} k_1 C_w(t) + k_2 \frac{N(t)w}{V} C_f(t) \quad (2)$$

$$\frac{d}{dt} C'_f(t) = k'_1 C'_w(t) - k'_2 C'_f(t) + k_3 C_f(t) \quad (3)$$

$$\frac{d}{dt} C'_w(t) = -\frac{N(t)w}{V} k'_1 C'_w(t) + k'_2 \frac{N(t)w}{V} C'_f(t) \quad (4)$$

The following symbols were used: t: time (h); C_f: concentration of the parent compound in one organism (μg/kg); C_w: concentration of the parent compound in water (μg/l); w: weight of one organism (kg); V: water volume (l); N: number of organisms;

k_1: uptake rate constant of the parent compound (l/kg/h); k_2: clearance rate constant of the parent compound; and k_3 biotransformation rate constant (1/h). The prime denotes the concentrations and rate constants of the metabolite.

The degradation process is assumed to be first order in the concentration of the parent compound in the organism. For compounds which metabolize slowly, k_3 approximates zero and only eqs. 1 and 2 are used. In this case, the steady-state bioconcentration factor (BCF: l/kg) equals k_1/k_2.

BCFs for tissues and organs were calculated from the mean total ^{14}C-concentrations in fish and water, respectively. Differences in the BCFs were tested with the Student's *t*-test.

Results

Levels of radioactivity in early juvenile trout exposed to zineb (Fig. 1) and guppies exposed to zineb and ziram, reached an apparent steady-state within approximately 24 h of exposure. In trout exposed to disulfiram and ziram, however, no steady-state conditions were reached, i.e., the total ^{14}C-activity in water initially decreased and subsequently increased again, whereas the total radioactivity levels in fish showed a reversed pattern of behaviour (Fig. 1).

This hindered the calculation of steady-state BCFs. The best fit to these experimental data was obtained by introducing three biotransformation-related constants (see data analysis). The k_3, k_1' and k_2' values (means ± SE) for disulfiram were 0.08±0.02, 40.4±58.8 and 0.88±1.6. The respective values for ziram were 0.21±0.03, 6.71±2.14 and 0.21±0.04. The bioaccumulative potential of all compounds was low (Table I). The lowest BCFs for zineb and ziram were found in tests with guppies.

TABLE I. Bioconcentration kinetics of dithiocarbamates in *Salmo gairdneri* and *Poecilia reticulata*.

Species	*Compound*	k_1±SE	k_2±SE	BCF[a]
S. gairdneri	disulfiram	75.9 ±11.6	0.21 ±0.08	225[b]
S. gairdneri	ETU	–	–	0.35[c]
S. gairdneri	zineb	2.41± 0.18	0.07 ±0.01	34
S. gairdneri	ziram	11.54± 0.48	0.002±0.47	90[b]
P. reticulata	zineb	1.86± 0.08	0.32 ±0.03	5.8
P. reticulata	ziram	2.99± 0.14	0.63 ±0.04	4.7

[a] The whole-body BCF represents the quotient of the k_1 and k_2 values.

[b] The experimental data indicated a substantial biotransformation of the parent compound. No steady-state conditions were reached (cf. Fig. 1). The 'BCF' of the compound was calculated from the measured radioactivity levels in fish and water. The highest C_f/C_w value is presented.

[c] Adequate estimations of the rate constants were not possible due to the low bioaccumulative potential of the compound. The highest C_f/C_w value is presented.

Upon exposure to zineb and ziram-contaminated water, radioactivity was rapidly disseminated through the tissues. The lowest levels of ^{14}C-activity were found in muscle, heart, brain and vertebral column. Liver and digestive tract contained the highest (Figs. 2 and 3). Zineb-derived radioactivity accumulated in liver accounted for about 60% of total radioactivity after 2 and 4 days, whereas the liver of ziram-treated fish accounted for approximately 20% of total radioactivity after comparable exposure times. Remarkably high residues were also detected in eyes and skin of ziram-treated fish. Radioactivity in the skin accounted for approximately 40% of total against 8% in zineb-treated fish. The initial concentrations of zineb and ziram in water of 225 ± 5 and 137 ± 2 μg/l, dropped by approximately 14% in 96 h.

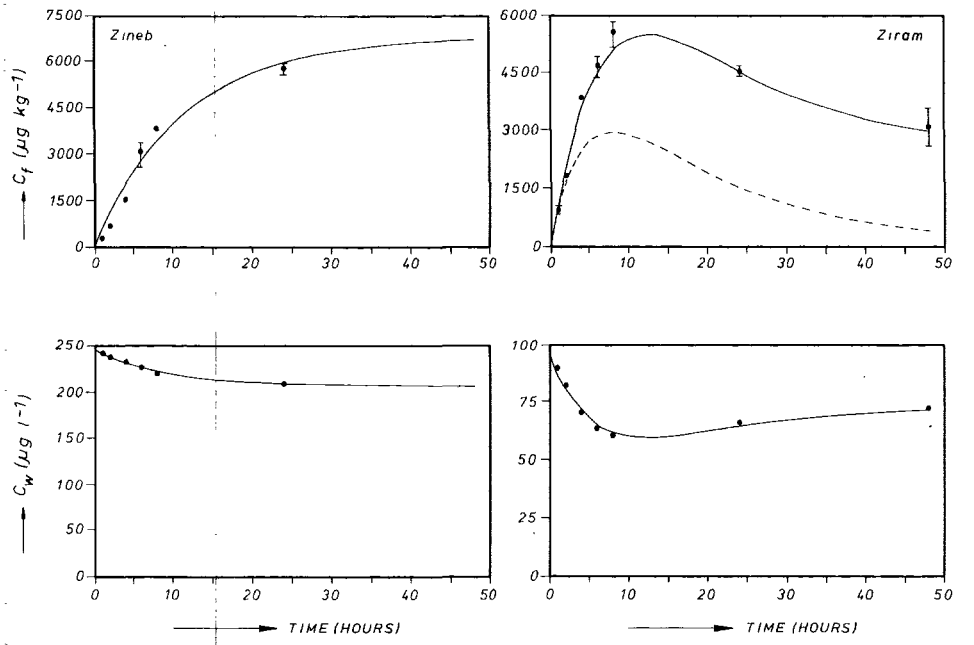


Fig. 1. Whole-body accumulation of zineb ($C_W(o)$ = 245 μg/l) and ziram ($C_W(o)$ = 97 μg/l) in early juvenile rainbow trout (*Salmo gairdneri*). . Points and associated vertical lines represent means ± SE of 5 samples. Lines are expected values based on the model calculations. A reasonable fit to the experimental data of ziram was obtained by assuming biotransformation of the parent compound to one degradation product. The expected concentrations of the parent compound are depicted by the dashed line.

Another accumulation experiment was conducted in order to study elimination. The initial concentrations of zineb and ziram in the medium in which the fish were exposed were 105 ± 2 and 118 ± 5 μg/l. This time, radioactivity in liver and gall bladder (contents included) were determined separately. The measurements revealed that the gall bladder was the major distribution site for the radiolabelled compounds and/or their degradation products (Fig. 4). After 96 h, the activity in the medium had dropped to 97 ± 2 and 93 ± 2 μg/l, respectively.

