

THE ENERGETICS OF STRESS TOLERANCE IN THE EARLY LIFE
STAGES OF THE GOLDFISH *Carassius auratus* L.

A Thesis submitted to the University of Stirling
for the Degree of

Doctor of Philosophy

By

MONORANJAN DAS

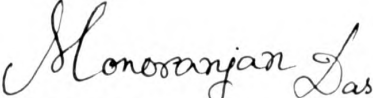
Institute of Aquaculture
University of Stirling
Stirling
Scotland

January 1995

DECLARATION

This thesis presents the research work conducted by the candidate at the Institute of Aquaculture, University of Stirling, during the period September, 1991 to January, 1995. The thesis has been composed by the candidate and no part of this work has been submitted for any other degree. All work referred to has been duly acknowledged.

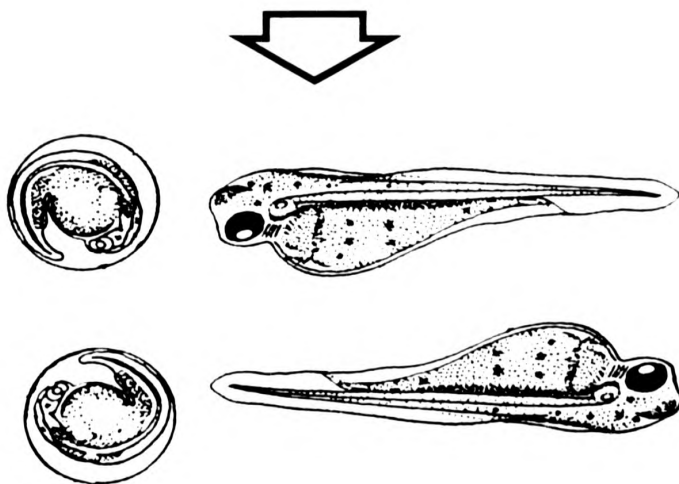
Signature of candidate:

A handwritten signature in cursive script that reads "Monoranjan Das". The letters are fluid and connected, with a prominent initial 'M'.

MONORANJAN DAS

DEDICATION

To baby fishes, whose sacrifice of life and pain of stress make this thesis possible



ACKNOWLEDGEMENT

I hereby express profound gratitude to my two supervisors Dr. Donald J Baird and Dr. Krishen J Rana for their advice, encouragement and supervision throughout the course of this research and for critically reviewing this thesis. I remain eternally grateful to both of my supervisors for their patience and understanding during the time of my difficulties.

I would like to acknowledge Dr. Fiona McLaren, Graham Taylor, and William Struthers for assistance with analytical work, Keith Ranson, Brian Howie, Drew Milroy, William Hamilton for assistance with system making and repairing, Sultan Mahmood, L.P. Jayatissa and Ann Nimmo for feeding fish in my absence and above all to all members of staff and colleagues of the Institute for their friendliness and necessary help.

I would also like to thank my friends at Stirling for their friendliness.

In addition I would like to express my deep sense of gratitude to my father, brothers, sisters and relatives for their blessings and regular writings, which helped me to feel relaxed particularly during my stress moments.

I am grateful to The Commonwealth Scholarship Commission in the United Kingdom, for financial support through the provision of a Commonwealth Academic Staff Scholarship, The Registrar, Bangladesh Agricultural University, Mymensingh, Bangladesh for granting my study leave and The British Council (Dhaka, London and Manchester) for look after my interests, during the period of this study.

Finally, I would like to thank my wife, Anjana, for her help, encouragement and lovely company. Thanks are due to my son, Anupam, and daughter, Mandira, for helping me in editing the manuscript/typescript and for sacrificing their due share of my time in favour of my study.

ABSTRACT

A short-term fish early life-stage toxicity test using sac-fry of the goldfish *Carassius auratus* L. was developed using a simple multichannel flow-through system. The physiological mechanisms behind the routinely used sublethal test endpoints of growth were investigated by examining energy partitioning within the developing sac-fry, and alternative endpoints of ammonia excretion and oxygen consumption were also investigated.

A technique was developed for goldfish broodstock management which gave the potential for year-round production of eggs and sac-fry for laboratory experimentation. This was achieved by holding two females and one male each in isolation in 120 litre tanks with 14L : 10D photoperiod at 20°C. Females were conditioned by induced ovulation by spiking the tank water with 3 µg.l⁻¹ of goldfish ovarian fluid, avoiding the need for stressful handling and/or temperature shocking normally required for induced spawning. Additionally, this technique allowed the repeated spawning of the same fish throughout the year. Eggs obtained by this method hatched after five days incubation at 20°C and reached a maximum body mass (in the absence of an external food supply) 4 days after hatching.

Three chemicals: cadmium chloride, 3,4-dichloroaniline (DCA) and chlorpyrifos, differing in water solubility and exhibiting different modes of action, were used as reference compounds to examine the physiological responses to toxic chemical stress in goldfish sac-fry during the period prior to first feeding. In preliminary studies of acute lethal toxicity, cadmium was the least toxic (96 h LC50 = 3404 µg.l⁻¹), DCA was intermediately toxic (96 h LC50 = 2359 µg.l⁻¹) and chlorpyrifos the most toxic (96 h LC50 = 22.6 µg.l⁻¹). Further studies with DCA which examined the effect of exposing from the egg stage (age 30 h) indicated that for this compound at least, including the egg stage reduced test sensitivity (96 h EC50 = 3332 µg.l⁻¹). It was concluded that the egg stage was generally resistant to toxic exposure when compared with the sac-fry stage, since the former was less likely

to accumulate toxic chemicals due to its possession of a relatively impermeable chorionic membrane.

A simple conceptual model of the effects of toxic chemical exposure on energy partitioning in developing fish sac-fry was developed and tested. Among the physiological endpoints investigated, growth, yolk utilization efficiency and oxygen to nitrogen ratio (O : N) were equally sensitive for all three chemicals tested, with no-effect concentrations (NOECs) of 500 $\mu\text{g.l}^{-1}$ for cadmium, 25 $\mu\text{g.l}^{-1}$ for DCA and 0.25 $\mu\text{g.l}^{-1}$ for chlorpyrifos. Oxygen consumption was significantly ($P < 0.05$) affected by two of the chemicals tested: cadmium (NOEC = 500 $\mu\text{g.l}^{-1}$) and DCA (NOEC = 25 $\mu\text{g.l}^{-1}$) but was unaffected at all concentrations of chlorpyrifos tested. In contrast, increased ammonia excretion was consistently the most sensitive parameter measured for all three chemicals tested: cadmium (NOEC = 250 $\mu\text{g.l}^{-1}$), DCA (NOEC = 25 $\mu\text{g.l}^{-1}$) and chlorpyrifos (NOEC = 0.25 $\mu\text{g.l}^{-1}$).

More detailed studies of patterns of oxygen consumption under sublethal chemical exposure revealed that oxygen consumption was indeed sensitive to low-level exposures. The complex of interacting factors determining oxygen consumption resulted in a non-monotonic response which was difficult to interpret due to confounding causal factors. In contrast ammonia excretion gave a simple monotonic response increasing with exposure, in line with predictions on increased protein breakdown under sublethal toxic exposure, indicating its potential value as an endpoint in fish early life-stage tests.

CONTENTS

	Page
Declaration	i
Dedication	ii
Acknowledgement	iii
Abstract	iv
Contents	vi
List of Tables	xi
List of Figures	xii
Terminology used in this Thesis	xvi
List of Abbreviation used in this Thesis	xvii
CHAPTER 1 GENERAL INTRODUCTION	1
1.1 The science of ecotoxicology	1
1.2 Environmental stress and response to stress	5
1.2.1 Stress concept	5
1.2.2 Response to stress: advantage of using physiological responses	7
1.3 The use of short-term fish early life-stage toxicity test in ecotoxicology	11
1.4 The goldfish as a test organism in aquatic toxicology studies	15
1.5 Toxicants	18
1.5.1 Cadmium	19
1.5.2 3,4-dichloroaniline	21
1.5.3 Chlorpyrifos	22
1.6 Fish early life-stage toxicity test endpoints	27

1.7 Energy budget: its advantage as stress indicator	30
1.8 Aims of the thesis	35
CHAPTER 2 GENERAL MATERIALS AND METHODS	36
2.1 Procurement of eggs and sac-fry	36
2.1.1 Brood stock rearing and egg incubation system	36
2.1.2 Fertilization and incubation of eggs	40
2.1.3 Selection of experimental sac-fry	41
2.2 Dilution water	45
2.3 Toxicants solution	47
2.3.1 Cadmium solution	47
2.3.2 3,4-dichloroaniline (DCA) solution	47
2.3.3 Chlorpyrifos solution	48
2.4 Flowthrough system	49
2.5 Preparation of the system for experiments	54
2.6 Monitoring toxicants concentration	54
2.7 Statistical analysis	59
CHAPTER 3 YEAR-ROUND SPAWNING OF AND EARLY LIFE HISTORY TRAITS OF GOLDFISH	60
3.1 Introduction	60
3.1.1 Year-round spawning of gold fish	60
3.1.2 Early life history traits of goldfish in the flow-through system	68
3.1.3 Aims	69
3.2 Materials and Methods	70
3.2.1 Techniques of ovulation of goldfish under controlled laboratory conditions	70
3.2.1.1 Preliminary experiments	70
(a): Ovulation of goldfish by raising water temperature and changing photoperiod	70
(b): Ovulation of goldfish by pimozide and LHRH-a injection	71
3.2.1.2 Ovulation of goldfish by inoculating ovarian fluid into the water of spawning tanks	72

3.2.2	Optimisation of broodfish stocking density for procuring all-year-round good quality eggs and sac-fry	73
3.2.3	Effects of selected spawning substrate on the goldfish early life-stages	74
3.2.4	Survival of goldfish sac-fry in the exposure chamber of the flowthrough toxicity testing system	74
3.2.5	Hatching success at different loading densities of eggs in the exposure chamber of the toxicity testing system	76
3.2.6	Oxygen and ammonia concentration at different densities in the exposure chamber of the toxicity testing system	76
3.2.7	Early life history growth traits of goldfish in the exposure chamber of the toxicity testing system	77
3.3	Results	78
3.3.1	Induced spawning of goldfish	78
3.3.2	Conditioning of goldfish brood stock at different densities under 20°C and 14L : 10D photoperiod	80
3.3.3	Performance of goldfish early life-stages in the incubation system and in the exposure chambers of the toxicity testing system	83
3.4	Discussion	91
3.4.1	Induced spawning of goldfish	91
3.4.2	Conditioning of broodfish at different stocking densities under 20°C and 14L : 10D photoperiod	93
3.4.3	Performance of goldfish early life-stages in the incubation system and in the exposure chambers of the toxicity testing system	94
CHAPTER 4 PATTERNS OF LETHAL TOXICITY IN GOLDFISH EGGS AND SAC-FRY		97
4.1	Introduction	97
4.1.1	Range finding test	99
4.1.2	Definitive test	100
4.1.3	Sensitivity of fish egg and sac-fry to stress	100
4.2	Materials and Methods	103
4.2.1	Lethal effects of cadmium, DCA and chlorpyrifos on goldfish sac-fry	103
4.2.1.1	Range finding test with cadmium DCA and chlorpyrifos	103
4.2.1.2	Definitive test with cadmium, DCA and chlorpyrifos on goldfish sac-fry to estimate LC50	104
4.2.2	Definitive test with DCA on goldfish eggs to estimate EC50	105
4.2.3	Statistical analysis	106

4.3 Results	106
4.3.1 Cadmium	107
4.3.2 3,4-dichloroaniline	107
4.3.3 Chlorpyrifos	108
4.4 Discussion	116
CHAPTER 5 SUBLETHAL PHYSIOLOGICAL EFFECTS OF TOXICANTS AND VALIDATION OF A MODEL OF FISH EARLY LIFE-STAGE SENSITIVITY	120
5.1 Introduction	120
5.1.1 Yolk absorption	123
5.1.2 Growth	124
5.1.3 Oxygen consumption	126
5.1.4 Nitrogen excretion	127
5.1.5 Oxygen to nitrogen ratio	128
5.1.6 Energy budget components of fish sac-fry	129
5.1.7 Aims of the study	130
5.2 Materials and Methods	131
5.2.1 Stock solution and exposure concentration of toxicants.	131
5.2.2 Exposure of sac-fry to sublethal concentrations	132
5.2.3 Oxygen consumption and ammonia excretion	132
5.2.4 Yolk utilization and larval growth	135
5.2.5 Oxygen to nitrogen ratio (O : N)	136
5.2.6 Energy budgets	136
5.2.7 Oxygen consumption over the period of sublethal exposure experiments	138
5.3 Results	142
5.3.1 Yolk utilization and somatic weight gain under sublethal stress	142
(a) cadmium	142
(b) DCA	143
(c) chlorpyrifos	143
5.3.2 The effects of sublethal stress of cadmium, DCA and chlorpyrifos on oxygen consumption, ammonia excretion and oxygen to nitrogen ratio (demand-side effect)	148
5.3.3 Energy budget of goldfish sac-fry under sublethal stress of cadmium, DCA and chlorpyrifos	153
5.3.4 Oxygen consumption over the period of sublethal exposure of cadmium, DCA and chlorpyrifos	158
5.4 Discussion	165

CHAPTER 6 GENERAL DISCUSSION

173

REFERENCES

184

List of Tables

Table 1.1	General information about cadmium.	24
Table 1.2	General information about 3,4-dichloroaniline (DCA).	25
Table 1.3	General information about chlorpyrifos.	26
Table 2.1	Chemical composition of dilution water (M7 medium)	46
Table 3.1	Rematuration of female goldfish at different combination of temperature and photoperiod (Calculated from Razani <i>et al.</i> , 1989).	65
Table 3.2	Ovulation success in <i>Carassius auratus</i> broodstock following different periods of low temperature conditioning.	81
Table 3.3	Ovulation success in <i>Carassius auratus</i> broodstock by injecting pimozide and LHRH-a at 20°C.	81
Table 3.4	Ovulation success in <i>Carassius auratus</i> broodstock held in different concentrations of ovarian fluid at 20°C.	82
Table 3.5	Rematuration of <i>Carassius auratus</i> stocked at different densities in the laboratory controlled condition (at 20°C; 14L : 10D) and ovulation using ovarian fluid.	82
Table 4.1	Acute toxicity of cadmium, DCA and chlorpyrifos on goldfish sac-fry.	109
Table 5.1	The observed actual cadmium concentration of the nominal exposure concentration during the exposure period of goldfish sac-fry. Mean given with \pm standard error, n = 6.	144
Table 5.2	The observed actual DCA concentration of the nominal exposure concentration during the exposure period of goldfish sac-fry. Mean given with \pm standard error, n = 6.	144
Table 5.3	The energy budget of <i>Carassius auratus</i> sac-fry under sublethal stress of cadmium, DCA and chlorpyrifos, expressed as a rate ($\text{mJ} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$).	154
Table 5.4	Sublethal responses in goldfish sac-fry toxicity tests with cadmium, DCA and chlorpyrifos. NOEC and LOEC were calculated by ANOVA at a critical probability $P \leq 0.05$.	163

Table 5.5 The sensitivity of sublethal physiological responses (LOEC) of goldfish in comparison to lethal responses (LC50) for different test species for the equivalent exposure period.