Whole-body elimination of zineb and/or its degradation product(s) was rapid during the first few days. After 4 days, only 25% of the initial residue was retained by the fish.

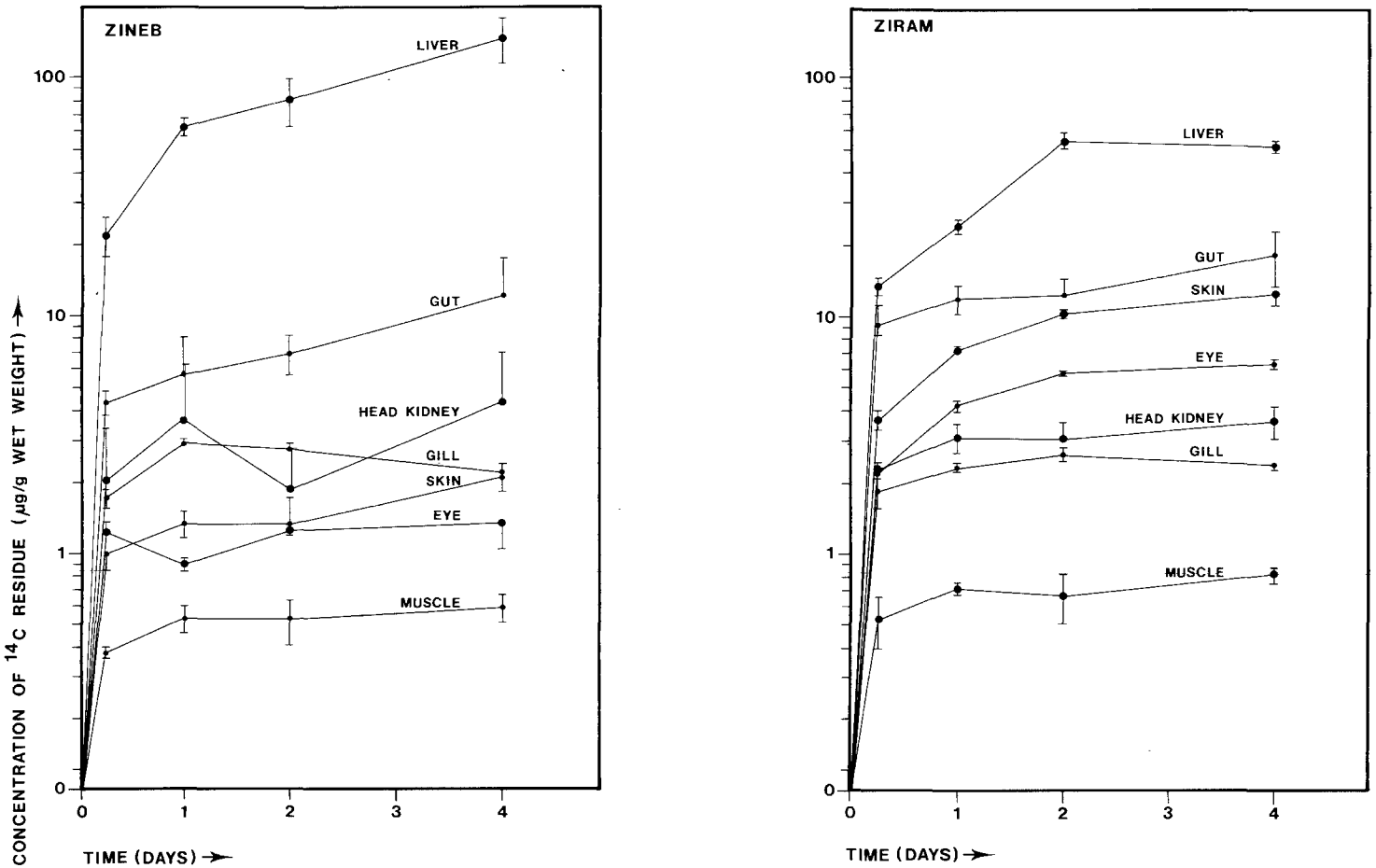


Fig. 2. Uptake of zineb and ziram in various tissues of *Salmo gairdneri*. Points and associated vertical lines represent means ± S.E.M. of 3-5 fish.

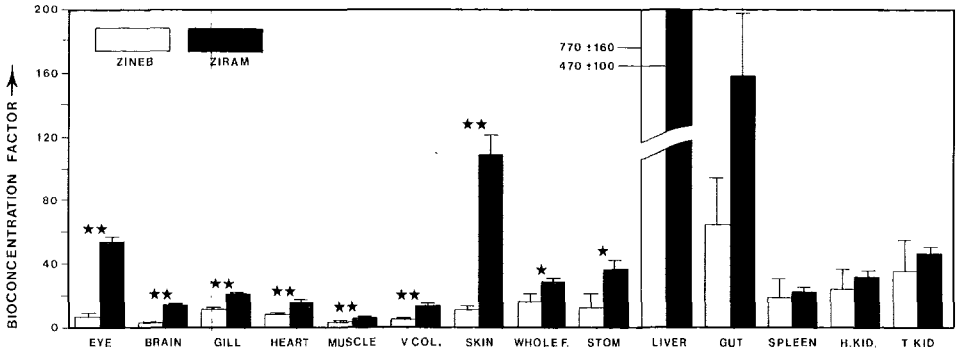


Fig. 3. Bioconcentration factors of ^{14}C-residues in whole fish and various tissues of *Salmo gairdneri* after 96 h exposure to zineb and ziram-contaminated water. Values and associated vertical lines represent means ± S.E.M. of 3-5 fish. Asterisks denote differences in BCFs at $P<0.05$ (*) and $P<0.01$ (**), respectively.

Further clearance was neglegible. The pattern of ziram elimination was similar, but slower than that of zineb. After 16 days, about 55% of the initial total ^{14}C-content in the body was eliminated. The loss of radioactivity in gill, blood, muscle, liver, gall-bladder and intestine (Fig. 4) was rapid. The concentration of zineb residues in the gall bladder had dropped by a factor 55, whereas that for ziram had decreased by a factor around 10 within 4 days upon transferring fish to clean water. The levels of radioactivity in the gall bladder varied considerably with time, which may be related to the large variations in the amount of bile excreted in this organ. Radioactivity levels in eyes and skin, however, remained almost constant for both compounds, but increased steadily by a factor around 3 in head and trunk kidney of ziram-treated trout during depuration. The latter points to a marked redistribution of radioactivity. The concentrations in the kidney of fish previously exposed to zineb remained approximately constant. After a 16-day elimination period, the ^{14}C-activity in eyes, skin and kidney of ziram-treated fish were respectively 7.5, 7.4 and 11.7 times higher than those in fish which had been exposed to approximately equal radioactivity levels of zineb. The importance of the skin in retention of radioactivity is depicted in Fig. 5.

The autoradiographic studies revealed a high labelling of the gall bladder and the intestinal lumen. Dissemination of ^{14}C-activity through the tissues was again rapid. A marked radioactivity was present also in pigmented tissues, i.e. the choroid-epithelium complex of the eye, skin and ophisthonephros; however, tissues of ziram-treated fish generally contained more radioactivity (Fig. 6). Detailed examination revealed that radioactivity was localized at distinct spots which coincided with pigment granules in melanophores. Upon transferring the fish to clean water, these spots retained their radioactivity for considerable periods of time. This was demonstrated for ziram and to a lesser extent for zineb-treated fish. After 16 days of depuration, radioactivity was almost entirely confined to the pigmented tissues (Fig. 7). Moderate to high levels of activity were also recorded in distinct spots in the subpharyngeal area, which corresponds to the location of thyroid follicles. High activity at these locations was still found

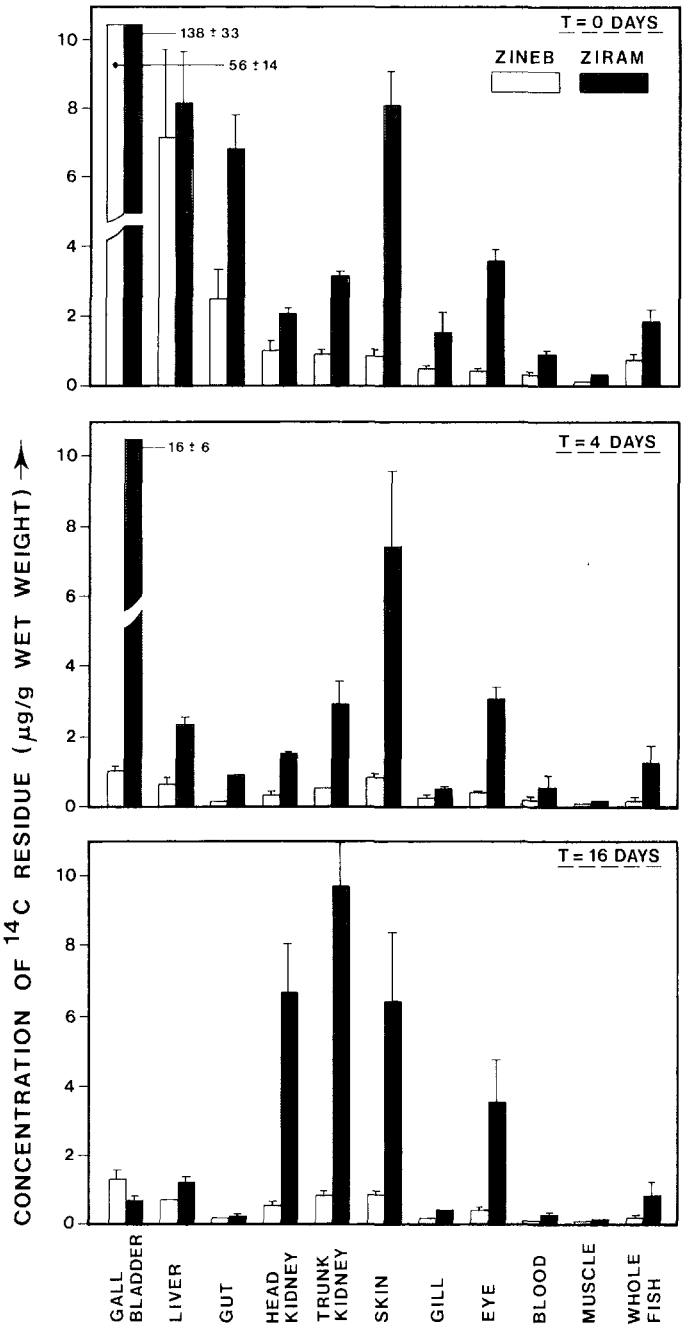


Fig. 4. Elimination of ^{14}C-residues in whole fish and various tissues of *Salmo gairdneri* after 96 h exposure to 105 and 118 µg/l zineb and ziram, respectively. Values represent means ± S.E.M. of 3-5 fish.

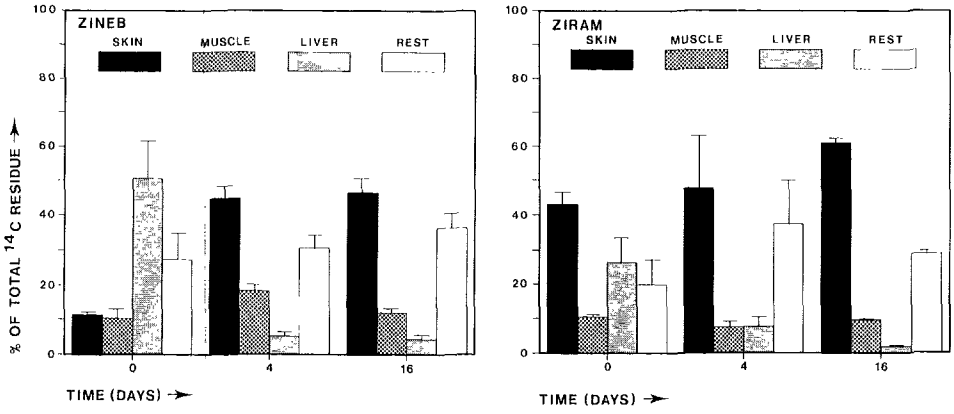


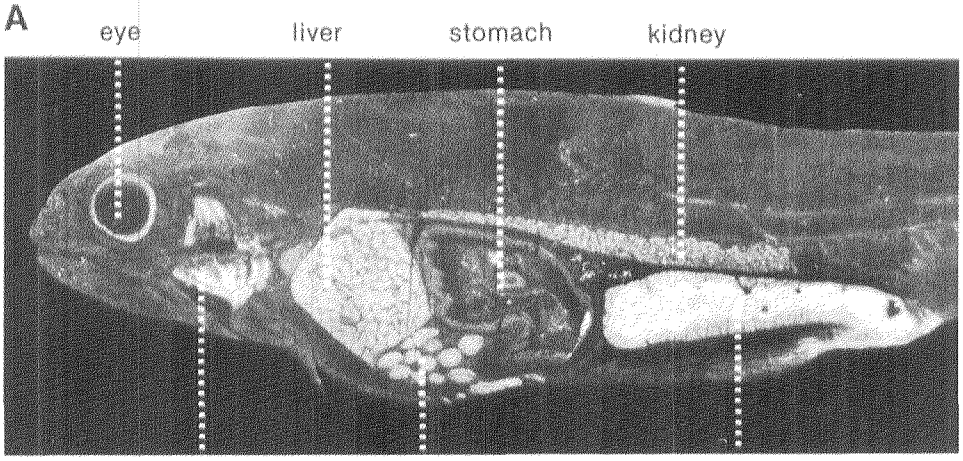
Fig. 5. Quantative distribution of zineb and ziram-residues in *Salmo gairdneri* during elimination. The values for the liver include those for the gall bladder.

after a 16-day elimination period, both in zineb and ziram-treated fish (Fig. 7). In all other tissues, radioactivity was rapidly lost.