164

List of Figures

- Figure 2.1 Water recirculatory system for rearing brood stock. The dashed line with arrow indicates direction of flow. 38
- Figure 2.2 Egg incubation recirculatory system. The dashed line with arrow indicates direction of flow. 39
- Figure 2.3 Egg incubation in an incubation chamber. Eggs attached to framed petridish. 42
- Figure 2.4 Development of goldfish egg and embryo in the laboratory, (a) fertilized egg (10-15 min), (b) first cleavage (30-40 min), (c) second cleavage (1-2 h), (d) advanced stage of cleavage (6-7 h), (e) blastula (9-10 h), (f) an embryonic stage (48 h), (g) eyed stage (72 h), (h) advanced stage of embryo (96 h), (i) sac-fry larvae shortly after hatching (125 h) and (Y) yolk mass. 43
- Figure 2.5 Goldfish sac-fry larvae after hatching, (a) normal sac-fry, (b) abnormal sac-fry, (c) lordosis, (d) scoliosis, (e) abnormal yolk sac (f) Pericardial oedema and (y) yolk. 44
- Figure 2.6 A schematic diagram of the flowthrough system in (a) plan view, showing three channels, and (b) elevation view showing a single independent channel. 53
- Figure 2.7 Relationship between nominal and actual concentration of cadmium. 57
- Figure 2.8 Relationship between nominal and actual concentration of DCA. 58
- Figure 3.1 A view of the eggs attached on the glass slides placed on a slide rack in a chamber in the incubation system. 75
- Figure 3.2 Percentage of fertilization, hatching and normal sac-fry emerged on hatching of gold-fish eggs attached to two substrates (Polystyrene petridish & Glass slides) incubated in to the incubation system. Bars are mean of three replicates given with + standard error of mean. 85
- Figure 3.3 Mortality pattern of goldfish sac-fry at different densities exposed to dilution water in the exposure chambers of the toxicity testing system. 86

Figure 3.4	Percentage of hatching success of goldfish eggs attached on glass slides and incubated into exposure chambers of the toxicity testing system at different densities. (Bar indicate \pm standard error).	87
Figure 3.5	Influence of stocking density of goldfish sac-fry on the oxygen concentration in exposure chamber of the flowthrough system. Data are means \pm standard errors. Critical level for toxicity tests is shown by a line.	88
Figure 3.6	Influence of stocking density of goldfish sac-fry on the ammonia concentration in exposure chamber of the flowthrough system. Data are means \pm standard errors. Critical level for toxicity tests is shown by a line.	88
Figure 3.7	Pattern of growth and yolk utilization of goldfish sac-fry expressed as (a) dry mass, (b) wet mass. Data are means \pm standard errors.	89
Figure 3.8	The ratio of dry mass to wet mass of gold fish sac-fry body and yolk over the yolk absorption period.	90
Figure 4.1	Mortality pattern of goldfish sac-fry exposed to different concentrations of cadmium with time. Group size = 90.	110
Figure 4.2	Mortality pattern of goldfish sac-fry exposed to different concentrations of DCA with time. Group size = 90.	111
Figure 4.3	Mortality pattern of goldfish sac-fry exposed to different concentrations of chlorpyrifos with time. Group size = 90.	112
Figure 4.4	LC50 values of cadmium at 48, 72, and 96 h for goldfish sac-fry. Note that 95% CL of the LC50 are small, and hence not easily visible on the plot. (See Table 4.1)	113
Figure 4.5	LC50 values of DCA at 48, 72, and 96 h for goldfish sac-fry. Note that 95% CL of the LC50 are small, and hence not easily visible on the plot. (See Table 4.1)	114
Figure 4.6	LC50 values of chlorpyrifos at 24, 48, 72 and 96 hours for goldfish sac-fry. Note that 95% CL of the LC50 are small, and hence not easily visible on the plot. (See Table 4.1)	115

- Figure 5.1 A conceptual model of the flow of energy through a sac-fry, showing that toxic effects of chemicals may result in reduced energy supply ("-ve") and/or in increased energy demand ("+ve"). 122
- Figure 5.2 The possible effect of (i) duration of toxicant exposure and (ii) intensity of toxicant exposure on oxygen consumption. A - alarm reaction, B - resistance, C - exhaustion. 141
- Figure 5.3 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on body weight gain of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 145
- Figure 5.4 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on yolk utilization by goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 146
- Figure 5.5 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on yolk utilization efficiency of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 147
- Figure 5.6 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on rate of oxygen consumption by a group of 30 goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 150
- Figure 5.7 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on rate of ammonia excretion by a group of 30 goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 151
- Figure 5.8 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on oxygen to nitrogen ratio of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference. 152
- Figure 5.9 Allocation of assimilated energy to growth, respiration and excretion by the goldfish sac-fry under sublethal (i) cadmium, (ii) DCA and (iii) chlorpyrifos exposure. U - excretion, R - respiration and P₂ - observed growth. 155

- Figure 5.10 Hourly energy budget showing percentage energy distribution of expected growth, respiration and excretion under different level of sublethal (i) cadmium, (ii) DCA and (iii) chlorpyrifos exposure. U - excretion, R - respiration, P_1 - expected growth (scope for growth). 156
- Figure 5.11 Relationship between observed growth and scope for growth of goldfish sac-fry under (i) cadmium, (ii) DCA and (iii) chlorpyrifos sublethal exposure. Growth and scope for growth are expressed as rate in energy unit ($\text{mJ}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}$). P_1 - scope for growth, P_2 - observed growth. 157
- Figure 5.12 Oxygen consumption pattern of goldfish sac-fry under sublethal exposure of (i) cadmium, (ii) DCA and (iii) chlorpyrifos. Data are means \pm standard errors. 161
- Figure 5.13 Effects of sublethal exposure concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on the oxygen consumption pattern of goldfish sac-fry. Data are means \pm standard errors. 162

Terminology used in this Thesis

Body	: the somatic tissue of the developing sac-fry including the yolk sac but excluding the yolk.
Lowest observed effect concentration	: refers to the lowest concentration which has significant effect in comparison to control at 95% level.
Embryo	: the developing fish before hatching.
Fry	: the developing fish after initiation of exogenous feeding.
Larvae	: the term larvae used loosely, which refers to sac-fry and/or fry.
Lethal response	: refers to the response determined by immobility of sac-fry and immobility plus nonviability of embryo at hatching.
No effect concentration	: refers to the highest concentration which has insignificant effect in comparison to control at 95% level.
Sac-fry	: the developing fish between hatch and completion of yolk.
Scope for growth	: energy available for growth calculated by solving energy budget equation.
Starved fry	: the developing fish after reaching maximum body mass on yolk and with no external source of food available.
Stress	: refers to a stimulus capable of disturbing physiological process.
Stress response	: refers to the reaction of the biological system when stress is applied.

List of Abbreviation used in this Thesis

C	: yolk energy consumed.
DCA	: 3,4-dichloroaniline.
EC50	: the concentration of a chemical that causes 50% an acute response other than death (<i>e.g.</i> egg nonviability).
EL	: embryo-larval.
ELS	: early life stage.
h	: hour.
LC50	: the concentration of a chemical that cause 50% lethal response in terms of death.
LOEC	: lowest observed effect concentration.
MATC	: maximum acceptable toxicant concentration.
NOEC	: no observed effect concentration.
O:N	: oxygen to nitrogen ratio (oxygen consumption to ammonia excretion calculated in atomic equivalents).
P	: energy incorporated in growth.
P ₁	: scope for growth.
R	: energy respired.
U	: energy excreted as ammonia.

CHAPTER 1

GENERAL INTRODUCTION

There are many ways in which we alter the freshwater environment. Agriculture is responsible for 68% of our water pollution (Caduto, 1990). Other sources of pollution include industry, paper mills, polluted precipitation and urban run-off. The world's chemical industries now produce over 50,000 chemicals in quantities greater than one ton per annum, and estimated 1000 new chemicals are added to the list each year (Bourdeau, 1984). Risk, which is defined as the scientific judgement of the probability of harm and safety, defined as the value judgement of the acceptability of a given risk (Cairns, 1989) to evaluate the chemicals remains the central point of the debate concerning the management of chemical impacts on the natural environment (Baird, 1994). The control and evaluation of the ecological effects of an ever-increasing list of chemicals has become a key environmental challenge to scientists. To take up this challenge a new scientific discipline 'ecotoxicology' has emerged.

1.1 The science of ecotoxicology

Ecotoxicology was first defined by Truhaut in 1969 (reported in Truhaut, 1977) as 'the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context'. Published definitions over

the past 20 years demonstrate that there is a difference in opinion concerning the scope and methodology of ecotoxicology. Moriarty (1991) defined ecotoxicology as the study of the effects of pollutants on ecosystems, where pollutants are defined as substances that occur in the environment at least partly as a result of man's activities, and which have deleterious effects on living organisms. Butler (1984) considered ecotoxicology as a study of the effects of released pollutants on the environment and on the biota that inhabit it. It could be said that man is the most important species of the earth's biota and thus the effects of pollutants on human health are central to ecotoxicology. There are also close connections between human activities and ecotoxicology: (a) human beings not only alter the environment but also produce and release pollutants and (b) all changes in the environment or its biota may affect directly or indirectly, the physical, economic or aesthetic well-being of mankind. Klaassen and Eaton (1991) defined ecotoxicology as a specialized area within environmental toxicology that focuses more specially on the impacts of toxic substances on population dynamics within an ecosystem. Forbes and Forbes (1994) defined ecotoxicology as the field of study which integrates the ecological and toxicological effects of chemical pollutants on populations, communities and ecosystems with the fate (transport, transformation and breakdown) of such pollutants in the environment.

The field of aquatic toxicology has evolved as the qualitative and quantitative study of the adverse or toxic effects of chemicals and other anthropogenic materials or xenobiotics on aquatic organisms. Toxic effects include both lethality (mortality) and sublethal effects such as changes in growth, development, reproduction,

pharmacokinetic responses, pathology, biochemistry, physiology and behaviour. Aquatic toxicology is also concerned with the concentration or quantities of chemicals that can be expected to occur in the aquatic environment in water, sediment or food (Rand and Petrocelli, 1985). Forbes and Forbes (1994) considered the definition of aquatic toxicology (given by Rand and Petrocelli, 1985) as equivalent to a definition of aquatic ecotoxicology.

Although ecotoxicology is a multidisciplinary subject which has its roots in both toxicology and ecology, ecotoxicology has a different research agenda (Butler, 1984). Ecotoxicological research is designed to achieve one or more of the following objectives, (1) to generate data to provide the basis for strategies of risk assessment and for environmental decision making, (2) to meet legal requirements regulating the development, manufacture or release of potentially dangerous substances and (3) to develop empirical or theoretical principles to further understanding of the behaviour and effects of chemicals in living systems (Forbes and Forbes, 1994).

In general, ecotoxicology can be defined as the science of studying potential chemical effects and fate of chemicals in the ecosystem. Thus an emphasis is needed on prediction rather than description, on solutions rather than problems. The need to predict the environmental impacts of chemical substances has led to the implementation of guidelines and legislation which require, among other things, that chemicals must pass an ecotoxicological evaluation programme before being marketed, in order to avoid damage to the ecosystem. The best way to predict the

effects of a chemical is the study of the effects on ecosystems but it is impossible to test the effects of all of the chemicals on ecosystems due to the long list of chemicals and also due to the complexity of ecosystems. Alternatively, prediction can be made by acquiring acute and chronic toxicity information about a chemical for organisms with a range of sensitivities or even for a few key species, however, this goal is seldom achieved (Giesy and Graney, 1989). Therefore, ecotoxicologists are forced to make predictions about chemicals from the short-term toxicity test results under laboratory conditions, although there is no one perfect, short-term test which will allow prediction of effects at the ecosystems level because there are complex interaction among individuals, species and their environment. Most of the laboratory based short-term toxicity tests still suffer from uncertainties and weaknesses to be ecologically relevant tests. There is still scope for improvement of laboratory based toxicity tests to make them more aligned to ecologically relevant tests, thus research is needed for the development of more relevant test concepts and methodologies, to be integrated into the ecotoxicological evaluation programme, and research is also needed to provide a sound scientific background for the test developed.

1.2 Environmental stress and response to stress

1.2.1 Stress concept

The concept of stress in biology has been defined by various authors in different ways. All definitions of stress incorporate the concept of a 'stimulus' and the effects of the applied stimulus on the biological system (the response) but the concepts used are often different. The word 'stress' was first used in medicine and human physiology. The classical work on the physiological response of an animal to stress was pioneered by Hans Selye, who defined stress as an applied stimulus and its response (Selye, 1946). He (Selye, 1950) applied it as the sum of all the physiological responses, by which an animal tries to maintain or re-establish normal metabolism in the face of a physical or chemical force. However, in a later work Selye (1974) redefined it as the response of the system to a stimulus, the stimulus being termed the 'stressor'.

Brett (1958) defined stress as any environmental factor (*i.e.* stimulus) which extends the normal adaptation response of an animal, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced. This definition did not recognize the effects that do not affect survival of an animal but still can affect the animal by reducing growth and/or reproductive capacity, which have consequences for long term survivorship of the species. Esch and Hazan (1978) defined stress as the effect (*i.e.* response) of any environmental alteration or force that extends homeostatic or stabilizing processes beyond their

normal limits, at any level of biological organization, whereas Ivanovici and Wiebe (1981) defined stress as 'response' in terms of reduced adenylate energy charge (see section 1.2.2).

Recently, the concept of stress was examined in a symposium organised by the Linnean Society of London, held in June 1988 to clarify and consider its use as a commonly defined meaning in ecology and evolutionary biology. Grime (1989) placed the idea of stress in its original physical context (force per unit area), for use in ecological and evolutionary research in the same manner as its use in physics to establish a useful commonality between stress analyses conducted upon living and non-living structure, and described stress as 'external constraints limiting the rates of resource acquisition, growth and reproduction of organisms'. Sibly and Calow (1989) defined stress in general as an environmental condition that, when first applied, impairs Darwinian fitness, by reducing survivorship and/or fecundity and/or increasing the time between life cycle events. Bradshaw and Hardwick (1989) defined stress as anything which reduces growth or performance. Koehn and Bayne (1989) consider stress as an environmental change that results in reduction of net energy balance. However, among the above definitions of stress, the definition given by Koehn and Bayne (1989) is the most appropriate in ecotoxicology to study the potential effects of chemicals in the ecosystem (for a more detailed explanation of the logic behind this concept, see section 1.2.2).

If energy resources are limiting either in terms of availability or in terms of an organism's capacity to acquire them, resources must be apportioned wisely into

physiological components. The fitness of a population depends on the interaction of individuals with their environment. A major part of this interaction involves the uptake and utilization of resources and depends largely on physiological functioning (Calow and Townsend, 1981). Therefore, in this thesis the word 'stress' will be used as a 'stimulus' which when applied is capable of disturbing normal physiological functioning of an organism related to energy acquisition and utilization, unless otherwise stated.

1.2.2 Response to stress: advantage of using physiological responses

Selye (1946, 1974, 1976) suggested that all noxious stimuli induce the same general response in mammalian systems and termed this response the General Adaptation Syndrome (GAS). The GAS proceeds through three stages, (1) the alarm reaction, immediately after the stimulus, followed by, (2) resistance, during which the organism continues to function despite the action of the stimulus, leading to, (3) exhaustion, and finally death if the stimulus is of sufficient intensity or duration. Animals exposed to continuous stress for long periods necessarily go through all three phases of GAS. This triphasic nature of GAS recognizes that the body's ability to tolerate stress, and the resources available to achieve this are finite (Selye, 1974, 1976).

Selye's GAS concept is further complicated by a sub-component of the general response, the Local Adaptation Syndrome (LAS)(Selye, 1976), in which stressors or groups of stressors produce specific localized responses. The overall response

is a blend of both specific and general components. Hence, GAS occurs irrespective of the stressors type but how it is manifested may depend on the type and magnitude of the localized specific action of the particular type of stressors. Although it is not universally agreed that GAS occurs in fishes it is widely accepted that the stress response as a whole is characterised by predictable physiological changes in fishes (Wedemeyer and McLeay, 1981). There is no reference to a non-stressed state in Selye's (1950) concept of stress, which makes quantitative and qualitative predictions difficult (Barber, 1990; Pickering, 1981). Subsequent definitions established a control or non-stressed condition that is the baseline response (Brett, 1958) and that stress increased mortality risk (Grime, 1979; Sibly and Calow, 1989). Others (Ivanovici and Wiebe, 1981; Lugo, 1978; Odum, 1967) have emphasized the stress response in terms of the energy demands imposed by stress. For example, Ivanovici and Wiebe (1981) used reduced adenylate energy charge (AEC) as an index of environmental stress which can be calculated from measured amounts of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) whereby

$$\text{AEC} = \frac{\text{ATP} + \frac{1}{2} \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}} \quad (1.1)$$

(Atkinson, 1977; Hochachka and Somero, 1984).

This value of AEC under steady state conditions of the components (ATP, ADP, AMP) was found to be 0.85 (Atkinson, 1977). With increased energy demand the high energy compound 'ATP' converts to ADP and release energy, ATP is produced in metabolism by phosphorylation of ADP.

However, such measurements at the biochemical levels require sophisticated instruments and highly specialized personnel; furthermore, they may not translate into effect at individual and population levels because of homeostatic compensation (Calow, 1989), thus making them difficult to implement in environmental monitoring programmes.

It is now generally accepted that stressed systems may exhibit a resultant decrease in energy availability and/or an increase in energy utilization. Koehn and Bayne (1989) considered the effects of stress primarily from the point of view of the physiological energetics of the organisms, *i.e.* the physiological traits (feeding, absorption and the metabolic costs of maintenance, growth and reproduction) that together comprise the energy budget.

Physiological responses have three important attributes, (1) they represent an integration of the cellular and biochemical processes that can alter the response to environmental changes, (2) they represent non specific (general) responses to the sum of environmental stimuli which are complementary to more specific responses at the biochemical level and (3) they are capable of reflecting deterioration in the environment before effects manifest themselves in the population or the community (Widdows, 1985). Therefore, an integrated measurement of physiological parameters related to energy acquisition and its utilization pattern will provide an important tool to quantify an organism's performance under conditions of environmental stress.

Quantification of an organism's physiological condition (*e.g.* O:N ratio; the ratio between oxygen consumed and nitrogen excreted, calculated in atomic equivalents), its performance (*e.g.* growth rate) and the efficiency (*e.g.* growth efficiency) in response to environmental stress fulfil most of the following criteria of environmental monitoring programmes (Widdows, 1985):

1. reflect a quantitative or predictable relationship with the stress.
2. have ecological significance and be shown or convincingly argued to be related to an adverse or damaging effect on the growth, reproduction or survival of the individual, the population and ultimately the well being of the community.
3. be sensitive to stress and to have a large scope for response throughout the range from optimal to lethal conditions.
4. have a relatively short response time, so that stress impact may be detected in its incipient stages.
5. be measurable with precision and with a high signal to noise ratio so that the effect (signal) may be easily detectable above the natural variability (noise).
6. be easily measured in the laboratory and under ambient conditions.