Discussion

Bioaccumulation of both zineb and ziram was low, but differences were found regarding their BCFs, internal distribution and retention, which may in part explain their differences in toxicity to fish (Van Leeuwen et al., 1985a, 1986a). The low bioaccumulative potential of both compounds, together with the low concentrations at which adverse effects become manifest, point at high intrinsic toxicity. The bioconcentration factors for zineb fell in the following order: 34 (early juvenile trout), 15 (juvenile trout) and 5.8 (guppy). The same held true for ziram. Their respective values were 90, 27 and 4.7. It is not unlikely that the overall bioaccumulation of zinc-containing DCs in fish, is mainly governed by a strong interrelationship involving the age (weight) of the animal, which dictates its ability to metabolize and excrete the compounds, and the temperature of the water which dictates, among other things, the rate of these processes. For both compounds high levels of radioactivity were registered in the viscera, which suggests a prominent role for biotransformation (Figs. 2 and 3). The rapid disappearance of radioactivity in fish via liver, bile and intestine is in support of this (Figs. 4 and 6). In rats, orally dosed with ziram, the formation of a host of degradation products such as thiram, the dimethylamine salt of dimethyldithiocarbamic acid, tetramethylurea, dimethylamine and carbondisulfide, has been reported. S-glucuronide formation of dimethyldithiocarbamate is another metabolic pathway which must not be excluded. Two major metabolites in the urine of zineb-treated rats were ethylenebisdiisothiocyanato sulfide and ETU (Paulson, 1977). Therefore it is likely that in our kinetic model the actual metabolic processes are oversimplified. Urinary excre-

A


eye
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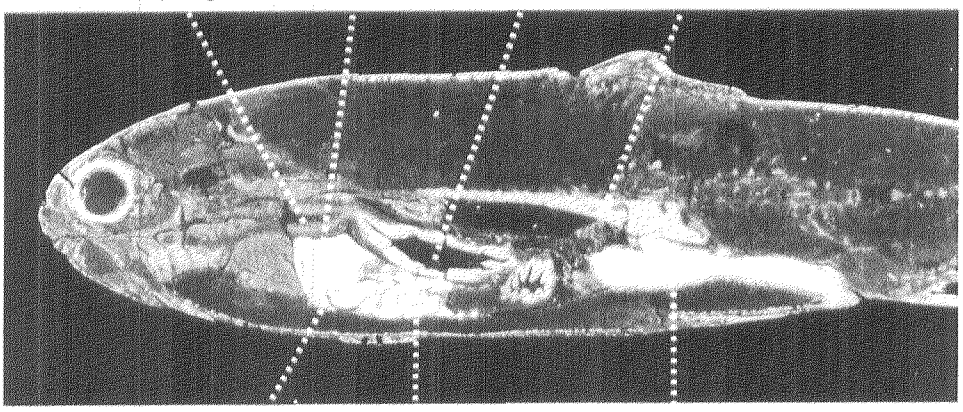
intestinal contents

B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gall bladder
caeca
intestine

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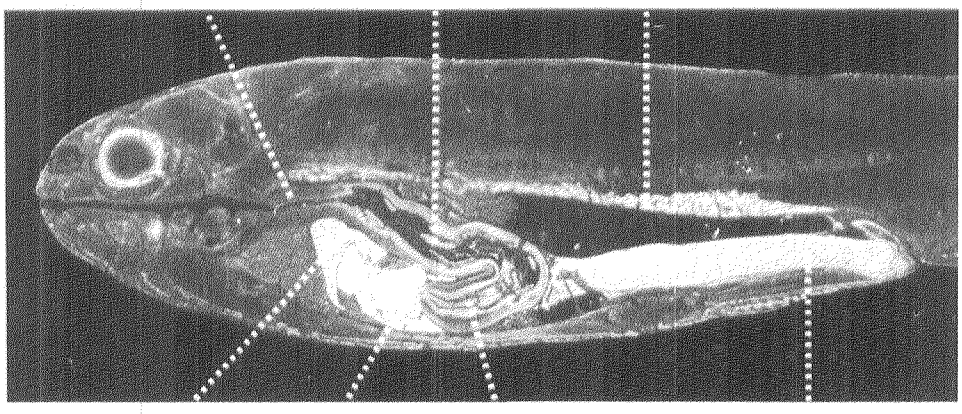
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caeca
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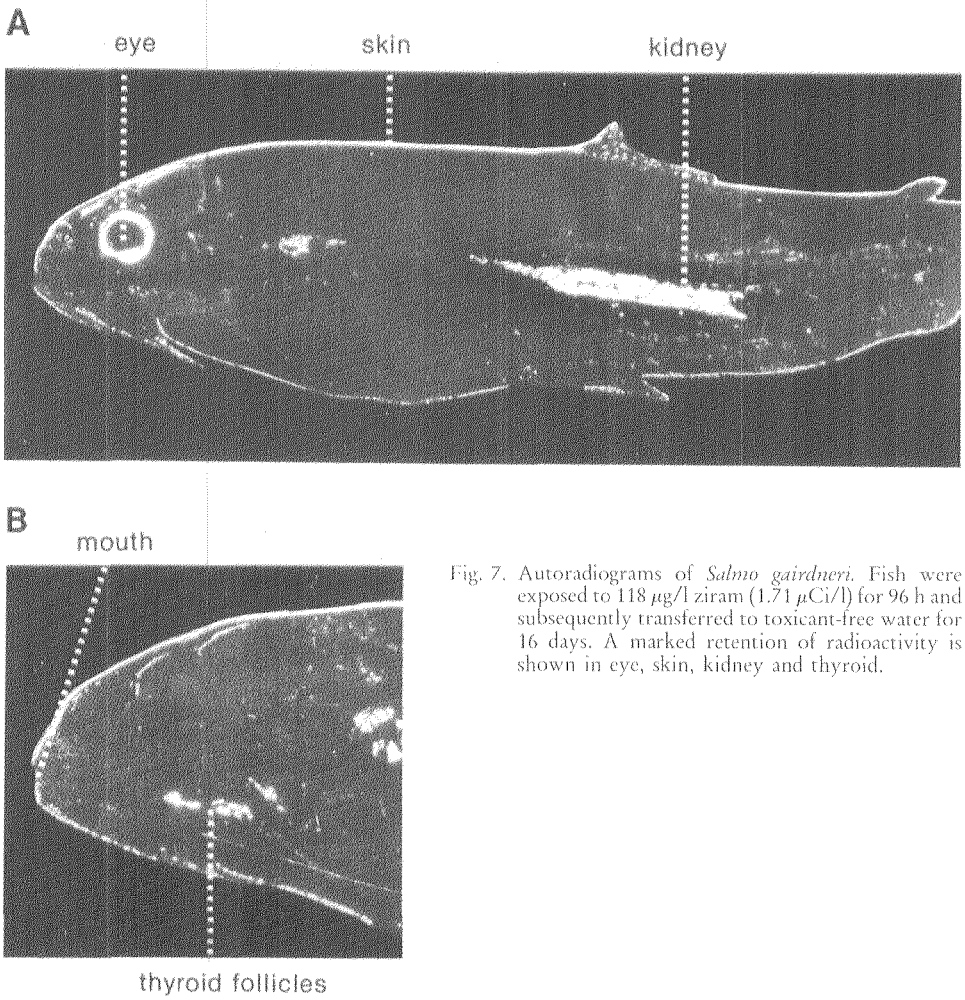

Fig. 7. Autoradiograms of *Salmo gairdneri*. Fish were exposed to 118 μg/l ziram (1.71 μCi/l) for 96 h and subsequently transferred to toxicant-free water for 16 days. A marked retention of radioactivity is shown in eye, skin, kidney and thyroid.

tion was not explicitly studied. This, together with faecal excretion and elimination via gill, suggests that by the end of the exposure period a substantial part of the concentration of radioactivity, both in water and fish, may originate from hydrophilic metabolites.

The whole-body autoradiography confirmed the results of the LSC. Ziram, and to a lesser extent zineb and/or their degradation products, appeared to accumulate

Fig. 6. Whole-body autoradiograms of *Salmo gairdneri* after exposure to 105 μg/l zineb (A; 1.22 μCi/l) and 118 μg/l ziram (B; 1.71 μCi/l) for 24 h. High levels of radioactivity can be seen in eyes, liver, pyloric caeca, kidney and intestine. There is also a marked labelling of the skin and stomach wall of ziram-treated fish. Low levels of radioactivity are seen in the brain. Autoradiogram C, a rainbow trout 6 h after intraperitoneal injection with 7 μg ziram (0.1 μCi) dissolved in 50 μl DMSO, reveals a similar distribution of radioactivity.

specifically in melanophores in the skin, the choroid-epithelium complex of the eye, and in other melanin containing tissues such as the kidney (Figs. 6 and 7). This may be attributed either to the removal of copper - the prosthetic group of phenoloxidase (tyrosinase), a metallo-enzyme involved in melanin synthesis - or to the attachment to copper, by which a dithiocarbamate-enzyme complex is formed (Eckert, 1957; Thorn and Ludwig, 1962; Wing and Rayford, 1982). These assumptions are supported by the finding that DDCs and various thioureas are potent inhibitors of phenoloxidase (Blagoeva and Stoichev, 1979; Chen and Chavin, 1978; Tomita and Hishida, 1961). This tentative hypothesis is also implicitly supported by Zavanella et al. (1984), who demonstrated a temporary reduction of melanogenesis in regenerating limbs of newt (*Triturus cristatus carnifax*) after exposure to maneb, and reports on melanosomes devoid of melanin in pigmented retinas of *Xenopus laevis* embryos treated with nabam and maneb (Bancroft and Prahlad, 1973; Prahlad et al., 1974). Similar effects were observed after exposure of amphibians to sodium diethyldithiocarbamate and its corresponding thiuramsulfides (Ghate and Mulherkar, 1980; Ghate, 1983), and in some embryolarval toxicity studies with *S. gairdneri* (Van Leeuwen et al., 1986a).

Interaction with melanin constitutes another mechanism by which high levels of radioactivity in melanophores may be explained. Melanin has a great affinity for metal ions, which is ascribed to its cation-exchange activity due to the free carboxyl groups present in the polymer (Larsson and Tjälve, 1978). Danielsson et al. (1984) showed a dramatically increased ^{203}Pb uptake in the melanin-containing structures in the eyes of mice after oral treatment with various DDCs, and postulated as the mechanism, the binding of Pb^{2+} to melanin. In our experiments dithiocarbamic acid was labelled and heavy metal ions were not, which suggests that other mechanisms such as nucleophilic substitution on the carbon of carboxyl groups of melanin may be involved (cf. Owens, 1969).

Relatively high levels of radioactivity were found in the thyroid follicles (Fig. 7). BDCs, ETU and CS_2, a degradation product of both BDCs and DDCs, are also known to accumulate in the thyroid (Bergman et al., 1984; Kato et al., 1976; Seidler et al., 1970). The precise mechanism remains conjectural, but may be related to the affinity for sulphydryl groups of thyroglobulin (Thorn and Ludwig, 1962; Vasák and Kopecky, 1967; Yoshida et al., 1978) or to the heavy metal sequestering properties of DCs and related compounds, as several metallo-enzymes and metal-requiring enzymes are involved in thyroxine synthesis (Bhattacharya and Datta, 1970; De Groot et al., 1984; Hati and Datta, 1967).

The present results show that dithiocarbamates localize selectively in various tissues, reported to be the target organs for the toxic action of DCs (Fishbein, 1977; Pacces Zaffaroni et al., 1978). Further experiments are required to investigate whether similar results would have been obtained if the labelling is attached to the CS_2 moiety, the active site of the DCs. It is also important to note that the results presented in this study may not fully apply to other metallo-dithiocarbamates and corresponding thiuramdisulfides, as the 'Verdrängungsreihe' of Eckert (1957) cannot be ignored.

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Summary and concluding remarks

Dithiocarbamates (DCs) are among the most widely used fungicides in The Netherlands as well as abroad. They are effective against a broad spectrum of fungi and plant diseases caused by fungi. They are also used in industry as slimicides in water-cooling systems, in sugar, pulp and paper manufacturing and as vulcanization accelerators and antioxidants in rubber. Because of their chelating properties they are also employed as reagents in heavy metal analysis and as heavy metal scavengers in waste water treatment. According to their structure the DCs can be roughly divided into two subgroups: the dialkyldithiocarbamates (DDCs) and ethylenebisdithiocarbamates (BDCs).

DCs, and BDCs in particular, are manufactured and formulated on a large scale. Dutch production is estimated at about 14,000 tonnes per year and about 400 tonnes (a.i.) are annually discharged into the aquatic environment of the Netherlands. Biomonitoring of waste water from DC-producing companies revealed that their waste water was highly toxic to fish, and the results stressed the need for further research. An extensive study of existing literature showed the lack of information about the aquatic toxicity of various DCs. Moreover, it was shown that their environmental behaviour is very complicated because they are subject to manifold degradation, polymerization, volatilization and complexation with heavy metals. Appropriate analytical methods on the ppb-level were in equally short supply. These deficiencies prevent an adequate evaluation of their aquatic ecotoxicological impact.

Given the above deficiencies and the importance of the subject, a research program was conducted in order to study aquatic toxicity, accumulation, elimination and biodegradation of various DCs and some degradation products. The investigations were carried out within the framework of the research and advisory task of the Institute for Inland Water Management and Waste Water Treatment; the legal framework for these investigations is provided by the Pollution of Surface Waters Act. These legislative and toxicological aspects are dealt with in more detail in chapter 1.

Short-term toxicity studies of 26 dithiocarbamates and related compounds are presented in chapter 2. Taken over the entire range of substances, sensitivity decreased in the following order: *Daphnia magna* (water flea), *Photobacterium phosphoreum* (a luminescent bacterium), *Chlorella pyrenoidosa* (a green alga), *Poecilia reticulata* (guppy) and *Nitrosomonas/Nitrobacter* (nitrifying bacteria). DCs and related compounds were found to be highly toxic. Quantitative structure-activity relationship (QSAR) studies revealed that toxicity to the various test species was a function of the *n*-octanol/water partition coefficient (P_{oct}). Effects on nitrification, however, could not be explained by this parameter.

In chapter 3 special attention is given to sublethal effects in rainbow trout *(Salmo gairdneri)* after acute exposure to tetramethylthiuram disulfide (thiram). Thiram affec-

ted both protein and non-protein-bound sulfhydryl levels in the fish and altered the lactate dehydrogenase and glucose-6-phosphate dehydrogenase activities, the blood glucose content and the hepatic lipid concentration. It also interfered with some hematological indices, inducing leucopenia, a fall in the hemoglobin content and an increase in the osmolarity of blood.