1.3 The use of short-term fish early life-stage toxicity test in ecotoxicology

Acute toxicity tests have been used extensively to determine the effects of potentially toxic materials such as pesticides, metals and industrial effluents on aquatic organisms during short-term (usually 96 h or less) exposure. Among the aquatic test organisms freshwater fishes have been used extensively in acute toxicity tests due to their importance in the aquatic ecosystem and also because there is more well documented biological information than for other aquatic organisms (Solbe, 1993). In aquatic acute tests, test results are expressed in terms of an LC50 (*i.e.* the concentration of the toxicant at which 50% of the test animals die within 96 or 48 h). Therefore, acute tests have been useful in providing rapid results with minimum cost but from an ecological point of view, acute toxicity may be of limited relevance in the sense that contaminants are frequently present in the natural water at concentrations too low to cause rapid mortality (Lloyd, 1992), but they may impair the physiological functioning of organisms. Many of the impairments resulting from low level exposure have ecological significance, in that they reduce the fitness of an organism in its environment by increasing the allocation of resources required for stress tolerance, reducing the available resource for growth or reproduction (Sibly and Calow, 1986).

A method of assessing low-level exposure effects is the chronic toxicity test. A chronic toxicity test is designed to expose all life-stages of the test animal to a range of chemical concentrations to bracket the threshold concentrations for significant deleterious effects (Petrocelli, 1985). One of the earliest reports of

chronic exposure to consecutive life-stages is Olson and Foster's (1956) summary of sodium dichromate toxicity to the eggs, fry and early juvenile stages of salmonids. An early published account of a full life-cycle toxicity test was that of Mount and Stephan (1967). In their study *Pimephales promelas* were exposed to a series of pesticides concentrations throughout their life-cycle and the effects of the toxicants on survival, growth and reproduction were measured and evaluated in terms of MATC (maximum acceptable toxicant concentration), which has been defined as the hypothetical toxic threshold concentration falling between the highest concentration showing no effect and the next higher concentration showing a significant toxic effect. Subsequently, life-cycle toxicity studies were conducted by many scientists with different fish and chemicals (Eaton, 1970, 1973, 1974; Hansen and Parrish, 1977; McKim and Benoit, 1971, 1974; Mount and Stephan, 1969; Smith, 1973) and no effect concentration (*i.e.* NOEC: no observed effect concentration) and effect concentration (*i.e.* LOEC: lowest observed effect concentration) measured. NOEC and LOEC are defined as the highest concentration of a material in a toxicity test that has no statistically significant ($P > 0.05$) adverse effect on the test organisms and the lowest concentration of a material in a toxicity test that has statistically significant ($P < 0.05$) adverse effect on the test organisms as compared with the controls, respectively (Rand and Petrocelli, 1985). NOEC and LOEC can not be given with statistics like LC50 with confidence limits. As the concentrations used in the test are predetermined, the measured value of no effect and effect concentrations are only approximation and can lie at any point within the range of NOEC and LOEC.

A life cycle test demands a minimum laboratory exposure of the animal from embryo to embryo, which can be six to twelve months in some fish but can last up to two years or longer in other species (Benoit *et al.*, 1982). The test organism may appear tolerant but a particular stage in the life-cycle may be particularly sensitive and this is the crucial stage in relation to the success of a population exposed to an environmental pollutant. Hynes (1960) and Tarzwell (1967) emphasised the necessity of conducting bioassays with the most susceptible life stage(s) of the test organism and in general embryonic developmental or larval stages of an animal are more sensitive than adult stages. The possibility of focusing research effort on more sensitive stages is one approach to reduce the duration of toxicity tests and hence improving utility and cost-effectiveness without sacrificing sensitivity (Kulshrestha *et al.*, 1986; McKim, 1985).

In fish, early life-stage exposure was originally defined in terms of exposure of the test organisms during most and if not all of the embryogenic period and exposure of fry for a certain period after hatch: 30 days for warm water species and 60 days for cold water species (Macek and Sleight, 1977). Early life-stages were shown to be the most sensitive in the life-cycle (Alderdice, 1985; Eaton *et al.*, 1978; McKim *et al.*, 1975, 1978; Pickering and Gast, 1972; Pickering and Thatcher, 1970; Sauter *et al.*, 1976). An early life-stage toxicity test with early developmental stages (embryo, larvae and early juvenile) of fish can be considered equivalent to a life-cycle test, since early life-stage toxicity tests measure toxicity of chemicals at levels which are similar or almost similar to levels that measured in life-cycle test

(Benoit *et al.*, 1982; McKim, 1977; Ward and Parrish, 1980; Woltering, 1984). Early life-stage (ELS) toxicity tests make it feasible to test important fish species that can not be studied in life-cycle tests because of problems of large size or age at maturity, spawning requirements or other factors uncondusive to laboratory culture. ELS tests greatly reduce the time needed to produce information on the toxicity of chemicals and thus can speed up the hazard assessment process (van Leeuwen *et al.*, 1990). Recently, 7-day larval growth and survival tests (Birge *et al.*, 1985; DeGraeve *et al.*, 1991; Norberg-King, 1989; Norberg-King and Mount, 1985) and 8-10 day embryo-larval growth and survival tests (Dave *et al.*, 1987; OECD, 1992) are being recommended as estimators of chronic effects. Developing embryo or larvae are the most sensitive stages in the life-cycle of teleosts, but differences in susceptibility exist between the different early developmental stages. In general, the younger embryonic stages are more vulnerable than the later embryonic stages (Kuhnhold, 1972; Sharp and Neff, 1980, 1982). Even though the relative susceptibility of early embryonic stages has been well documented, the yolk-sac or alevin stage is known to be the most sensitive stage in the life-cycle (Linden, 1974; Paflitschek, 1979; Schimmel *et al.*, 1974; van Leeuwen *et al.*, 1985). For this reason, it is possible that simple, but reliable and cost-effective toxicity tests with the most sensitive life-stages (*i.e.* sac-fry) can be used in place of more complex embryo-larval tests by measuring the short-term sensitive physiological parameters.

One interesting feature of such tests is that the sac-fry is completely dependent on the food in its yolk-sac for energy. Although the effects of toxic chemicals on external food supply or feeding rate are immaterial at this stage, yolk must be assimilated, which is analogous to feeding if we consider the yolk-sac to be external to the 'body' of the developing larvae. If the developing sac-fry has to utilize some of its fixed energy supply for elevated activity resulting from an avoidance reaction, for detoxification of chemicals or for the repair of tissue damage, the consequence will be reduced growth. If environmental factors affect larval growth due to increased metabolic costs, the size of the larvae at first feeding will be reduced, hence it could be more susceptible to predation (Blaxter, 1988; May, 1974). In natural populations, the timing of first feeding is of importance for the survival of larvae (Heming and Buddington, 1988), and any changes in this timing of first feeding due to disturbed yolk utilization could induce increased mortality by breaking natural synchrony of 'critical period' (changes from internal to external feeding) with the availability of food of the appropriate size and quantity for larval feeding (Blaxter, 1988; Rosenthal and Alderdice, 1976), which could affect the population as a consequence of this increased mortality.

1.4 The goldfish as a test organism in aquatic toxicology studies

Fish are found in almost all aquatic environments and show great variety in their means of adaptation to different habitats. Beyond their economic value, fish play an important role within aquatic food webs and established knowledge about fish is much more diverse, detailed and comprehensive than that of other aquatic

animals, which is why fish, especially freshwater fish, have been used extensively in aquatic toxicity studies (Solbe, 1993).

The choice of test organisms to create standard methodologies that will be usable on a wide geographic scale in ecotoxicological research programmes is of great importance. Rand and Petrocelli (1985) considered several criteria for the suitability of a species in toxicity testing: species should encompass a broad range in sensitivity, they should be widely available and abundant, indigenous or representative of natural systems, recreationally, commercially or ecologically important, amenable to routine maintenance in the laboratory, techniques should be available for culturing and rearing them in the laboratory and adequate background information should be available in selecting organisms for toxicity testing. Boudou and Ribeyre (1989) added other criteria for the selection of test species: the average life span of individuals, the size of the fish and growth rate, the species susceptibility to handling stress and social behaviour.

It is difficult to find a species of fish with all of the above properties. The most commonly used fish for toxicity tests are those which can be readily obtained from a commercial supplier (*e.g.* rainbow trout) or which can be readily bred in the laboratory (*e.g.* zebrafish). However, these are not always the most sensitive species of fish (*e.g.* zebrafish is relatively less sensitive, Nagel *et al.*, 1991). The ideal fish should be convenient to maintain in the laboratory by way of size and requirements and which can be bred round the year in the laboratory (Lloyd, 1992).

The goldfish (*Carassius auratus*) lives in eastern Asia from Amur to eastern India (Grzimek and Ladiges, 1973), inhabiting standing and slowly flowing waters. Goldfish have acquired a global distribution through widespread introduction for ornamental purposes. Escapes and deliberate releases have resulted in natural populations in over 20 countries and its actual distribution is probably far wider (Welcomme, 1988). Goldfish (*Carassius auratus*) are members of the carp family (Cyprinidae) of fishes. Carps are widely distributed throughout the world and are of importance as aquaculture species in Eastern Europe and many Asian countries, such as China, India and Bangladesh.

Goldfish are eurytolerant fish, and can be reared at a wide range of water temperatures (Cossin *et al.*, 1977), are found in waters of varying salinities and are tolerant to handling stress (Grzimek and Ladiges, 1973). The fish has been bred for 800-1000 years in China and Japan, it reaches its breeding age at the end of its first year of life and has the potential to breed repeatedly up to year seven (Grzimek and Ladiges, 1973). There is a possibility of obtaining fully mature goldfish at any desired time of the year from laboratory rearing (Razani and Hanyu, 1986a and 1986b; Razani *et al.*, 1989; Yamamoto *et al.*, 1966), and mature goldfish can be induced to artificial spawning in the laboratory (Sokolowska *et al.*, 1984; Stacey *et al.*, 1979). As many as 15000 eggs can be obtained from a single spawning (Horvath *et al.*, 1992). These make goldfish highly suitable for extensive use in regulatory testing of chemicals. There are already a great deal of background data on the response of goldfish to chemical stress. In one of the earliest published

toxicity testing studies, Powers (1917) found that the survival time of the goldfish had a clear dependence on the concentration of a variety of toxic substances.

1.5 Toxicants

There is obvious value in monitoring the effects of known stressors on the physiology of organisms: such studies provide a standard by which the severity and significance of other environmental stressors can be measured (Leatherland and Sonstegard, 1984), because organisms show general response to stress irrespective of the type of stress (Selye, 1976). Therefore to study general stress responses in goldfish sac-fry, three reference toxicants: a water-soluble heavy metal, an intermediately water-soluble aniline and a poorly water-soluble organophosphate were chosen. The toxicants used were cadmium chloride, 3,4-dichloroaniline (here after called DCA) and chlorpyrifos. Cadmium was selected by considering its suitability in laboratory studies (known and easy handling and analytical techniques) DCA and chlorpyrifos were selected as a part of an EC research project on an integrated approach to the development and validation of ecologically relevant toxicity tests for the impact of poorly water-soluble compounds on aquatic ecosystems. These toxicants have three different mode of action: cadmium affects enzyme, damage skin and gill, DCA has non-specific anaesthetizing effect and chlorpyrifos affects acetylcholinesterase. Any response common to cadmium, DCA and chlorpyrifos exposure will be considered as a general response, because these chemicals represent three different broad groups of chemicals, are different in water-solubility and also different in their mode of action on a biological system.

1.5.1 Cadmium

Cadmium is a biologically non-essential heavy metal (Table 1.1). It is a nondegradable cumulative pollutant which interferes with the metabolism of essential metals in animals and human beings (Allen, *et al.*, 1974). Cadmium is normally present in soil and water at low concentrations. Due to mining activities and its use in the manufacture of a wide range of industrial products (Mance, 1987) it may be present in much higher concentrations in natural waters: for example in Srisagar Lake, India, concentrations of up to 1 mg.l^{-1} have been recorded (Agarwal, 1978). Due to its increasing use in industries cadmium poses a serious potential hazard (Duffus, 1983). Cadmium also has the potential to become bioconcentrated in aquatic food chains, with a high bioaccumulation factor *i.e.* a ratio of cadmium in the tissue to cadmium level in the water of up to 10,000 has been previously reported (Pascoe and Matthey, 1977).

Cadmium is reported to be lethal to fish early life-stages both at low level (*e.g.* $2.4 \text{ } \mu\text{g.l}^{-1}$, Carrol *et al.*, 1979; for brook trout fry) and high level (*e.g.* $9200 \text{ } \mu\text{g.l}^{-1}$, van Lccuwen *et al.*, 1985; for rainbow trout eyed egg). Cadmium level as low as $0.47 \text{ } \mu\text{g.l}^{-1}$ can impair yolk utilisation and has been found to have negative effects on growth of Atlantic Salmon sac-fry (Rombough and Garside, 1982). In developing fish embryos, cadmium induces abnormal development of the spinal column (Rombough and Garside, 1982) by impairing calcification through interference with calcium metabolism (Eaton, 1974; Rombough and Garside, 1984). Cadmium can

also interfere with osmoregulation and has been shown to delay water hardening and the process of water uptake in the eggs of the Pacific herring, *Clupea pallasii* (Alderdice *et al.*, 1979). Garpike (*Belone belone*) embryos reduced their heart rate when exposed in water containing 1.0 mg.l⁻¹ of cadmium (von Westernhagen *et al.*, 1975). *Clupea harengus* embryos exposed to cadmium concentrations higher than 1 mg.l⁻¹ showed a reduction in eye diameter (von Westernhagen *et al.*, 1974), and in another study on this species, cadmium concentrations as low as 3.0 µg.l⁻¹ reduced larval growth (Ojaveer *et al.*, 1980). Cadmium, like many other metals, such as zinc may damage mitochondria and impair energy transfer and metabolism of protein (Somasundaram *et al.*, 1984). Cadmium is also reported to interfere with insulin secretion and to influence carbohydrate metabolism by reducing muscle glycogen and increasing blood glucose (Forlin *et al.*, 1986) and this effect may become persistent. Haux and Larsson (1984) have reported persistent effects of cadmium on carbohydrate metabolism after one year of exposure in rainbow trout.

1.5.2 3,4-dichloroaniline

3,4-dichloroaniline (DCA) is a halogenated aromatic amine (Table 1.2), which is used as an intermediate in the production of herbicides, pigments and pharmaceuticals. The total annual worldwide production of DCA during the last decade was between 42,000 and 47,000 metric tons (Livingston and Willacy, 1991). DCA is released to the environment through the degradation of herbicides used in agriculture, of which the most widely used are propanil, diuron, linuron, and neburon (Chow and Murphy, 1975; DiMuccio *et al.*, 1984; El-Dib and Aly, 1976; Hutson and Roberts, 1987; Kalsch *et al.*, 1991; Still and Herrett, 1976; Tatsumi *et al.*, 1992; Viswanathan *et al.*, 1978). It is also present in the effluents from dye manufacturing plants (Adema and Vink, 1981; Crossland and Hillaby, 1985; Games and Hites, 1977). It has been shown to be present in surface waters (Wegman and deKorte, 1981) and in rice field water (Deuel *et al.*, 1977). It is resistant to biodegradation (Kuiper and Hanstveit, 1984; Wolff and Crossland, 1985), but subject to photo-degradation (Miller *et al.*, 1980; Wolff and Crossland, 1985) and has a half-life in shallow temperate ponds of 2 - 6 days (Wolff and Crossland, 1985). DCA can cause an imbalance in enzyme activity and can cause respiratory uncoupling resulting in reduced ATP production, it may also decrease the oxygen carrying capacity of the haemoglobin which could result in impaired growth (Chow and Murphy, 1975). Adema and Vink (1981) reported lethal values of 0.1 mg.l⁻¹ to 20 mg.l⁻¹ and sublethal values of 0.01 mg.l⁻¹ to 1 mg.l⁻¹ of DCA for a range of organisms.

Fathead minnow eggs exposed to the herbicide propanil at concentrations of 3.8 $\mu\text{g.l}^{-1}$ showed significantly reduced egg hatching (Call *et al.*, 1983). However, early life-stage exposure (28 d) of fathead minnow eggs showed no significant effect on egg hatchability and survival up to 5 days at concentrations up to 160 $\mu\text{g.l}^{-1}$ but mean total biomass per chamber (integrated effect of survival and weight) was significantly reduced by exposure to concentrations as low as 7.1 $\mu\text{g.l}^{-1}$ of 3,4-dichloroaniline (Call *et al.*, 1987). A bioaccumulation factor of 45 has been reported for DCA in juvenile rainbow trout (Crossland, 1990). DCA is of particular interest to ecotoxicologists because of its potential toxicity due to its unusually high acute to chronic ratio (ACR), which was found to be as much as 1200 (Call *et al.*, 1987). DCA is considered as a useful model of chlorinated hydrocarbons (Meer *et al.*, 1988) and it has been recently used in an interlaboratory calibration exercise for a toxicity test using early life-stages of the zebrafish (Nagel *et al.*, 1991).