From the ecological point of view the ultimate aim in aquatic toxicity testing should be the study of adverse effects on the ecosystem level. Nevertheless, our understanding of what is really happening in the real world, is still too incomplete to allow for adequate predictions. This problem can be partly overcome by studying toxicity at a lower level of complexicity, i.e. the population level. In chapter 4 two procedures for chronic toxicity testing with *D. magna* are presented and discussed, together with the current methods for this purpose. In 21-day life-table experiments with cohorts (isolated generations), separate measures of age-specific survival and reproduction may be linked together and used to estimate population growth in an unlimited environment (exponential growth). The semistatic method presented is simple, accurate and allows for statistical evaluation of the test results. Chemical analyses of the concentrations of the test compound are not *per se* required. In routine toxicity testing, population growth is only studied with algae. The second method is a mere extension of this approach and was designed in order to study population growth of daphnids in a limited environment (logistic growth). The latter type of experiment is performed in an intermittent-flow system and is initiated with small populations composed of cohorts of different ages. The method is ecologically more attractive, more laborious as well, and chemical analyses are required. Finally it is stated that the introduction of the concepts of population dynamics in reproduction tests with *D. magna* is a realistic step towards ecotoxicology.

Due to chemical-analytical constraints, the semistatic life-table procedure was applied for chronic toxicity testing of DDCs, BDCs and major transformation products. The results are presented in chapter 5. Survival, reproduction and individual growth of *D. magna* were clearly reduced at levels of approximately 10 and 80 μg/l, respectively. QSARs were calculated for a limited number of the compounds involved. Over the broad range of lipophilicities (10^7), toxicity could best be described by a sigmoid function of P_{oct}.

Differences in the susceptibility of early life stages of *S. gairdneri* are the subject of chapter 6. It is becoming increasingly apparent that with regard to toxic effects of environmental pollutants, early life stages constitute the critical 'sensitive links' in the life cycle of fish. Although embryolarval tests as compared to life cycle studies reduce the time to produce information on the toxicity of chemicals, they remain laborious. A further reduction of exposure times, i.e. short-cut methods, are needed in view of the lack of fish toxicity data. In order to detect susceptible stages during the ontogenesis of fish, simultaneous static accumulation experiments and acute toxicity tests have been undertaken together with fat content and fresh-weight determinations. In conclusion it is stated that short-term exposure of early fry is a practical approximation in establishing toxicant concentrations acceptable for fish. For compounds having a high bio-

accumulative potential, the exposure period should also include the sac fry stage. When teratogenesis has to be studied, the exposure period should include the early embryonic stages as well.

DC-induced dysmorphogenetic effects have been observed in mammals, birds and amphibians No reports, however, have appeared on their teratogenic effects in fish. For this reason early life stages of *S. gairdneri*, from the fertilized egg to the early fry stage, were continuously exposed to various DDCs, BDCs and a number of degradation products. The results are presented in chapter 7. Both BDCs and DDCs induced high embryotoxicity, especially during late gastrulation, when somites began to form and during early organogenesis. DDCs were generally found to be more toxic than BDCs. For some DDCs adverse effects below ppb-level were observed. All compounds, including carbon disulfide, were shown to have teratogenic properties. The most striking effects were vertebral abnormalities, which compare favourably with those observed in warm-blooded animals and amphibians.

Chapter 8 deals with the histopathological effects of DCs. Among the survivors of *S. gairdneri* exposed during embryogenesis, a high incidence of malformations was observed. These malformations were almost exclusively confined to the notochord which increased both in length and diameter. As a result the normal rod-like appearance was completely disturbed; it was twisted and distorted in dorsal, ventral and lateral directions. In the affected notochords ectopic osteogenesis was observed. Other effects, e.g. the disruption of the histoarchitecture of myomeres and myosepta and organ dislocations were closely related to these malformations. X-ray analyses revealed compression and fusion of vertebrae and 'wavyness' of various skeletal elements. Semi-chronic exposure of juvenile rainbow trout to various DCs induced a concentration-related decrease in hepatic glycogen storage. Proliferation of bile duct epithelium cells and necrosis of individual hepatocytes were observed at high exposure levels. Some compounds induced extensive hemorrhages in the brain as well as spinal extravasates of blood cells.

Finally, in chapter 9, data are presented on the bioaccumulative potential of zinc-derivatives of both dialkyldithiocarbamic acid (ziram) and ethylenebisdithiocarbamic acid (zineb) in fish. Whole-body accumulation of the ^{14}C-labelled compounds was low with bioconcentration factors (BCFs) < 100. Radioactivity was rapidly disseminated through the tissues. In ziram and zineb-treated *S. gairdneri* relatively high radioactivity levels were detected in the liver, gall bladder and intestinal contents, which suggests a prominent role for hepatic biotransformation and biliary excretion. In ziram-treated trout, eyes and skin also appeared to be major distribution sites. Tissue disposition of radioactivity differed for the two compounds, with ziram having the higher tissue-BCFs. After transfer of pre-exposed fish to toxicant-free water, there was an initial rapid rate of whole-body elimination, followed by a slow rate. The results were confirmed by whole-body autoradiography. It is stated that the observed differences in toxicokinetics between zineb and ziram may, in part, explain the difference in toxicity to fish between the BDCs and DDCs.

The agricultural and industrial benefits of dithiocarbamates as well as their economic

impact are great. Nevertheless, from these investigations and other studies it follows that DCs are cytotoxic and must be regarded as broad-spectrum biocides. Nitrification and other microbiological processes may be disturbed if DCs or their degradation products are discharged into the aquatic environment. The compounds are toxic to green algae and very toxic to freshwater crustaceans. Moreover, teratogenic effects in fish are already induced at extremely low concentrations. Reports have appeared on the mutagenicity and carcinogenicity of some of these compounds. Hence, DCs may seriously pose a hazard to the functioning of aquatic ecosystems. The compounds are not expected to accumulate to a great extent. They are moderately persistent in aquatic environments. Tentative water quality standards are proposed of 1.0 and 0.1 μg/l for BDCs and DDCs, respectively. Toxic concentrations to non-target organisms are likely to be achieved by the current practice of production, formulation and use of DCs. This urges the need for appropriate measures. The following regulatory actions have been taken:

- Within the framework of the Dutch Pollution of Surface Waters Act, sanitation measures have been imposed on DC-producing companies in order to reduce aquatic pollution caused by these substances.
- Within the framework of the Dutch Pollution of Surface Waters Act, biomonitoring of waste water from these and other pesticide producers is required.
- The Institute for Inland Water Management and Waste Water Treatment has advised against the employment of DCs as heavy metal scavengers in waste water treatment, as the compounds are generally more toxic than the metals they must remove.
- Within the framework of the Netherlands Pesticide Act the policy is to restrict the use of dithiocarbamates as slimicides and biocides in water-cooling systems and pulp and paper mills; their use is allowed only in situations where application, water purification facilities and discharge conditions are such that damage to aquatic ecosystems as a result of these compounds is not likely to occur. Moreover, all biocides meant to be used in process and cooling water with dithiocarbamates as active ingredients are being labelled as hazardous to fish and other aquatic organisms.
- In the framework of the Paris Convention a discussion has been initiated to include dithiocarbamates in the black list.

Samenvatting en slotopmerkingen

Dithiocarbamaten (DC's) vormen een belangrijke groep van pesticiden die al sinds 1934 in gebruik zijn voor de bestrijding van verschillende soorten bacteriën, schimmels, wormen, mossels, slakken en insecten. Zij worden toegepast in de land-, tuin- en bosbouw alsmede in de industrie. De goed in water oplosbare natrium derivaten worden vanwege hun grote affiniteit voor zware metalen gebruikt als reagentia voor de chemische bepaling van zware metalen en in de humane geneeskunde in gevallen van intoxicaties met onder andere nikkel. De laatste jaren worden DC's steeds meer toegepast voor de verwijdering van zware metalen uit afvalwater. Een bijzondere toepassing vormt het gebruik van disulfiram (tetraethylthiuram disulfide) als therapeuticum voor chronisch alcoholisme, sinds twee Deense artsen, in experimenten met zichzelf, poogden de wormverdrijvende eigenschap van deze stof vast te stellen maar ziek werden tijdens een cocktail party.

Op basis van hun chemische structuur kunnen DC's onderverdeeld worden in twee groepen t.w.: dialkyldithiocarbamaten (DDC's) en ethyleenbisdithiocarbamaten (BDC's). DC's en met name BDC's worden op grote schaal geproduceerd en geformuleerd. De nationale productie wordt geschat op 14.000 ton per jaar en daardoor geraakt jaarlijks circa 400 ton in het Nederlandse aquatische milieu. Toxicologisch onderzoek bracht aan het licht dat afvalwater van deze bedrijven zeer toxisch was voor vissen en deze constatering maakte vervolgonderzoek noodzakelijk. Een uitgebreide literatuurstudie leerde dat nauwelijks relevante informatie voorhanden was om adviezen op te baseren. Bovendien bleek het chemisch gedrag van deze stoffen uitermate gecompliceerd te zijn. De stoffen hydrolyseren en (bio)degraderen tot een groot aantal producten, waaronder ook vluchtige verbindingen. Sommige uitgangsstoffen en enkele degradatie producten hebben een polymeer karakter en complexeren met zware metalen. Adequate chemisch analytische methoden voor de bepaling van deze stoffen op ppb-niveau zijn eveneens niet beschikbaar.

Bovenstaande overwegingen alsmede de taak die de Dienst Binnenwateren/RIZA vervult met betrekking tot de Wet Verontreiniging Oppervlaktewateren, hebben geleid tot de start van een onderzoek naar de aquatische toxiciteit, bioaccumulatie en persistentie van deze stoffen en enkele degradatie producten. Diverse wettelijke en toxicologische aspecten komen ter sprake in hoofdstuk 1.

In hoofdstuk 2 worden de resultaten van kortdurende toxiciteitsstudies besproken met 26 dithiocarbamaten en verwante verbindingen. Het blijkt dat de gevoeligheid voor de stoffen afneemt in de volgorde: *Daphnia magna* (watervlo), *Photobacterium phosphoreum* (een bioluminescerende bacterie), *Chlorella pyrenoidosa* (een groenalg), *Poecilia reticulata* (gup) en *Nitrosomonas/Nitrobacter* (nitrificerende bacteriën). De

onderzochte bestrijdingsmiddelen hebben een hoge toxiciteit. Bovendien blijken enkele degradatie producten aanzienlijk toxischer dan de uitgangsproducten. De toxiciteit van de verbindingen voor de verschillende testorganismen correleert in hoge mate met hun partitiecoëfficient *n*-octanol/water (P_{oct} waarde). Uit quantitatieve structuur-activiteitsrelatie (QSAR) studies blijkt dat de toxiciteit goed te beschrijven is als een functie van de P_{oct} waarde met uitzondering van de effecten op de nitrificatie.

Subletale effecten in de regenboogforel (*Salmo gairdneri*) na acute belasting met thiram (tetramethylthiuram disulfide) worden beschreven in hoofdstuk 3. Thiram blijkt veranderingen te induceren in zowel de proteïne-gebonden als de niet proteïne-gebonden sulfhydryl niveaus, alsmede in de lactaat dehydrogenase en glucose-6-fosfaat dehydrogenase activiteit en de bloedglucose spiegels. Ook blijkt het lipid gehalte in de lever te worden verhoogd. Verder induceert thiram leukopenie, een daling in het hemoglobine gehalte en een toename van de osmotische waarde van het bloed.

In hoofdstuk 4 worden twee toxicologische testprocedures besproken voor chronisch onderzoek met *D. magna*. Met de eerste methode, een 21-daagse semistatische toets met organismen van één leeftijdsklasse (cohorten), kan aan de hand van dagelijks geregistreerde effecten op de overleving en reproductie de exponentiële populatiegroeiconstante berekend worden. De methode is simpel, accuraat en statistische bewerking van de resultaten is mogelijk. Chemisch-analytische verificatie van de testconcentraties is niet strict noodzakelijk.

Een tweede methode is toegepast om de effecten van toxische stress te bestuderen in een gelimiteerd milieu (logistische groei). Dit type experiment wordt uitgevoerd in een doorstroomopstelling. Begonnen wordt met kleine populaties met een stabiele leeftijdsstructuur die bestaan uit cohorten van verschillende leeftijd. De methode is in oecologisch opzicht relevanter dan de eerste, maar bewerkelijker en chemisch analytische verificatie van de concentraties van de testverbindingen is noodzakelijk. Geconcludeerd wordt dat de introductie van populatie-dynamica in chronische toetsen met *D. magna* een belangrijke rol kan vervullen in het ecotoxicologisch onderzoek.

De chemisch-analytische beperkingen zijn er de reden voor dat de chronische toxiciteit van DC's voor *D. magna* getoetst is op de semistatische wijze. De resultaten worden in hoofdstuk 5 beschreven. Overleving, reproductie en individuele groei worden gereduceerd door DDC's en BDC's bij concentraties van respectievelijk circa 10 en 80 μg/l. QSAR's zijn berekend voor een beperkt aantal van de getoetste stoffen. Over de brede range van lipofiliteit (10^7), blijkt dat de toxiciteit te beschrijven is als een sigmoide functie van de verdelingscoëfficiënt *n*-octanol/water.

Verschillen in gevoeligheid van de vroege levensstadia van de regenboogforel is het onderwerp van hoofdstuk 6. Steeds duidelijker wordt dat met betrekking tot de toxische effecten van verontreinigende stoffen, vroege levensstadia de kritieke schakels vormen in de levenscyclus van vissen. In vergelijking met toetsen die de gehele levenscyclus van vissen omvatten betekent het uitvoeren van embryolarvale testen een aanzienlijke tijdbesparing, zonder dat dit ten koste gaat van een verlies aan gevoeligheid.