1.5.3 Chlorpyrifos

Chlorpyrifos is a poorly water-soluble organophosphate insecticide (Table 1.3), with a water solubility at 25°C of 0.2 mg.l^{-1} (Kersting and van Wijngaarden, 1992). It is used to control mosquitoes, fire ants, household insects, turf and ornamental plant insects, agricultural crop insects and for other purposes (Berg, 1983; Worthing and Walker, 1983). It is an important threat to the aquatic environment due to its widespread use as mosquito larvicide (Jarvinen *et al.*, 1983), and may be applied directly to ponds, lake margins and wetland areas. Chlorpyrifos like other

organophosphate insecticides exerts a toxic effect through an acetylcholinergic mode of action (Rodrigues *et al.* 1983). Its effects have been studied on different groups of organisms and it has been found to be highly toxic at low level exposure; for example, the 96 h LC50 for *Daphnia longispina* is 0.3 $\mu\text{g.l}^{-1}$, for *Gammarus pulex* it is 0.07 $\mu\text{g.l}^{-1}$ and for *Gasterosteus aculeatus* it is 8.5 $\mu\text{g.l}^{-1}$ (van Wijngaarden *et al.*, 1993). Its toxicity to both freshwater and saltwater estuarine fish is also documented. The 96 h LC50 for freshwater fish varies from 2.4 $\mu\text{g.l}^{-1}$ (bluegill: *Lepomis macrochirus*) to 280 $\mu\text{g.l}^{-1}$ (channel catfish: *Ictalurus punctatus*) (Johnson and Finley, 1980) and for saltwater fish varies from 1.3 $\mu\text{g.l}^{-1}$ (*Menidia peninsulae*) to 520 $\mu\text{g.l}^{-1}$ (*Opsanus beta*) (Clark *et al.*, 1985). NOEC and LOEC derived in ELS tests with estuarine atherinids exposed to technical grade chlorpyrifos ranged from 0.28 to 0.75 $\mu\text{g.l}^{-1}$ and 0.48 to 1.8 $\mu\text{g.l}^{-1}$, respectively (Goodman *et al.*, 1985). NOEC and LOEC derived from ELS tests with fathead minnows (*Pimephales promelas*) exposed to technical grade chlorpyrifos ranged from 1.6 to 3.2 $\mu\text{g.l}^{-1}$ (Jarvinen and Tanner, 1982). The acute (96 h LC50) to chronic (geometric mean of NOEC and LOEC, measured on fish early life stage test) ratio for chlorpyrifos is very low 2.4 to 4.6 (calculated from data reported by Clark *et al.*, 1985 and Goodman *et al.*, 1985).

Table 1.1 General information about cadmium.

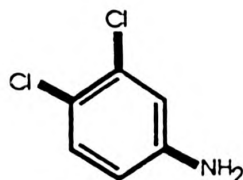
Chemical structure	$\overset{+2}{\text{Cd}}-$
Formula (chemical used)	$\text{CdCl}_2 (\text{Cd}^{2+} + 2\text{Cl}^{-})$
Molecular weight	183.3 (112.4 + 70.9)
Water solubility	>150 g.l ⁻¹
<i>Daphnia magna</i> LC50 (48 h)	4.1 µg.l ⁻¹ *
<i>Oreochromis niloticus</i> (sac-fry) LC50 (96 h)	20.6 µg.l ⁻¹ **
<i>Tilapia zilli</i> (sac-fry) LC50 (96 h)	143.5 µg.l ⁻¹ **
<i>Salmo gairdneri</i> (fry) LC50 (96 h)	10.0 µg.l ⁻¹ ***

* from Barber (1990)

** from Siriwardena (1993)

*** from van Leeuwen *et al.* (1985)

Table 1.2 General information about 3,4-dichloroaniline (DCA).

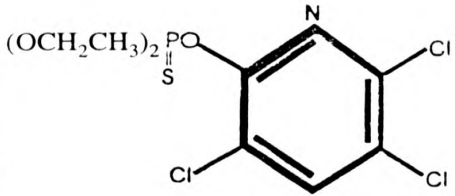
Chemical structure	
Formula	$C_6H_5Cl_2N^*$
Molecular weight	162.0*
Partition co-efficient octanol/water at 20°C	2.7
Water solubility (20°C)	600 mg.l ⁻¹ *
<i>Daphnia magna</i> LC50 (48 h)	0.23 mg.l ⁻¹ **
<i>Daphnia magna</i> LC50 (96 h)	0.16 mg.l ⁻¹ **
<i>Salmo gairdneri</i> LC50 (96 h)	2.7 mg.l ⁻¹ *
<i>Brachydanio rerio</i> LC50 (96 h)	8.5 mg.l ⁻¹ ***

* from Crossland (1990)

** from Adema and Vink (1981)

*** from Nagel *et al.* (1991)

Table 1.3 General information about chlorpyrifos.

Chemical structure	 <p>The chemical structure shows a pyridine ring with a nitrogen atom at the top. At the 2-position, there is a phosphorus atom double-bonded to a sulfur atom and single-bonded to two ethoxy groups (OCH₂CH₃). At the 3, 4, and 6 positions, there are chlorine atoms.</p>
Formula	C ₉ H ₁₁ O ₃ Cl ₃ NPS
Molecular weight	350.6
Partition co-efficient octanol/water at 20°C	5.11
Water solubility (25°C)	0.2* - 2** mg.l ⁻¹
Half-life in water (pH 8, 25°C)	1.5 days**
Half-life in water with phosphate buffer (pH 7, 15°C)	100 days**
<i>Daphnia magna</i> LC50 (96 h)	0.1 µg.l ⁻¹ ****
<i>Daphnia longispina</i> LC50 (96 h)	0.3 µg.l ⁻¹ *****
<i>Gasterosteus aculeatus</i> LC50 (96 h)	8.5 µg.l ⁻¹ *****
<i>Ictalurus punctatus</i> LC50 (96 h)	280 µg.l ⁻¹ *****

* from Kersting and van Wijngaarden (1992)

** from Worthing and Walker (1983)

*** from Soares (pers. comm.)

**** from van Wijngaarden (1993)

***** from Johnson and Finley (1980)

1.6 Fish early life-stage toxicity test endpoints

Toxicity test endpoints are defined in terms of a response that is the reaction of the test organism to a stimulus (Solbe, 1993). The basis for substituting early life-stage tests for life-cycle tests is the need to derive a more rapid, less costly, and yet reliable means of estimating the chronic toxicity of test chemicals. However, tradition doesn't necessarily provide the most useful approach as has been pointed out by Dave (1993) and it is appropriate and timely to re-evaluate the selection of toxicity test endpoints.

Survival has been the most frequently studied endpoint in both acute and chronic studies of the effects of toxicants on aquatic organisms. More recently, other endpoints relating to the impairment of growth and/or reproduction have been measured. Impairment of growth in length and/or weight has become the sublethal response of choice in most fish early life stage toxicity tests. The reasoning behind this choice is that many otherwise different undetected effects of toxicants may show up as an impaired ability to convert food into tissue growth (Dave, 1986; Dave *et al.*, 1987). Growth, being a sublethal response, is believed to be more sensitive than survival. However, in a review of 173 chronic, subchronic and early life-stages toxicity tests (Woltering, 1984) larval survival was the most sensitive endpoint in 57% of the early life-stage toxicity tests. In a later review of 136 tests Kristensen (1990) concluded that growth was the most sensitive endpoint in 70% of the early life-stage tests when the juvenile stage was included. But growth of fish early life-stage toxicity test has been reported in different ways (*e.g.* length,

wet weight, dry weight, individual weight, treatment weight) by different authors. Reporting of test endpoints without detailed information of the test methodology can be misleading: the reporting of growth in terms of average individual growth, average treatment growth, dry weight or wet weight should be explained clearly and standardized. It is possible that the same data on growth reported in different ways can lead to different conclusions *e.g.* lower treatment growth with fewer surviving larvae can give higher individual growth in larval growth test.

Recently, a 7-day growth and larval survival test using the fathead minnow (*Pimephales promelas*) has been advocated (DeGraeve *et al.*, 1991; Norberg-King, 1989; Norberg-King and Mount, 1985). But differences in growth response might have arisen in this type of test due to the influence of ration and individual interaction during group rearing as affected by partial kills (Dave, 1986). To avoid the effect of ration, an embryo-larval test with embryo and sac-fry has been suggested (Dave, *et al.*, 1987; OECD, 1992). Although growth is a widely used endpoint for fish embryo-larval toxicity test the underlying mechanisms of growth are poorly studied. The possible endpoints regarding yolk (limited resource) utilization and the efficiency of yolk utilization are yet to be addressed systematically in fish early life-stage toxicity tests. Sublethal effects are defined as "those responses to environmental changes: histological, morphological, physiological or ethological- that may be induced in one stage of development but be expressed at a later stage of organization or development in terms of reduced survival potential", (Rosenthal and Alderdice 1976). This definition emphasizes the

effects influencing survival potential of organisms but does not recognize those effects which do not affect survival of the exposed organisms but still have ecological relevance *e.g.* impairment of reproductive capacity. Sublethal effects consider those which do not cause rapid death but impair the functioning of organisms at the biochemical, physiological, behavioral or life-cycle level (Boudou and Ribeyre, 1989). For practical convenience, an organism's response to a potential stress may be viewed on different time scales and sublethal responses can be categorized as primary responses, which includes release of hormones from endocrine tissue; secondary response, which includes changes in blood chemistry, depletion of nutrient stores and metabolic changes such as negative nitrogen balance; and tertiary response which involves impaired growth and reproduction, and increased vulnerability to diseases (Wedemeyer and McLeay, 1981). Detection of some effects in suborganismal responses can be difficult and may require sophisticated instrumentation and would be difficult to implement in environmental monitoring. But individual physiological responses, such as feeding rate, oxygen consumption, oxygen to nitrogen ratio are sensitive to environmental change (Widdows, 1985) and comparatively easy to measure. Hence, increasing attention has been directed towards the measure of sublethal effects by employing physiological indices (Koehn and Bayne, 1989; Larsson *et al.*, 1988). The determination of a physiological endpoint related to yolk utilization of yolk feeding sac-fry and its use in short term fish early life-stage toxicity test could prove useful for regulatory purposes.

1.7 Energy budget: its advantage as stress indicator

The law of conservation of energy (energy is constant, and can neither be created nor be destroyed) was first applied in physiology during the 18th century (Cathcart, 1953), and can be expressed as:

$$\text{energy input} = \text{energy output} \quad (1.2)$$

Organisms build, maintain and reproduce themselves from resources obtain from food. In growing organisms the energy equation can be expressed as:

$$\text{food input} - \text{faecal output} = \text{absorbed energy} = \text{production of tissue} + \text{heat} + \text{excretory loss} \quad (1.3)$$

This simple energy budget model has been used successfully in agriculture (Brody, 1945), in fish biology (Winberg, 1960) and in describing the economy of ecological systems at both community (Lindeman, 1942) and population levels (Phillipson, 1966; Ricker, 1968).

Energy flow through a biological system can be represented by using standard symbols used by different authors (Brett and Groves, 1979; Klekowski, 1970; Klekowski and Duncan, 1975; Petruszewicz, 1967; Ricker, 1968).

$$C = P + R + U + F \quad (1.4)$$

$$C - F = A = P + R + U \quad (1.5)$$

Where C = food energy consumed

P = energy incorporated in production

R = energy respired

U = energy excreted

F = energy lost as faeces.

A = assimilated energy

The energy budget can be solved either on an instantaneous or cumulative basis for short or long periods of time (Knight, 1985). In an instantaneous energy budget, parameters are expressed in energy units (J) per unit time (*e.g.* hour, day, year). In a cumulative energy budget (first proposed by Klekowski *et al.*, 1967; developed by Klekowski, 1970; and Klekowski and Duncan, 1975) all the parameters are cumulated from the beginning of the period of the life-cycle to the end of an identifiable period of the life cycle. Its parameters are expressed in energy unit per life period. In studies of fish energetics it is usual to express the energy transformations in terms of physiological rates, with the flow of materials being calculated in energy units (KJ) (Jobling, 1994). Direct energy losses (heat) of fish are difficult to measure using direct methods such as calorimetry: usually they are measured indirectly using an oxycalorific value based on oxygen consumption.

Fish rely, predominantly, upon lipid and proteins as respiratory substrates, and the approximate oxycaloric co-efficient for use in estimating energy metabolism in fish has been suggested to as 13.59 KJ/g or 19.4 KJ// oxygen consumed (Jobling, 1994; Kamler, 1992). The use of an oxycaloric co-efficient to estimate energy expenditure is based on the principle that metabolism occurs aerobically (Dabrowski, 1989; Rombough, 1988).

It is usual to find one of the major components of a balanced energy equation estimated by difference in order to produce a balance (Jobling, 1994). All errors associated with the determinations of the measured components, however, become a pooled error in the component estimated by difference. Further problems may arise because it is not possible to obtain information about the energy content of the particular experimental organism both before and after an experiment.

Assumptions have been made to calculate the bioenergetics equation *e.g.* energy value of yolk mass over the period of endogenous feeding considered constant (Lasker, 1962; Quantz, 1985; Raciborski, 1987) to study energetics early life-stages of fish. It has become common practice in bioenergetics studies to borrow parts of a physiological process from well-studied, closely related fish species or size-classes to a complete bioenergetics model of a poorly investigated fish species, *e.g.* Boisclair and Sirois (1993) borrowed caloric density value of sockeye salmon for the calculation of energetics of brook trout.

Yolk is the source of energy and materials for growth of fish sac-fry. The mobilization of yolk reserves in teleostean sac-fry occurs through the vitelline syncytium, a thin tissue which envelops the whole yolk mass. Yolk utilization is analogous to feeding of external feeding animals, *i.e.* food absorption through the gut wall being analogous to yolk absorbed through yolk syncytium. Yolk absorbed by a sac-fry larva provides materials to be deposited in the growing tissue and supplies energy for respiration. No faeces are produced by fish larvae prior to initiation of exogenous feeding, only metabolites are excreted. Therefore, the energy consumed from yolk is partitioned as follows:

$$C = P + R + U \quad (1.6)$$

$$\text{or} \quad P = C - (R + U) \quad (1.7)$$

Where, C is the energy consumed from yolk and P, R, U are production (somatic growth), respiration and excretion respectively in terms of energy.

Each component of the right hand side of the energy budget equation (equation 1.7) is a physiological process that can be readily measured and converted into energy equivalents ($J \cdot h^{-1}$). The energy budget thus provides a means of integrating these basic physiological processes (yolk absorption, respiration and excretion), an index of the energy available for growth. Due to the applied stress, a trade-off occurs (Sibly and Calow, 1986) between growth and stress tolerance *i.e.* by increasing R or U of the equation 1.6, resulting in negative effects on net energy balance (Koehn

and Bayne, 1989) *i.e.* reduced growth (P). The available energy for growth (net energy balance) measured from the energy budget has been referred to as 'scope for growth' by Warren and Davis (1967). The advantages provided by this energy budget approach are, (1) sensitivity in the ability to detect immediately the more subtle effects of an environmental change and (2) an understanding of the bioenergetics of production in an assessment of the energy status of the individual and the various components of growth under different environmental conditions (Bayne *et al.*, 1985).

The use of scope for growth has proved to be useful both in the laboratory (Bayne, 1975; Widdows, 1978; Widdows and Bayne, 1971) and in the field (Bayne and Widdows, 1978; Widdows *et al.*, 1981) with bivalves, and more recently with other species such as crustacea (Naylor *et al.*, 1989). With fish, most of the studies employing growth as an index of toxic effect have used the direct measurement of growth rather than determining it together with the balanced energy equation. The knowledge of measured components of the basic energy equation together with environmental factors can be used to demonstrate the organism's performance under environmental stress, so can be used in ecological modelling (Borgmann and Whittle, 1992, Brandt and Hartman, 1993; Madon and Culver, 1993; Ney, 1993; Vaughan *et al.*, 1984; Weininger, 1978) for environmental management.

1.8 Aims of the thesis

Fish sac-fry are generally the most sensitive stage of the life-cycle of fish (see section 1.3), and can be useful indicator of low-level toxic effects. However, little effort has been devoted towards an understanding of the physiological mechanisms behind toxicity test endpoints or towards the development of rapid, short-term measures of sublethal physiological responses to stress. Considering these limitations of the existing fish early life-stage toxicity test a research programme was planned towards the development of a short-term toxicity test with goldfish (*Carassius auratus*) sac-fry. To achieve this, the following aims were set:

1. to develop a brood-fish management system for the year-round production of goldfish eggs and sac-fry from the same set of brood fish.
2. to derive rapid sublethal endpoints for use in fish early life-stage toxicity tests.
3. to develop a conceptual model of the physiological effects of sublethal exposure to toxicants on the developing sac-fry.
4. to validate the model by studying sublethal exposure responses.

CHAPTER 2

GENERAL MATERIALS AND METHODS

Techniques common to all experiments in the present study are described below. Methodology specific to individual experiments are described in the relevant chapters.

2.1 Procurement of eggs and sac-fry

2.1.1 Brood stock rearing and egg incubation system

Two groups of goldfish *Carassius auratus* L. (comet variety), each composed of males and females of ages 0+ (30.83 ± 5.14 g, 8.93 ± 0.71 cm) and 1+ (137.2 ± 59.06 g, 14.54 ± 1.86 cm), imported from Israel were obtained from C. Murray Aquatics, Glasgow, Scotland. Fish were maintained in a recirculatory system in a $20 \pm 1^\circ\text{C}$ constant temperature room, at the Institute of Aquaculture, University of Stirling. The recirculatory system (Figure 2.1) was comprised of four 120 l glass aquaria and each received water at a rate of $1.7 \text{ l}\cdot\text{min}^{-1}$ through a delivery pipe fitted with a valve to control the flow rate. The outflows from individual tanks were channelled to a sump tank through a series of settling tanks with biofilter rings. A pump (Otter 750, Beresford, UK) was used to pump the water from the sump tank to the holding tanks and excess water was collected in the sump tank through a bypass pipe fitted with a valve. Water quality was monitored at

fortnightly intervals and water quality levels remained within the following values: pH 7.4 - 7.8 (measured by using a digital pH meter, Model PW 9409, Philips, UK), oxygen 7.6 - 8.2 mg.l⁻¹, and NH₃ 0.08 - 0.15 mg.l⁻¹ (for analytical methods, see section 2.2 and 3.2.6). A photoperiod regime of 14L : 10D was maintained for all experiments and broodstock manipulations, with light intensity on the water surface of 80 - 120 Lux (measured by using an optometer, Model 40X, United detector Technology Inc., UK). Fish holding tanks were aerated by air-stone, and additional aeration was provided in the sump tank.

The fish were fed ad libitum twice daily with trout pellet (BP standard expanded diet No.3; protein content 40%; BP Nutrition (UK) Ltd., Inverbreakie, Scotland). Waste materials were cleaned by siphoning and the system refilled with fresh water on a daily basis.

The egg incubation system consisted of five 10" x 7" x 12" chambered glass tank fitted to a recirculatory system (Figure 2.2). Water was delivered to each chamber through delivery pipes, water being pumped from the reservoir sump tank by a Fluval 303 pump to the incubation chamber via an eight watt UV steriliser. The outflow from the chamber was directed through a fine net (400 µm mesh) that fitted on the spillway to a drain and ultimately collected in the sump tank.

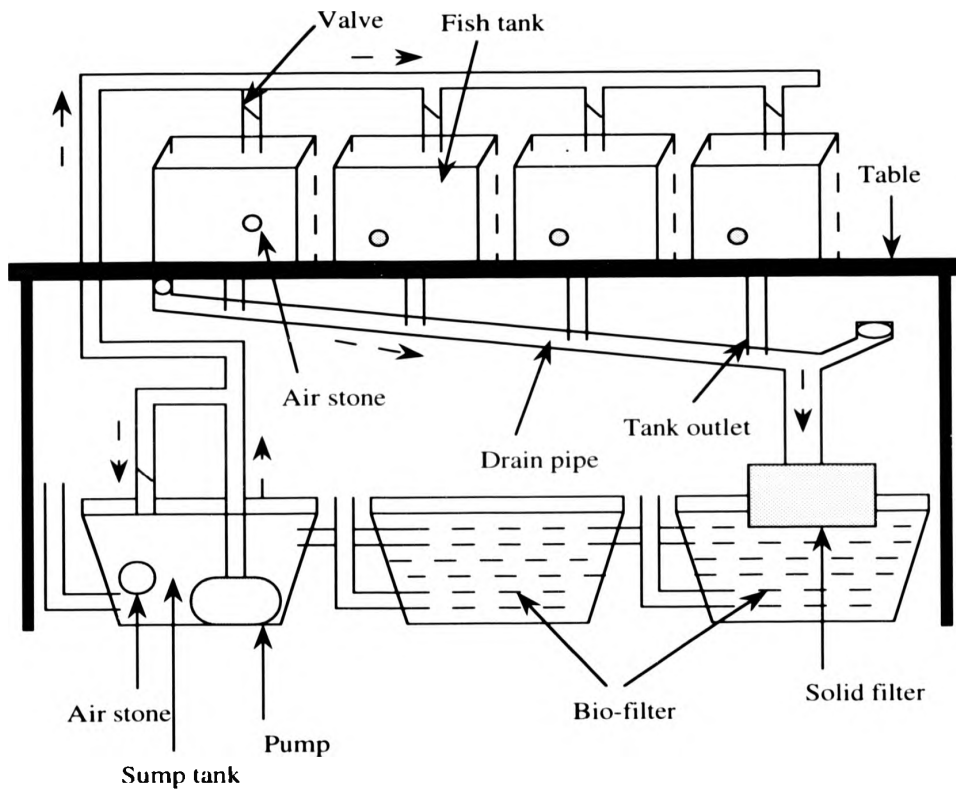


Figure 2.1 Water recirculatory system for rearing brood stock. The dashed line with arrow indicates direction of flow.

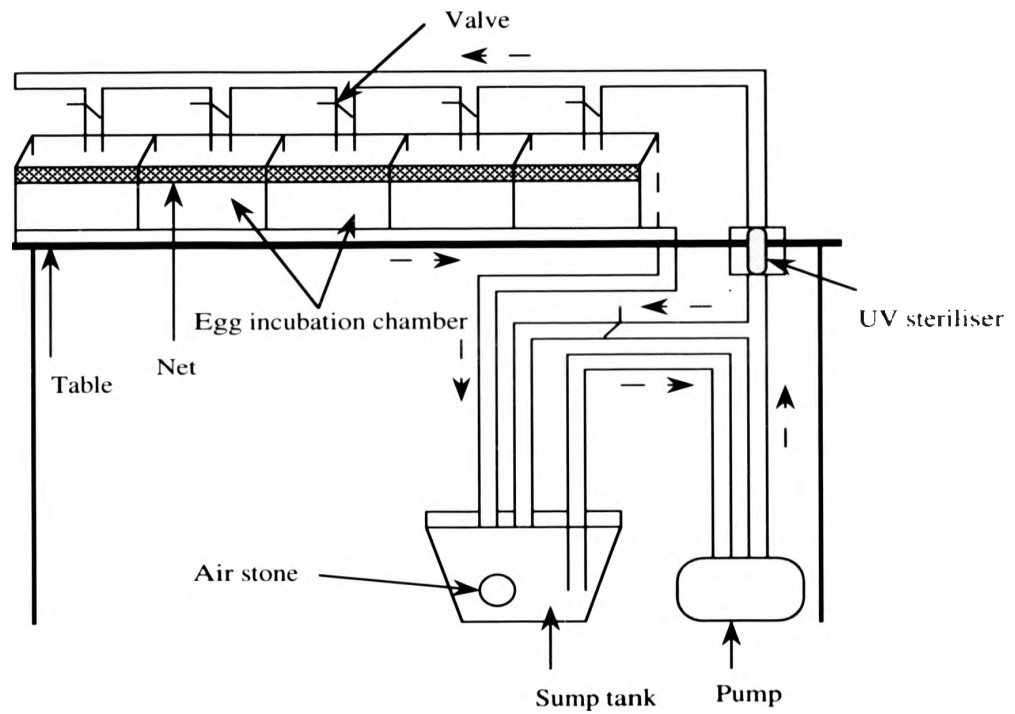


Figure 2.2 Egg incubation recirculatory system. The dashed line with arrow indicates direction of flow.

2.1.2 Fertilization and incubation of eggs

Milt was collected from male fish into capillary glass tubes by applying gentle pressure on the abdomen. Artificially ovulated female fish were anaesthetized in a 1:10,000 benzocaine solution. The anaesthetized fish were rinsed with fresh water and then dried with soft paper tissue to remove excess water. The eggs from ovulated female were then extruded by applying gentle abdominal pressure and collected in a Petri dish. Between 5000 and 42,000 eggs were collected from each fish at each spawning, which were then divided among several Petri dishes (incubation plates). The milt was diluted in a microcentrifuge tube with teleost physiological saline (Burnstock, 1958) at a ratio of 1 part milt to 5 parts saline. A drop of diluted milt was then poured on the eggs in each Petri dish, and small amounts of water (15 - 20 ml) added, after which the Petri dish was shaken gently by circular movement to ensure effective fertilization. Eggs were spread in a monolayer so that they did not overlap. Petri dishes were kept on the table for a few minutes to allow water hardening of the eggs, and to allow the eggs to stick to the surface of the Petri dish. Eggs were subsequently washed with water several times and placed in an incubation chamber (Figure 2.3) maintained at $20 \pm 1^{\circ}\text{C}$. After 24 hours, white opaque eggs were dislodged from the Petri dish by rolling them gently with the help of a Pasteur pipette under a magnifying glass, and discarded. Fertilization rate was calculated at that point, although it was recognised that this would tend to overestimate infertility, since no distinction could be made between unfertilized eggs and those dying during early development. The development of embryos proceeded as follows: cleavage occurred around 30 - 40

minutes after fertilization, with the blastula stage being reached after 9 - 10 hours (Figure 2.4). Eyed stage was reached after 72 hours and hatching of the embryos as yolked sac-fry began after 110 hours and 90% of the embryos hatched out as yolked sac-fry after 125 hours of incubation.

2.1.3 Selection of experimental sac-fry

Sac-fry obtained from a single female were examined under a dissecting microscope at hatching and transferred to a Petri dish containing dilution water. Sac-fry were judged to be normal if they showed no obvious morphological deformities and if the angle between the longitudinal axis of the head and that of the body was approximately 135° or greater under a dissecting microscope (Olympus, Japan). If this angle was less than 135° but the sac-fry showed no deformities, it was considered premature (Wiegand *et al.*, 1988). Deformed sac-fry, which included structurally deformed and premature individuals were separated from normal sac-fry (Figure 2.5) and discarded. Normal sac-fry were then randomly allocated in groups of five to each exposure chamber until the designed total number of sac-fry per chamber was reached.

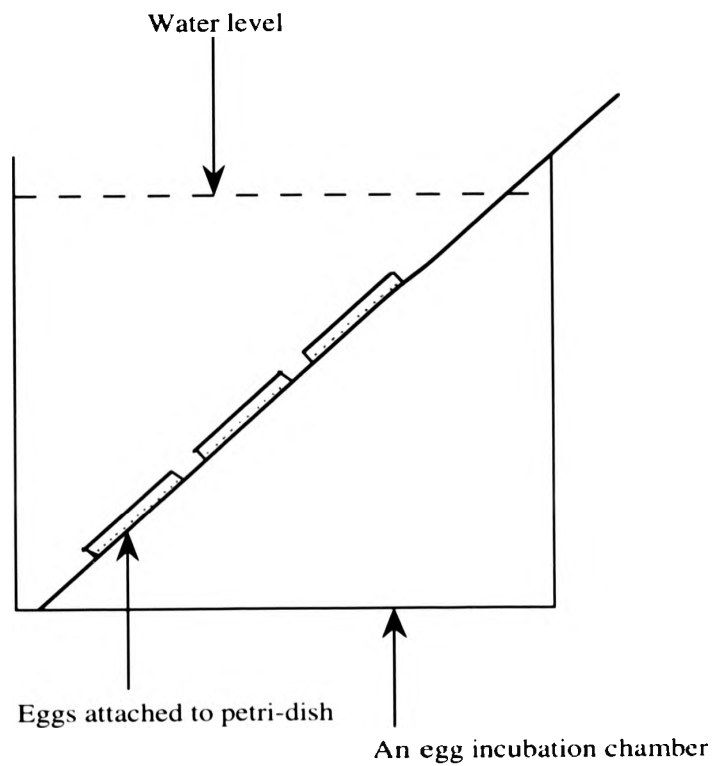


Figure 2.3 Egg incubation in an incubation chamber. Eggs attached to framed petridish.

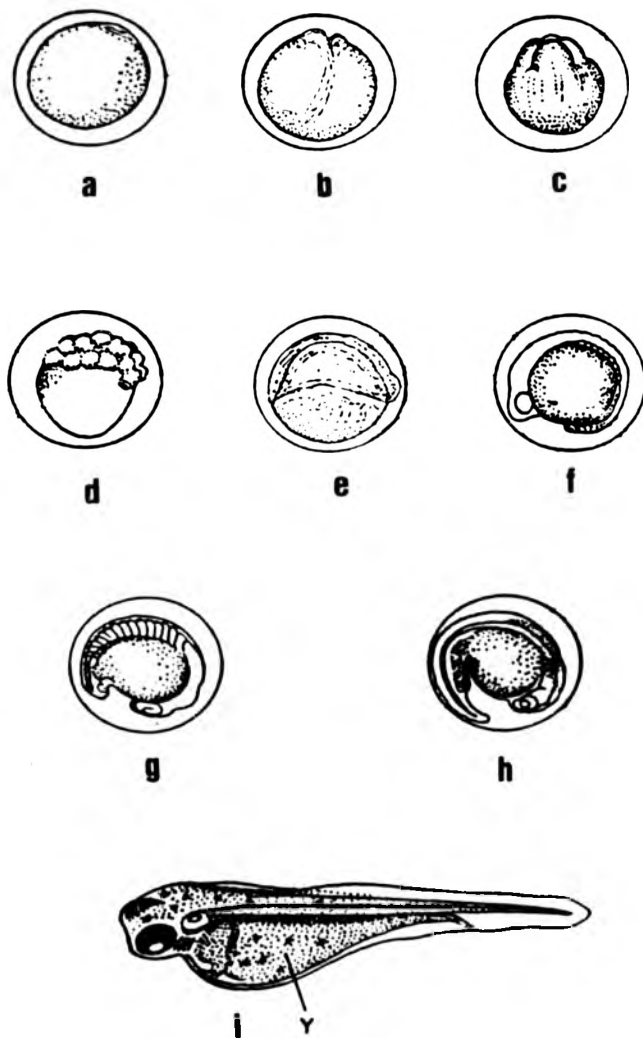
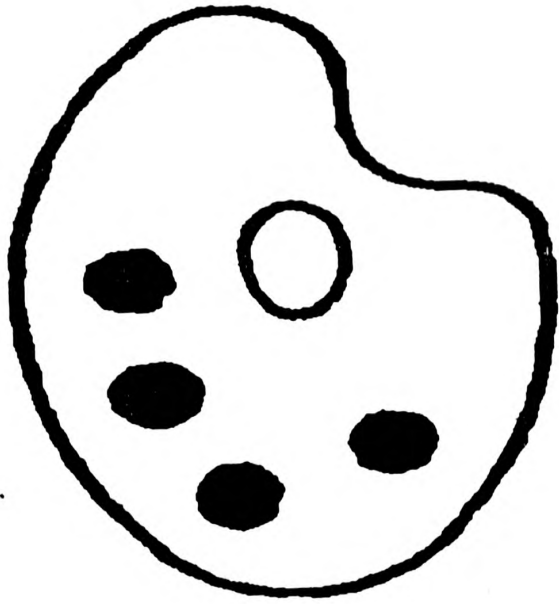


Figure 2.4 Development of goldfish egg and embryo in the laboratory. (a) fertilized egg (10-15 min), (b) first cleavage (30-40 min), (c) second cleavage (1-2 h), (d) advanced stage of cleavage (6-7 h), (e) blastula (9-10 h), (f) an embryonic stage (48 h), (g) eyed stage (72 h), (h) advanced stage of embryo (96 h), (i) sac-fry larvae shortly after hatching (125 h) and (Y) yolk mass.

ORIGINAL IN COLOUR

P 44



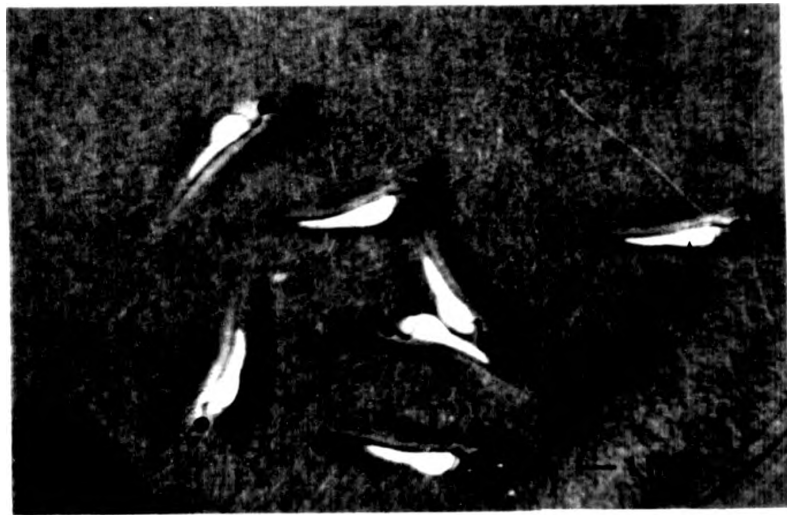


Figure 2.5 Goldfish sac-fry larvae after hatching, (a) normal sac-fry, (b) abnormal sac-fry, (c) lordosis, (d) scoliosis, (e) abnormal yolk sac (f) Pericardial oedema and (y) yolk.

2.2 Dilution water

For toxicity tests, soft or moderately hard artificial waters are commonly used (*e.g.* those described in ASTM, 1980). Here M7 artificial water (Anonymous, 1991) was selected on the assumption that it contains all the macro and micronutrients required for the culture of most aquatic organisms. M7 water was used routinely both for egg incubation media and in toxicity tests, in contrast to broodstock, which were maintained in mains tap water. The dilution water was prepared according to the recipe given in Table 2.1, using nanopure water (conductivity $<0.05 \mu\text{s cm}^{-1}$, resistivity 18.3 megohm cm, organic level <20 ppb total organic carbon). Nanopure water was obtained from a filtration system (Barnstead D4752). Prepared dilution water was aerated for at least 24 hours before use in the constant temperature room to ensure it reached air saturation, and a constant temperature of 20°C . The water hardness ($238 - 253 \text{ mg CaCO}_3\text{.l}^{-1}$), pH (7.9 - 8.2) and oxygen ($9.0 - 9.2 \text{ mg.l}^{-1}$) were monitored using standard methods (APHA, 1989), digital pH meter (Model PW 9409, Philips, UK) and oxygen meter (Model 781, Strathkelvin Instruments, UK) respectively.