Embryolarvale toetsen met *S. gairdneri* echter, blijven bewerkelijk en vistoxiciteits-

gegevens schaars, zodat een verdere reductie van tijd benodigd voor het verkrijgen van deze gegevens noodzakelijk is. Ter detectie van het meest gevoelige stadium in de ontogenese van vissen zijn simultaan accumulatie- en toxiciteitsproeven uitgevoerd. Geconcludeerd wordt dat kortdurende experimenten met jong broed, na dooierzak resorptie, een praktische benadering zijn om grenswaarden van stoffen voor vissen vast te stellen. Voor stoffen die sterk kunnen accumuleren moet echter de toets in het dooierzaklarvale stadium worden gestart. Wanneer teratogene effecten moeten worden bestudeerd dient de blootstellingsperiode ook de vroeg embryonale stadia te omvatten.

Dithiocarbamaten zijn vruchtbeschadigend in zoogdieren, vogels en amfibieën. Er waren echter geen gegevens voorhanden over hun teratogene effecten in vissen, wat de aanleiding was tot het verrichten van embryolarvaal onderzoek met *S. gairdneri*. De resultaten zijn beschreven in hoofdstuk 7. Zowel BDC's als DDC's blijken vooral voor de jonge levensstadia van de regenboogforel zeer toxisch te zijn. De toxiciteit die zich met name manifesteert gedurende de eerste helft van de embryonale ontwikkeling gaat naast sterfte, gepaard met een grote mate van beschadiging van de vrucht. Enkele DDC's vertonen embryotoxiciteit beneden het ppb-niveau. Alle stoffen met inbegrip van koolstofdisulfide blijken teratogeen en misvormen met name de wervelkolom. Deze deformaties lijken sterk op de teratogene effecten die ten gevolge van deze stoffen zijn waargenomen bij warmbloedigen en amfibieën. Vissen met grote skelet deformaties blijken niet in staat zich op normale wijze voort te bewegen, hetgeen, wanneer de testen niet tijdig worden beëindigd, leidt tot uithongering gevolgd door sterfte.

Hoofdstuk 8 beschrijft de histopathologische effecten van enkele dithiocarbamaten. In forellen, belast gedurende de embryolarvale ontwikkeling, blijken de misvormingen zich primair te beperken tot de chorda dorsalis. Geconstateerd is dat zowel de lengte als diameter van dit embryonale steunorgaan toeneemt. In plaats van staafvormig was het orgaan in alle mogelijke richtingen geknikt en gebogen. Ook is ectopische botvorming waargenomen. Andere effecten zoals de misvorming van de histoarchitectuur van myomeren en myosepta en orgaandislocaties blijken nauw gerelateerd aan de effecten op de chorda. Via röntgenanalyses is vastgesteld dat er compressie en fusie van wervels en andere abnormaliteiten in het skelet optreden. Subchronische belasting van juveniele regenboogforellen met verschillende DC's blijkt een concentratie afhankelijke afname in het lever glycogeen gehalte te induceren. Proliferatie van galgang epitheel en necrose van individuele hepatocyten is waargenomen bij relatief hoge blootstellingsniveaus. Enkele verbindingen veroorzaken aanzienlijke bloedingen in hersenen en ruggemerg.

In hoofdstuk 9, worden resultaten gepresenteerd van het bioaccumulatie onderzoek met zink derivaten van de twee groepen, te weten ^{14}C-gelabeld ziram (zink dimethyldithiocarbamaat) en zineb (zink ethyleenbisdithiocarbamaat). De bioaccumulatie, betrokken op de gehele vis, blijkt laag met bioconcentratie factoren kleiner dan 100. De radio-activiteit verspreidt zich snel door de weefsels. Relatief hoge radioactiviteitsniveaus zijn gedetecteerd in de lever, gal en darminhoud zowel voor zineb als ziram, hetgeen duidt op biotransformatie in de lever en excretie via gal. In de experimenten met ziram blijkt ook veel radio-activiteit in ogen en huid te accumuleren. De interne distributie voor deze twee stoffen is verschillend. Ziram heeft de hoogste

weefselconcentratie factoren. De eliminatie van radio-activiteit verloopt in twee fasen, waarbij een initiële snelle fase wordt gevolgd door een beduidend langzamere. De resultaten zijn bevestigd middels autoradiografisch onderzoek. De waargenomen verschillen in toxico-kinetiek tussen zineb en ziram verklaren mogelijk ten dele de verschillen in toxiciteit voor vissen tussen BDC's en DDC's.

Uit het gepresenteerde onderzoek alsmede uit andere publicaties blijkt dat deze stoffen cytotoxisch en nauwelijks selectief zijn, zodat DC's beschouwd moeten worden als breed-spectrum biociden:

- dithiocarbamaten zijn nauwelijks bioaccumulatief en matig persistent;
- nitrificatie en andere microbiële processen kunnen worden gestoord als DC's of hun degradatie producten geloosd worden in het aquatische milieu;
- de verbindingen zijn toxisch voor algen en zeer toxisch voor kreeftachtigen;
- dithiocarbamaten blijken teratogeen in visbroed bij extreem lage concentraties;
- mutagene en carcinogene effecten zijn gerapporteerd voor enkele van deze stoffen.

Dit betekent dat DC's een serieuze bedreiging vormen voor het functioneren van aquatische oecosystemen. Op basis van dit onderzoek worden als voorlopige waterkwaliteitscriteria voor BDC's en DDC's concentraties van respectievelijk 1 en 0.1 μg/l voorgesteld. De huidige praktijk van productie en gebruik maakt het waarschijnlijk dat in het ontvangende water toxische concentraties worden bereikt. Dit maakt een restrictief beleid noodzakelijk. Tot nog toe heeft dit geleid tot de volgende acties:

- in het kader van de Wet Verontreiniging Oppervlaktewateren zijn saneringsmaatregelen opgelegd aan dithiocarbamaat-producerende bedrijven teneinde te komen tot een aanzienlijke beperking van de watervervuiling veroorzaakt door de lozing van deze stoffen;
- in het kader van de Wet Verontreiniging Oppervlaktewateren wordt vereist dat afvalwater van bovengenoemde en andere bestrijdingsmiddelen producenten alsmede formuleringsbedrijven regelmatig wordt getoetst middels bioassays;
- De Dienst Binnenwateren/RIZA heeft de toepassing van dithiocarbamaten als complexvormers voor de behandeling van afvalwater verontreinigd met zware metalen ontraden. Dit, vanwege het feit dat het middel veelal erger is dan de kwaal;
- in het kader van de Bestrijdingsmiddelenwet wordt een restrictief beleid gevoerd aangaande het gebruik van dithiocarbamaten als slijmbestrijdingsmiddel en koelwaterbiocide. Het gebruik van deze stoffen wordt alleen toegestaan indien schade aan aquatische systemen kan worden voorkomen. Dit hangt o.a. af van de wijze van toediening, het aanwezig zijn van zuiveringstechnische voorzieningen en de actuele condities waaronder lozingen plaatsvinden. Bovendien zullen etiketten van biociden welke dithiocarbamaten als actieve stoffen bevatten worden voorzien van een waarschuwing betreffende het potentiële gevaar voor vissen en andere waterorganismen;
- in het kader van het Verdrag van Parijs is een discussie geopend omtrent de plaatsing van dithiocarbamaten op de zwarte lijst.

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