Table 2.1 Chemical composition of dilution water (M7 medium)

Chemicals	Amount in stock solution (mg.l ⁻¹)	Volume of stock solution (ml added to 10 l of nanopure water)	Concentration in dilution water (mg.l ⁻¹)
Micronutrients			
H ₃ BO ₃	7148.7	1	0.715
MnCl ₂ .4H ₂ O	901.2	1	0.090
LiCl	765.0	1	0.077
RbCl	177.5	1	0.018
SrCl ₂ .6H ₂ O	380.0	1	0.038
NaBr	40.0	1	0.004
Na ₂ MoO ₄ .2H ₂ O	157.5	1	0.016
CuCl ₂ .2H ₂ O	42.0	1	0.004
ZnCl	32.5	4	0.013
CoCl.6H ₂ O	25.0	4	0.010
KI	8.1	4	0.003
Na ₂ SeO ₃	5.5	4	0.002
NH ₄ VO ₃	1.4	4	0.001
NaEDTA.2H ₂ O	5000	-	0.625
FeSO ₄ .7H ₂ O	1991	-	0.249
FeEDTA ^(a)	-	2.5	-
Macronutrients			
CaCl ₂ .2H ₂ O ^(b)	29380.0	100	293.800
MgSO ₄ .7H ₂ O ^(b)	12330.0	100	123.300
NaHCO ₃ ^(b)	6480.0	100	64.800
KCl ^(b)	14500.0	4	5.800
NaSiO ₃ .5H ₂ O ^(b)	10000.0	10	10.000
NaNO ₃	685.0	4	0.274
KH ₂ PO ₄	357.5	4	0.143
K ₂ HPO ₄	460.0	4	0.184
Vitamins ^(c)			
Thiamine-HCl	750.0	-	0.075
Cyanocobalamine	10	-	0.001
Biotin	7.5	1	0.001

(a) Both solutions were prepared separately, mixed and autoclaved immediately in order to produce the FeEDTA solution

(b) Prepared when required, used within a week.

(c) Vitamin stock solutions were always stored frozen. Vitamin stock solutions were added to the medium shortly before use.

2.3 Toxicants solution

All toxicant stock solutions were prepared immediately prior to the start of each experiment.

2.3.1 Cadmium solution

Cadmium chloride (anhydrous CdCl_2 , Analar grade, Sigma Chemicals Ltd., USA) was used to prepare the stock solution. 163.2 mg of anhydrous CdCl_2 was weighed out and added to 100 ml of dilution water (M7) in a 1000 ml volumetric flask. The weighing vessel was rinsed three times and washings added into the flask, the volume was then made up to 1000 ml to produce a 100 mg.l^{-1} (as Cd^{2+}) stock solution for range finding and LC50 tests. Similarly, 32.64 mg of CdCl_2 was dissolved in 1000 ml of dilution water to produce a 20 mg.l^{-1} (as Cd^{2+}) stock solution for sublethal studies. The appropriate amount of stock solution was mixed with prepared dilution water to make up the required exposure concentrations.

2.3.2 3,4-dichloroaniline (DCA) solution

3,4-dichloroaniline (DCA) was obtained from Aldrich Chemicals Ltd. UK. The DCA was powdered using a pestle and mortar to make it more easily soluble. 50 mg of powdered DCA were weighed out and added to 500 ml of dilution water in a 1000 ml conical flask. The weighed vessel was rinsed out and washings added into that conical flask and this was repeated three times to ensure that all chemicals

were added. The water in the flask was then made up to one litre by adding dilution water. The flask was shaken in a dark orbital incubator (XX1. C, Gallenkamp, UK) at 20°C at 180 rpm for 24 hours to make stock solutions of 50 mg.l⁻¹ for range finding and LC50 tests. Similarly, a second, 10 mg.l⁻¹ stock solution was made by weighing 10 mg of powdered DCA for sublethal studies. The conical flasks containing DCA stock solutions were wrapped with aluminium foil to avoid photodegradation. Exposure concentrations were made by mixing appropriate quantities of stock solution with dilution water. Stock solutions were kept in a refrigerator in the dark except when in use.

2.3.3 Chlorpyrifos solution

Chlorpyrifos (99% pure chlorpyrifos standard) was obtained from the University of Coimbra, Portugal. Chlorpyrifos was powdered by using pestle and mortar. 1000 µg of powdered chlorpyrifos was weighed out and propan-2-ol (1 ml) was added into a 5 ml volumetric flask and shaken slowly until completely dissolved. When dissolved the solution was poured into a one litre volumetric flask with 100 ml dilution water. The flask was then shaken well for mixing. The 5 ml flask was rinsed with dilution water five times into the one litre volumetric flask. The volume was finally made up to 1000 ml gradually by adding dilution water to make a stock solution of 1000 µg.l⁻¹ with a carrier of propan-2-ol (1 ml.l⁻¹). The exposure concentrations were made by mixing appropriate quantities of the stock solution with the dilution water. The stock solution was kept in a refrigerator in the dark except when in use.

2.4 Flow-through system

To maintain a constant exposure level and prevent the build-up of waste products and also to minimize the design complexity of commonly used flow-through systems, a simple flow-through system was developed. The design was based on inexpensive off-the-shelf components, which were potentially disposable, if required. It consisted of a series of truly independent channels allowing independent channel maintenance and providing test data which were suitable for analysis by inferential statistics (*e.g.* ANOVA). The basic design of the test system is represented in schematic plan view in Figure 2.6(a) illustrating its simple, modular construction, based on independent channels linked to a multichannel peristaltic cassette pump which could accommodate up to 16 channels simultaneously. A single channel of the system in elevation view consisted of a reservoir holding the premixed test compound in its test medium linked to an exposure chamber containing the test organisms (Figure 2.6(b)). The flow-through system was completely closed, since substances with a low water solubility may become concentrated in the microlayer at the water air interface (Lloyd, 1992) if any air was allowed to enter the system.

Toxicant reservoir

Fifteen 1 l amber glass bottles were used to deliver three replicate treatments of four different concentrations of toxicants and the dilution water as control. They were randomly allocated to different treatments. The reservoirs were filled with

freshly prepared toxic solutions of different concentrations and dilution water at the start of each experiment and thereafter refilled with prepared toxic solutions and dilution water on a daily basis.

Toxicant delivery system

Toxicant solutions were delivered to individual exposure chambers using a variable speed peristaltic multichannel cassette pump (Model 202 S, Watson Marlow, UK). Siliconized tubes (Manifold pump tubing of 1.52 mm internal diameter, Watson Marlow, UK) connected to individual channel frames (cassette) were used to deliver the toxicant solutions or dilution water constantly, at a regular speed. The speed of the pump and the internal diameter of the tubing determined the flow rate. Both free ends of the tubes were connected to translucent silicone tubing. One end of the tube was connected to the individual reservoir containing exposure solutions or dilution water, the other end was embedded in a bored silicone bung to fit into the open end of the exposure chamber. Construction materials of the system that came into contact with test solutions or dilution water were all either glass or silicone. Despite their relatively high cost these were selected on the basis of their relative non-reactivity, as they did not contain any substances which could be leached into aqueous solutions. The use of relatively inert substances also minimized adsorption of test chemicals from the solutions or dilution waters to contacting surfaces.

Exposure chamber

The exposure chambers comprised fifteen 130 ml capacity glass syringes connected to 0.8 mm diameter (internal) silicone tubes. The outlets to which the silicone tubes were attached were reduced by fixing silicone glue to prevent fry escape. Cylinders were arranged side by side on a grooved plastic gutter placed on a table. Outflowing toxicant solution and dilution water were collected in a 50 l plastic container for disposal. In a previous study Siriwardena (1993) maintained out flow from the exposure chamber by using clamps fitted to the outflow tube that comes from the tissue culture flask (exposure chamber) against the constant inflow maintained by peristaltic pump. It was found that exposure chambers sometimes cracked due to pressure when sealed tightly and to avoid this, they were sealed loosely to allow overflow. To overcome these problems, in this study, glass cylinders, which can endure high pressure without deformation were used, keeping the system completely closed, but enabling precise flow rate control without any requirement for clamps on the outflowing tube.

Flow rate of the system

Siriwardena (1993) reported a flow rate of $1.12 \text{ ml}\cdot\text{min}^{-1}$ in tilapia sac-fry flow-through toxicity tests. He reviewed the flow rates used in fish ELS toxicity tests and reported $1.12 \text{ ml}\cdot\text{min}^{-1}$ to be the lowest ever reported flow rate for fish early life-stage toxicity test. The flow rate of the present system was maintained at $0.51 \text{ ml}\cdot\text{min}^{-1}$, with a measured intrachannel co-efficient of variation $<1\%$ and

interchannel co-efficient of variation 4.1%. Daily flow per channel was 734 ml, equivalent to 5.65 volumes of exposure chamber per day which is within recommended values of water exchange (*e.g.* 4.8 -8.0 volumes: Sprague, 1969; 5 volumes: OECD, 1992). Flow rate depends on size of the exposure chambers and loading density. To minimize the use of toxic media, smaller size exposure chambers (130 ml) were used. Loading density was maintained within the recommended value (less than 0.5 g.l⁻¹ per 24 h and not exceeding 5 g.l⁻¹ of solution at any time; OECD, 1992). In fact densities were maintained (0.29 g.l⁻¹ wet weight; 0.05 g.l⁻¹ dry weight) well below OECD (1992) recommended maximum level for both lethal and sublethal tests. The use of less toxic media for toxicity tests is important to minimize quantities of chemical used, risks of contamination and costs, although the flow rate should be at a level that is suitable for the requirements of the test organisms.

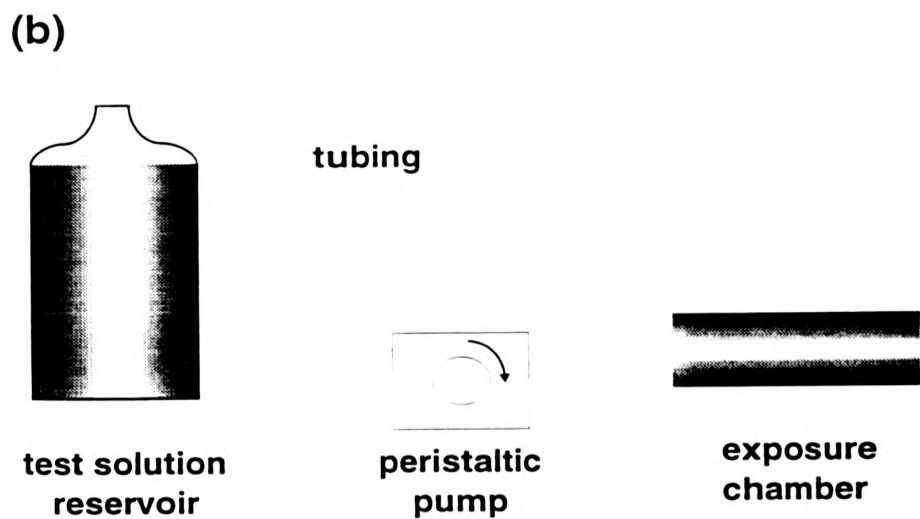
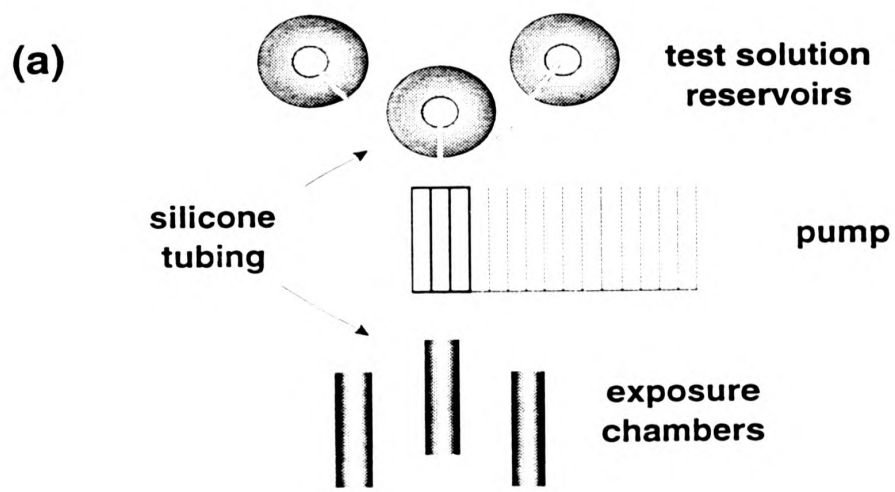


Figure 2.6 A schematic of the flow-through system in (a) plan view, showing three channels, and (b) elevation view, showing a single independent channel.

2.5 Preparation of the system for experiments

New reservoirs and exposure chambers were cleaned to remove any chemical or other residues remaining from the manufacture or accumulated during construction and storage. Hypochlorite at 200 mg.l⁻¹ (5 ml of household bleach in one litre of water) was put into the reservoir bottle and the chambers were immersed in the solution for six hours followed by several cycles of rinsing with tap water to disinfect. Nitric acid (5%) was then used in a similar way for five to six hours to remove mineral deposits and any metal residues. The system was also run for 2 to 3 hours with 5% nitric acid to clean any residues in the tubing. Again reservoirs and exposure chambers were rinsed with nanopure water and the system was run for five to six hours with nanopure water. In case of DCA and chlorpyrifos the system was run for an additional five to six hours with 2% acetone to remove any organics. Further, with chlorpyrifos the whole system was washed with 20% cyclohexane. Then the whole system was rinsed with nanopure water for five to six hours. Finally the system was washed with dilution water and the flowthrough system was run overnight with dilution water before the sac-fry were introduced into the exposure chambers.

2.6 Monitoring toxicants concentration

The nominal concentrations of the toxicants used in the present study were maintained by regulating the concentration of the toxicants in the stock solutions. The actual concentration of the toxicants in the exposure chamber during each

experiments were analyzed as follows:

Cadmium

Samples were acidified with 1% nitric acid and analyzed using an atomic absorption spectrophotometer, Perkin-Elmer 2280 (Golterman *et al.*, 1978). The actual concentration of cadmium did not vary more than $\pm 10\%$ of the nominal concentrations. The lowest detection level of cadmium was $0.5 \mu\text{g.l}^{-1}$ (W. Struthers, personal communication). The relationship between nominal and actual cadmium concentrations is shown in Figure 2.7.

3,4-dichloroaniline

Water samples were analyzed for 3,4-dichloroaniline (DCA) by using a HPLC (LDC Analytical, USA) at 242 nm with a 500 μl loop and 25 cm Partisil ODS 3 retention column using an eluent of 70% acetonitrile and 30% nanopure water. Calibration was made with $100 \mu\text{g.l}^{-1}$ DCA in mobile phase. A peak was resolved in 4.25 - 4.53 minutes. Actual concentrations did not vary more than 20 % of the nominal concentration in any of the experiments. The relationship between nominal and actual DCA concentrations is shown in Figure 2.8.

Chlorpyrifos

Analysis of either chlorpyrifos stock solutions or exposure concentrations for the actual of the nominals were not possible in this study, but stock solutions prepared

following the same procedure and analyzed over the period showed an appreciable change in concentration (Naylor, pers.comm.) with 63% recovered after 48 h and 59% after 9 days. Soares (pers.comm.) recovered 65% after 10 days under similar conditions to those used in this study. The concentration used in this study can be transformed into 60% (approximately) of the nominal concentrations, based on above data.

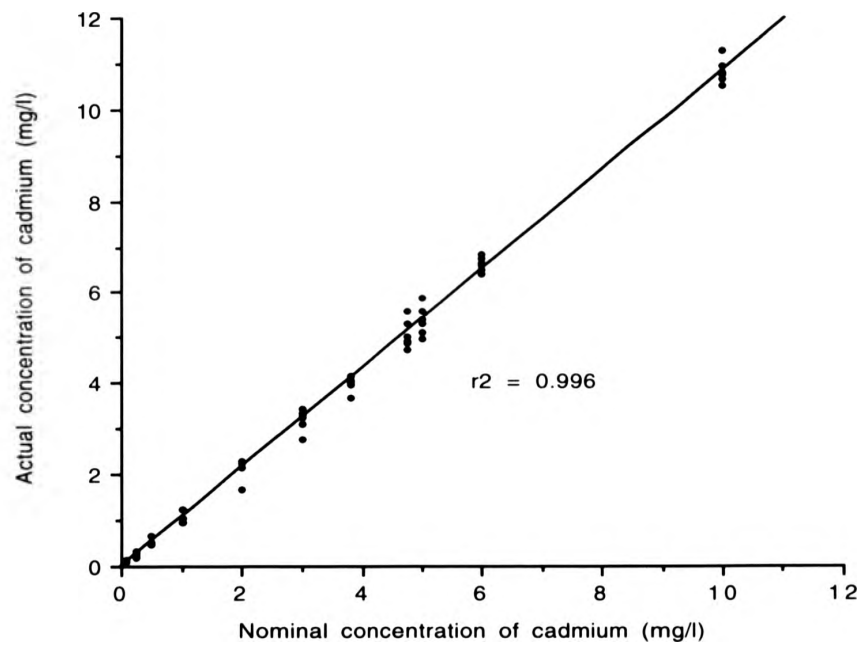


Figure 2.7 Relationship between nominal and actual concentration of cadmium.

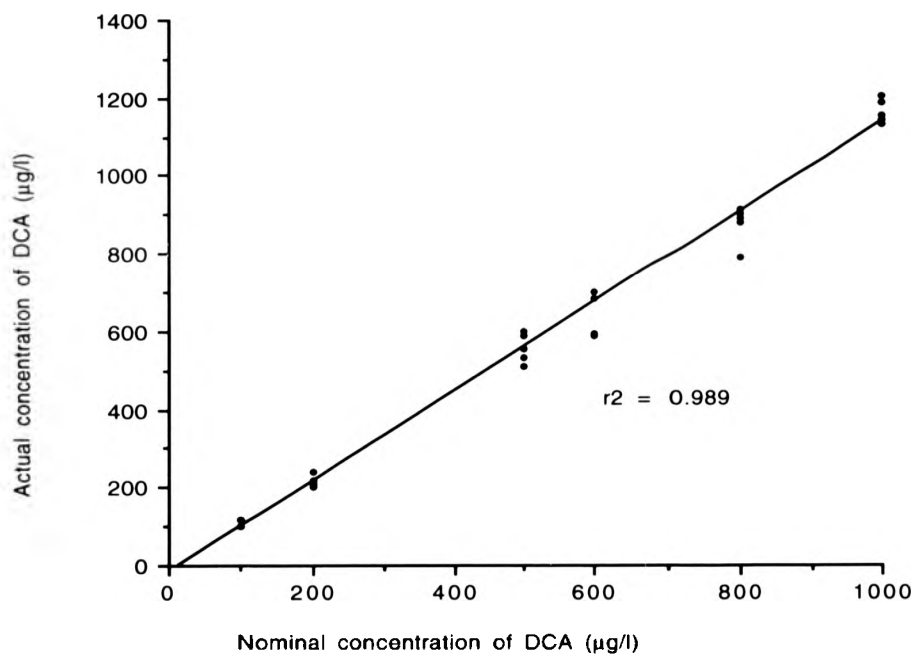


Figure 2.8 Relationship between nominal and actual concentration of DCA.

2.7 Statistical analysis

LC50 was estimated from a computer programme based on probit analysis (Finney, 1971). Parametric analyses were carried out using one way analysis of variance, and between-treatment differences were resolved using Tukey HSD test (Zar, 1984).

CHAPTER 3

YEAR-ROUND SPAWNING OF AND EARLY LIFE HISTORY TRAITS OF GOLDFISH

3.1 Introduction

3.1.1 Year-round spawning of gold fish

Under natural conditions, the timing of reproduction in fish is controlled by changes in environmental conditions, enabling the production of young to be synchronized with periods of maximal food supplies. This seasonality of reproduction, which is of considerable adaptive significance to wild stocks of fish, is often a disadvantage in research studies where supplies of eggs and fry may be required throughout the year. For the routine study of fish early life-stages to evaluate the toxicity of the ever increasing number of chemicals for regulatory purposes, a constant supply of fish eggs and larvae of consistent quality throughout the year is a prerequisite.

Aquatic ecotoxicologists commonly carry out fish early life-stage toxicity tests using fish obtained from commercial suppliers or which have been bred under laboratory conditions (Lloyd, 1992). For example rainbow trout is widely farmed in temperate areas and eggs can be obtained from the supplier. But such supplies are often unreliable and may only be available at certain times of the year and it

is often difficult to know the history of the material supplied. On the other hand some laboratories use the tropical zebrafish (*Brachydanio rerio* Hamilton Buchanan) for early life-stage toxicity tests. These fish are obtained from commercial suppliers and bred as required under laboratory conditions. *B. rerio* has been chosen as a standard species by the International Organization for Standardization (ISO, 1984) for toxicity studies due to its small size and its all-year-round availability. Interlaboratory studies of early life-stage toxicity tests have also been performed (Dave *et al.*, 1987; Nagel *et al.*, 1991) on this species. However, it is a warm water fish and requires high temperatures (+25°C) to breed, inviting dubious relevance in the toxicity test results (Dave *et al.*, 1987) which may be difficult to interpret, due to variable and high rates of larval deformity, and high egg and larval mortalities (Dave, 1993). Its sensitivity to stress is also poor when compared with other species (Nagel *et al.*, 1991).

Furthermore, zebrafish produce only a small number of eggs in a spawning. For the procurement of required amount of eggs and larvae of zebrafish for early life-stages toxicity tests it is necessary to allow the fish to perform group spawning (5 - 10 fish in a batch: OECD, 1992). Eggs and larvae thus produced from group spawning may be heterogenous and may produce different responses when exposed to toxicants. It is widely accepted that organisms from the different environment and different gene pools may vary in their response to stress *e.g.* Baird *et al.* (1991), found different response to toxicants by different clones of *Daphnia magna*. Therefore it is important to use the eggs and larvae from the same set of brood-fish

for the consistency of test results.

Goldfish (*Carassius auratus*) is a well known fish of the Cyprinidae family which can produce as many as 15,000 eggs in a single spawning (Horvath *et al.*, 1992). It has the potential to breed repeatedly over the course of its life span and throughout the year under controlled laboratory conditions (Razani and Hanyu, 1986a, Razani *et al.*, 1989). But detailed information on the conditioning of laboratory-held brood fish for the production of a continuous supply of eggs and sac-fry of consistent quality is lacking. Finding an appropriate combination of factors for stimulating full maturation of ovaries and induced breeding is of importance for the continuous supply of eggs and larvae for early life-stage toxicity tests.

In the natural environment increasing day-length and temperature stimulate ovarian growth, maturation and ovulation in goldfish (Gillet *et al.*, 1978). Oocyte growth starts during winter and progresses to ovulation by spring when the water temperature reaches a level of 18 - 20°C (Melotti, 1986). In the laboratory female goldfish maintained in cold water (13 - 14°C) develop to a pre-ovulatory condition (Yamazaki, 1965), and from females with mature ovaries, ovulation can be induced within two days at a temperature increase from cold (13 - 14°C) to warm water (20°C) (Yamamoto *et al.*, 1966). This technique has been used in several studies for the ovulation in this species (*e.g.* Stacey *et al.*, 1979). Other external factors such as photoperiod (Stacey *et al.*, 1979), presence of active males (Hervey and

Hems, 1968), presence of aquatic vegetation (Stacey *et al.*, 1979) and standing water (Peter *et al.*, 1978) also regulate the ovulation of goldfish. Critical photoperiod for ovarian maturation and ovulation in goldfish reared in warm water (24°C) lies between 13L : 11D to 14L : 10D photoperiod (Razani *et al.*, 1987) but there is no critical level of such photoperiod for ovarian maturation and ovulation in fish reared in cold water (16°C) (Razani and Hanyu, 1986a, 1986b). The critical temperature for ovarian maturation of goldfish has been suggested to lie between 18 and 21°C regardless of photoperiod (Razani *et al.*, 1987). Male goldfish remain mature all-year-round when maintained in cold water (13 - 14°C) and can produce sufficient milt within a day for fertilization of ovulated eggs by transferring to tank water at 20°C (Yamamoto *et al.*, 1966). Milting condition of males held at 20 ± 1°C can be maintained by routine injection of human chorionic gonadotropin (Wiegand *et al.*, 1988) or carp pituitary extract (Wiegand *et al.*, 1989).

In most published research studies on goldfish, spawning involves collection of mature goldfish from a supplier and rearing for a short conditioning period. Fish are then spawned by altering environmental factors such as temperature, photoperiod and the presence of spawning substrate (Yamamoto *et al.*, 1966; Stacey *et al.*, 1979) and/or by hormonal induction (Moriwaki *et al.*, 1991; Peter *et al.*, 1987; Sokolowska *et al.*, 1984, 1985; Suzuki *et al.*, 1988). Although hormonal treatment is a relatively straightforward method to spawn fish (*e.g.* Das *et al.*, 1992; Halder *et al.*, 1991; Peter *et al.*, 1988), the repeated use of hormone and in particular Human Chorionic Gonadotropin (HCG) can induce ovarian refractoriness

in brood stock (VanDer Kraak *et al.*, 1989) which may develop some antigenicity to HCG (Donaldson and Hunter, 1983; Fukein- Chiangsu- Chekiang- Sanghai Cooperative Group, 1977) and ultimately fish may become unresponsive to HCG injections (Fukein-Chiangsu-Chekiang- Sanghai Cooperative Group, 1977). Despite these difficulties, efforts have been made to obtain year-round rematuration and spawning of goldfish in the laboratory by hormone injection. Razani *et al.* (1988a, 1988b, 1989) conducted studies on the maturation of both male and female goldfish under different combinations of temperature and photoperiod over a 12-month period (July to June) and found that at low temperature (16°C) males remain mature all the year-round irrespective of photoperiod whereas at high temperature (24°C), maturity, expressed in terms of milt production was dependent on photoperiod. Females matured, rematured and spawned all-year-round (Table 3.1) at low temperature (16°C), but spawned only for a short period (July-August) at high temperature (24°C).

Table 3.1 Rematuration of female goldfish at different combination of temperature and photoperiod (Calculated from Razani *et al.*, 1989).

Temperature	Photoperiod	Average spawning/ individual/ Year	Spawning season
16°C	16L : 8D	2.5	Year round
16°C	12L : 12D	2.25	July-Aug. & Feb-June
24°C	16L : 8D	1.3	Mostly July-Aug.
24°C	12L : 12D	0.2	July-Aug.
Natural	Natural	1.16	May-Aug.

Considering the critical range of temperature (18 - 20°C: Razani *et al.*, 1987) for goldfish maturation, natural history of spring (>12 h light period in a day) spawning (Hrevey and Hems, 1968; Melotti, 1986) and in view of proper utilization of laboratory space, brood fish were reared at 20°C and 14L : 10D photoperiod. It was also desirable to produce eggs and sac-fry under the same laboratory conditions, with both fish and larvae located in the same laboratory. In this study, in addition to the brood fish rearing at 20°C and 14L : 10D photoperiod to prepare them for spawning, several preliminary experiments on goldfish spawning by using the technique of temperature change only (Stacey *et al.*, 1979) and changing temperature in combination with hormone injection (Sokolowska *et al.* 1984) with minor modification on the basis of laboratory facilities available were also planned to select simple induced spawning techniques for goldfish under laboratory conditions to procure eggs and sac-fry.

In this study fish were maintained in tanks of a recirculatory system separating them by sex. Once they had reached milting condition males remained in that condition and required no hormonal treatment. Females, which it had been originally planned to spawn over a period of time, one by one, on the other hand, ovulated in groups in the morning without any treatment. These females rematured after three months and same phenomenon (group spawning) happened again. As a result of these synchronized ovulation, routine experiments suffered, leaving long gaps without a supply of material (eggs).

To obtain eggs throughout the year alternative approaches were investigated. From reproductive physiological reports (Idler *et al.*, 1987; Scott *et al.*, 1991) it was evident that reproductive activity might be controlled by some pheromone: defined here as a chemical communication signal and considered to be the evolutionary consequence of interactions with other individuals (Stacey, 1991).

Recently, the study of pheromones and their role in maturation in fish has significantly advanced (Cardwell *et al.*, 1991; Sorensen, 1991; Sorensen and Stacey, 1987; Sorensen *et al.*, 1991; Stacey *et al.*, 1987). In all cases reported so far, teleost reproductive hormones and their metabolites released by fish into the surrounding water exhibit some pheromonal activity (Stacey and Sorensen, 1991). Goldfish sex pheromones have been identified as a mixture of unmodified hormones and metabolites (Sorensen *et al.*, 1991). Goldfish females sequentially release two steroid hormonal pheromones: a primer pheromone (slower physiological responses) and a releaser pheromone (rapid behavioral responses). Water-borne preovulatory primer pheromone released by female increases milt volume by acting via olfactory receptors to increase blood GtH (Gonadotropic Hormone) in males, which in turn stimulates the testes (Sorensen and Stacey, 1990). Water-borne postovulatory releasers induce the male to engage in courtship (Sorensen and Stacey, 1990). The female primer pheromone system seems common to all members of the carp family so far studied (Cardwell *et al.*, 1991). Recent evidence suggests that gonads produce nonsteroidal, water-soluble proteins which specifically regulate the production and secretion of gonadotropin in mammals (Ying, 1988). Inhibin is

identified as a suppressor of pituitary FSH (Follicle Stimulating Hormone) secretion, whereas activin has been identified as a stimulator of FSH secretion. Peter *et al.* (1991) extracted both inhibin and activin from the goldfish ovarian extract and found that both type of proteins acutely stimulate GtH secretion in goldfish.

Pheromones can provide information about reproductive state and gender but it has not yet been experimentally proven whether hormonal pheromones can provide species-specific information, particularly in sympatrically-spawning species (Cardwell *et al.*, 1991). In natural environments, closely related species may live sympatrically and yet maintain differences in spawning period. Considering the pheromonal sensitivity and their species-specificity and also considering the complexity and incompatibility of the existing techniques of goldfish spawning, an experimental programme was planned to evaluate goldfish ovarian fluid as a stimulant for ovulation of goldfish as an easily workable, dependable, yet simple method of goldfish spawning under laboratory conditions.

3.1.2 Early life history traits of goldfish in the flow-through system

Before carrying out toxicity tests using a new test organism and/or a new test exposure system, it is first necessary to demonstrate that the test organisms perform satisfactorily within the system in 'unstressed' conditions (*i.e.* with no toxicant added). The loading density of the test organisms, defined as the ratio of sac-fry

biomass to the volume of solution in an exposure chamber of the toxicity testing system should be such that they can maintain 'normal' physiological functions. There should be no stress due to produced metabolites (*e.g.* ammonia), or impairment by reduced oxygen supply. It is also important to determine an appropriate duration for the test exposure, on the basis of the physiological status of the experimental organisms. In the case of fish sac-fry, held without external food supply over the chosen exposure period, it is important to determine the stage at which the maximum dry body mass is attained. Larval growth in terms of dry mass is more relevant than the wet mass as water content in the tissue may vary under different conditions (*e.g.* water content of the tissue may increase under starvation, Hemming and Buddington, 1988) possibly resulting in dubious results of yolk utilization efficiency. Energy equations for fish early life-stages change during the later part of yolk utilization (Kamler, 1992). Sac-fry growth progresses as long as absorbed yolk energy is surplus to metabolic costs and its body mass reaches its maximum level at a certain point. After this the sac-fry enters a state of starvation with regressed growth without external food supply.

3.1.3 Aims

The aims of the work described in this chapter were:

1. to develop a brood stock management system for the continuous supply of good quality eggs and sac-fry of goldfish from a small stock of brood fish.
2. to establish the time taken to attain maximum dry body mass of

goldfish sac-fry in the absence of external food supply.

3. to determine a loading density of fish sac-fry in each exposure chamber of the test system suitable for conducting early life-stage toxicity tests.

3.2 Materials and Methods

3.2.1 Techniques of ovulation of goldfish under controlled laboratory conditions

3.2.1.1 Preliminary experiments

Preliminary experiments were conducted to induce ovulation to search for a reliable production method for consistent quality eggs and sac-fry:

(a) Ovulation of goldfish by raising water temperature and changing photoperiod

Changing in temperature (from cold to warm) was initially used to ovulate female goldfish (Yamamoto *et al.*, 1966; Stacey *et al.*, 1979). In this study mature +1 female goldfish were reared individually in a 120 l glass tank maintained at 15°C and subjected to 12L : 12D photoperiod for either two, three, four or five weeks during April and June, 1992. Mature females from which a few eggs could be extruded by gentle pressure on the abdomen were selected for the experiments. The

fish were transferred to a tank set at 20°C and 14L : 10D photoperiod. On the second day a male was added to the tank a few hours after the onset light and removed near the end of light period to ensure female was ready to spawn during early hours of the light period, rather than spontaneously spawning during the latter part of the dark period which would otherwise occur in the continuous company of the male fish (Hervey and Hems, 1968; Melotti, 1986). This was repeated on succeeding days. Using this procedure females ovulated in one to five days. Ovulated females were stripped in the morning (7 am to 10 am) and eggs fertilized and incubated as described in section 2.1.2. Fertilization and hatching performance were recorded.

(b) Ovulation of goldfish by pimozide and LHRH-a injection

Ovulation by temperature manipulation proved to be unreliable. Spawning trials were therefore conducted using pimozide and LHRH-a (des-Gly¹⁰ [D-Ala⁶]-Luteinizing Hormone Releasing Hormone ethylamide) injection. LHRH-a (Sigma chemical Co. Ltd., St. Louis, USA) was dissolved in freshwater teleost physiological solution (PS; Burnstock, 1958). A synthetic dopamine antagonist 'Pimozide' (Sigma chemical Co Ltd., St. Louis, USA) was suspended in a vehicle containing 0.7% NaCl and 0.1% sodium metabisulphite (Sokolowska *et al.*, 1984). Mature females were selected from fish reared at 15°C and 12L : 12D photoperiod from which a few eggs had been extruded by gentle pressure on the abdomen to confirm the preovulatory maturation state. Fish were transferred to tanks maintained at 20°C and subjected to a 14L : 10D photoperiod. Fish and mature females

maintained at 20°C were injected intraperitoneally with pimozone at the dose of 10 $\mu\text{g}\cdot\text{g}^{-1}$ body weight (10 $\mu\text{l}\cdot\text{g}^{-1}$ body weight) and LHRH-a at the dose of 0.1 $\mu\text{g}\cdot\text{g}^{-1}$ body weight (5 $\mu\text{l}\cdot\text{g}^{-1}$ body weight) as per Sokolowska *et al.*(1984), at the base of the pectoral fin of anaesthetized fish at 2 pm and 5 pm, respectively, on the second day of temperature shock. Ovulation was checked on the following morning (7 am to 10 am). Ovulated females were stripped and eggs fertilized and incubated as described in section 2.1.2. Fertilization and hatching performance were recorded.

3.2.1.2 Ovulation of goldfish by inoculating ovarian fluid into the water of spawning tanks

Eggs from pimozone and LHRH-a injected female from an earlier experiment were stripped in a petridish and the ovarian fluid was pipetted with a digital Finnpiquette (5 - 40 μl) in microcentrifuge tubes. The ovarian fluid was frozen at -70°C for later use. Thereafter ovarian fluid was collected following manual stripping of fish ovulated by this technique. Preovulated females were kept in a spawning tank maintained at 20°C and the tank water was spiked with the thawed ovarian fluid (stored at -70°C for up to 1 month) at 10 - 11 am, to give a concentration of 2 μl , 3 μl , and 4 μl of ovarian fluid per litre of tank water. A male was added with a female in a tank just after spiking of ovarian fluid and kept in the tank for the light regimes only. Ovulation was checked for the next five consecutive mornings. Ovulated females were stripped and eggs fertilized and incubated as described in section 2.1.2. Fertilization and hatching performance were recorded.

Between 5000 and 42,000 eggs were collected from a single spawning by manual stripping from females of 58 g to 310 g body weight. From the whole volume of eggs, measured in a graduated cylinder, 3 samples of 1 ml of eggs were transferred to Petri dishes, water was added and eggs were spread in a monolayer. After water hardening the number of eggs in each sample was counted under a dissecting microscope. The total number of eggs was calculated by using the following formula.

$$N = \frac{n \times V}{v} \quad (3.1)$$

where N = total number of eggs in a spawning

n = average number of eggs in a sample

V = total volume of eggs in a spawning

v = volume of the sample used

Numbers were rounded to the nearest thousand.

3.2.2 Optimisation of broodfish stocking density for procuring all-year-round good quality eggs and sac-fry

Both 1+ age group males and females were kept separately in a 120 l tank fitted in a recirculatory system (see 2.1.1) at $20 \pm 1^\circ\text{C}$ and 14L : 10D photoperiod. Female fish were maintained in three separate tanks at densities of three, two and one fish per tank (120 l) for three months. The readiness of the female fish for induced ovulation was checked weekly by gentle abdominal pressure. The fish from

which a few ova could be extruded by abdominal pressure were induced to ovulate by inoculating ovarian fluid into the water of the spawning tanks.

3.2.3 Effects of selected spawning substrate on the goldfish early life stages

Artificially ovulated eggs collected in a Petri dish by manual stripping were fertilized and spread on glass slides and polystyrene Petri dishes. Petri dishes were attached to a polystyrene pipette frame for easy handling in the incubation system (see Figure 2.3). Glass slides were placed on a slide racks and hung in the incubation system (Figure 3.1). Fertilization and hatching performance were recorded.

3.2.4 Survival of goldfish sac-fry in the exposure chamber of the flowthrough toxicity testing system

Normal sac-fry (see Figure 2.5a) hatched in the incubation system were stocked at 30, 40, and 50 sac-fry per exposure chamber in the flowthrough system. The sac-fry were exposed to dilution water (M7 medium: see section 2.2) in the exposure chambers by daily refilling of reservoirs with dilution water. Trials were terminated when mortality (lack of reaction to mechanical stimulus) exceeded 50% in all the chambers. Mortality was checked daily. Dead sac-fry, if any, were removed from the exposure chamber every day, preferably at the same time of the day (between 10 and 12 am).

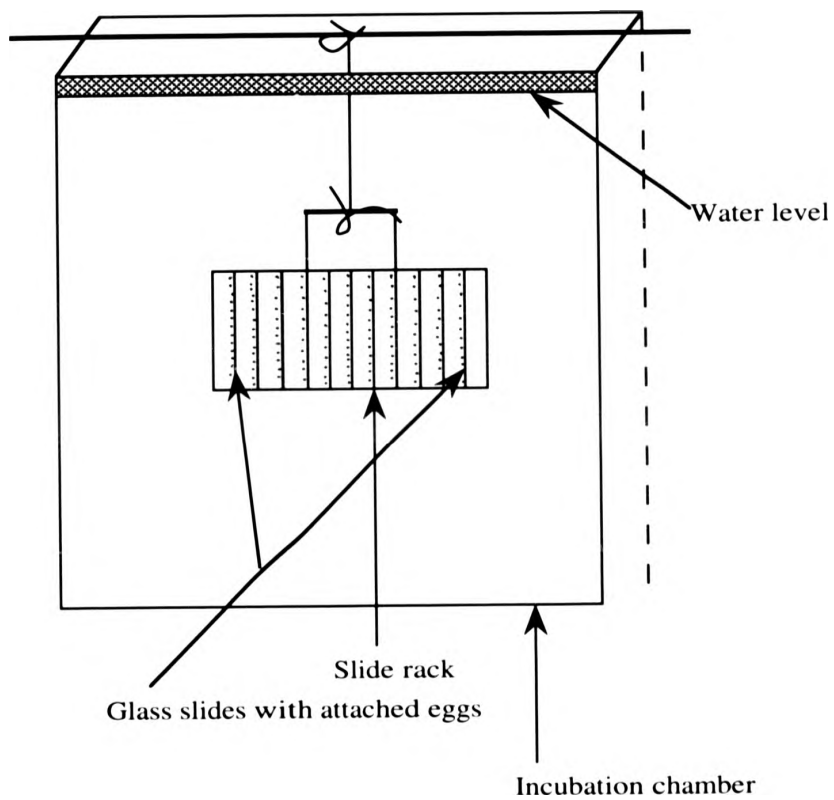


Figure 3.1 A view of the eggs attached on the glass slides placed on a slide rack in a chamber in the incubation system.

3.2.5 Hatching success at different loading densities of eggs in the exposure chamber of the toxicity testing system

Artificially ovulated eggs collected in a Petri dish by manual stripping were fertilized and spread on glass slides. Subsequently, the glass slides were placed in slide racks and placed in an incubation chamber (Figure 3.1) in the incubation system for 24 hours. The rate of fertilization was recorded by counting transparent eggs (live) and white eggs (dead) under a magnifying glass. Dead eggs and/or excess eggs on the selected slide were gently removed with the help of a Pasteur pipette. Five triplicate densities of eggs (30, 40, 50, 60 and 70 eggs.glass slide¹) were placed in exposure chambers in the flow-through system. The rate of hatching and presence of abnormalities among the hatched sac-fry were recorded after a total of 125 hours of incubation by counting emerged sac-fry and identifying them as normal and abnormal (see section 2.1.3) under a dissecting microscope (Olympus, Japan).

3.2.6 Oxygen and ammonia concentration at different densities in the exposure chamber of the toxicity testing system

To find out the suitable loading density of sac-fry in the exposure chambers for toxicity testing, oxygen and ammonia concentrations were measured in the outflowing water from the test chambers for different densities of sac-fry. Three replicate densities of sac-fry (30, 40 and 50 sac-fry per chamber) were introduced into the exposure chamber of the flow-through toxicity testing system. At 24, 48

and 96 hours after the introduction of newly hatched sac-fry into the exposure chambers, oxygen levels were determined by withdrawing water samples from the exposure chamber outflow tube with a syringe, and injecting the sample into a jacketed flow-cell microelectrode (TC 500, Strathkelvin Instruments, UK) connected to a dissolved oxygen meter (Model 781, Strathkelvin, Instruments, UK). At 24 and 96 hours after introduction of newly hatched sac-fry into the exposure chambers, 5 ml of outflowing water from each test chamber was transferred into a clean plastic vial, stoppered and frozen for analysis of ammonia at a later date. Ammonia was measured colorimetrically in an autoanalyser by standard methods (Technicon Sampler IV, Gradco International Ltd., UK). Measured oxygen content and ammonia content of the exposure medium with different loading densities were compared with the recommended values of OECD (1992) and Widdows (1985) respectively for toxicity test conditions.

3.2.7 Early life-history growth traits of goldfish in the exposure chamber of the toxicity testing system

Before carrying out toxicity tests with sac-fry, and considering physiological aspects of growth as test endpoints it is important to know the growth performance of sac-fry within the test system with no toxicant added, for the valid interpretation of test results. Thirty newly-hatched sac-fry were exposed to dilution water in each exposure chamber. From 20 randomly selected newly hatched sac-fry, 10 whole sac-fry and 10 sac-fry with yolks removed by dissection were placed individually in previously weighed (using a microbalance of 1µg accuracy, MT5, Mettler, UK)

cones of aluminium foil after removing surface water by tissue blotting, weighed and stored in microcentrifuge tubes, labelled and frozen individually at -70°C . After 24, 48, 72, 84, 96, 108 and 120 h, respectively, pooled samples of 20 fish larvae were taken from two randomly selected exposure chambers. Ten of the 20 larvae were dissected under dissecting microscope to remove their yolk mass, if present. The larvae were then dried on absorbent paper, placed individually in a preweighed aluminium foil cone, weighed and placed in individually labelled microcentrifuge tube and frozen at -70°C . To obtain a dry mass estimate, those samples were freeze dried to a constant weight using a freeze dryer (Modulyo 4K, Edwards, UK) and re-weighed. Dry mass estimation was carried out on individuals, using the microbalance. Yolk mass was calculated indirectly by subtracting the body mass from the whole mass.

3.3 Results

3.3.1 Induced spawning of goldfish

Low temperature (15°C) conditioning for different periods to prepare the fish for induced ovulation (Table 3.2) indicated that two out of three fish conditioned for 28 days and three out of four fish conditioned for 35 days successfully ovulated as a result of changing temperature and photoperiod and adding males during the light regime. Fish conditioned for less than 28 days did not ovulate (Table 3.2). Ovulation occurred within five days of temperature shock.

Three out of five fish conditioned at low temperature (15°C) and injected with pimozide and LHRH-a successfully ovulated (Table 3.3) but in the case of fish maintained at 20°C constant temperature and injected with pimozide and LHRH-a, only two fish out of five ovulated.

Inoculating tank water with ovarian fluid at concentrations of either 2 µl, 3 µl or 4 µl ovarian fluid per litre of tank water gave 100% success with fish maintained at a constant temperature of 20°C (Table 3.4). To test the repeatability of using goldfish ovarian fluid to induce ovulation of goldfish as a successful technique, a total of 15 fish were induced by inoculating the intermediate concentration of ovarian fluid (3 µl.l⁻¹ of tank water). Thirteen fish out of 15 females ovulated as a result of this technique. Ovulation occurred within three days of being exposed to ovarian fluid and the addition of a male during the light regime.

Arcsine transformed values of percentage of fertilization success, hatching success, and proportion of normal sac-fry emerged on hatching of eggs (see section 2.1.3) were analyzed by one way ANOVA. There were no significant differences between fertilization success ($F = 0.81$; $df = 4, 40$; $P > 0.05$), hatching success ($F = 0.04$; $df = 4, 40$; $P > 0.05$) and proportion of normal sac-fry emerged on hatching of eggs ($F = 0.52$; $df = 4, 40$; $P > 0.05$) obtained by different ovulation techniques. The percentage of fertilization success, hatching success and normal sac-fry emerged on hatching of eggs was always above 90% in all the techniques used.

3.3.2 Conditioning of goldfish brood stock at different densities under 20°C and 14L : 10D photoperiod

The rate of maturity (preovulatory stage: a few eggs could be extruded by gentle abdominal pressure) was found to be between 2.7 and 25.3 per individual per year (Table 3.5) among the groups reared at densities one - three fish per 120 l capacity tank maintained at 20°C and 14L : 10D photoperiod fitted in a recirculatory system. The re-maturation of goldfish females reared at density of one fish per tank of 120 l capacity gave maximum spawning success (Table 3.5). Fish reared individually in a tank rematured every ten to eighteen days. Fish reared for three months at the density of 3 fish per tank spawned 2.7 times per year per individual, 2 fish per tank gave 4.7 ovulation per year per individual and 1 fish per tank gave 25.3 ovulation per year per individual (Table 3.5). Fish stocked individually in a tank inoculated with ovarian fluid (3 µg.l⁻¹ of tank water) was subsequently used for induced spawning of goldfish. From September 1993 to February 1994, each fish spawned 12, 11 and 10 times, respectively, by this technique.

Table 3.2 Ovulation success in *Carassius auratus* broodstock following different periods of low temperature conditioning

Days held at 15°C	Proportion of successful ovulations
14	0 out of 3
21	0 out of 3
28	2 out of 3
35	3 out of 4

Table 3.3 Ovulation success in *Carassius auratus* broodstock by injecting pimozide and LHRH-a at 20°C

Fish matured under different conditions	Proportion of successful ovulations
Temperature conditioning plus Pim. & LHRH injection	3 out of 5
Pim & LHRH injection without temperature conditioning	2 out of 5

Table 3.4 Ovulation success in *Carassius auratus* broodstock held in different concentrations of ovarian fluid at 20°C

Ovarian fluid $\mu\text{g.l}^{-1}$ tank water	Proportion of successful ovulations
2	3 out of 3
3	3 out of 3
4	3 out of 3

Table 3.5 Rematuration of *Carassius auratus* stocked at different densities in the laboratory controlled condition (at 20°C; 14L:10D) and ovulation using ovarian fluid

No. of fish/tank	Total no. of fish	Total no. of ovulation	Ovulation/female/year
3	9	$6=(1 \times 2 + 4 \times 1 + 4 \times 0) *$	2.7
2	6	$7=(2 \times 2 + 3 \times 1 + 1 \times 0) *$	4.7
1	3	$19=(2 \times 7 + 1 \times 5) *$	25.3

* Figure in parenthesis represent the number of fish x frequency of spawning in 90 days.

3.3.3 Performance of goldfish early life-stages in the incubation system and in the exposure chambers of the toxicity testing system

The percentage of fertilized eggs, percentage of hatching and percentage of normal sac-fry emerged on hatching of eggs using two substrates (polystyrene petridish and glass slide) are shown in Figure 3.2. The percentage of fertilization ($F = 4.09$; $df = 1, 4$; $P > 0.05$), percentage of hatching ($F = 0.00$; $df = 1, 4$; $P > 0.05$) and percentage of normal sac-fry emerged on hatching of eggs ($F = 5.45$; $df = 1, 4$; $P > 0.05$) were not significantly different between the two substrate used. The percentage of fertilization, percentage of hatching and percentage of normal sac-fry emerged on hatching of eggs in all trials was over 90%.

Sac-fry exposed to dilution water at 30, 40, and 50 per chamber showed no mortality until the 11th day of exposure under starvation. For fry starved at 50 sac-fry per chamber, more than 50% mortality occurred on day 12, whereas at 30 and 40 sac-fry per chamber this occurred on day 13 (Figure 3.3).

The hatching success of eggs in the exposure chamber of the toxicity testing system is shown in Figure 3.4. Hatching success for eggs stocked at 30 eggs per chamber was 90% but this reduced as the density was increased (Figure 3.4) and only 79.5% eggs hatched when stocked at 70 eggs per chamber. Hatching was negatively correlated ($r = -0.78$, $p < 0.05$, $n = 15$) with the density of eggs incubated in the flowthrough system. There were significant differences in hatching success ($F = 4.09$; $df = 4, 10$; $P < 0.05$) between 30 and 70 fry per chamber.

Oxygen and ammonia concentrations in the exposure system loaded with different densities of sac-fry are shown in Figure 3.5 and Figure 3.6. Oxygen levels of the exposure water decreased with increased sac-fry loading density and also within each density over time as expected. Oxygen levels were always above OECD (1992) recommended value (Figure 3.5). Oxygen levels in chambers having density of 30 sac-fry per chamber were above 90%. In other chambers it was above 90% for up to 24 h period and then went down with time although it never reached below 80% of the saturation level. On the other hand ammonia levels of the exposure water increased with higher sac-fry densities and also within each density over time (Figure 3.6) but remained below $140 \mu\text{g.l}^{-1}$ in all cases. Ammoniacal nitrogen concentration $>140 \mu\text{g.l}^{-1}$ is considered inhibitory (Widdows, 1985).

The yolk utilization patterns of goldfish sac-fry are shown in Figure 3.7. Goldfish sac-fry attain maximum dry body mass within 96 h after hatching at 20°C (Figure 3.7a), although some yolk remained at this stage. Whole dry mass also attained its maximum at 96 hours after hatching. Whole wet mass attained a maximum at 108 h after hatching, whereas wet body mass attained a maximum at 120 h after hatching (Figure 3.7b). The ratio of dry mass to wet mass declined with time within the experimental period (Figure 3.8) *i.e.* water percentage increased with the age of goldfish sac-fry. Yolk contained less water than body.

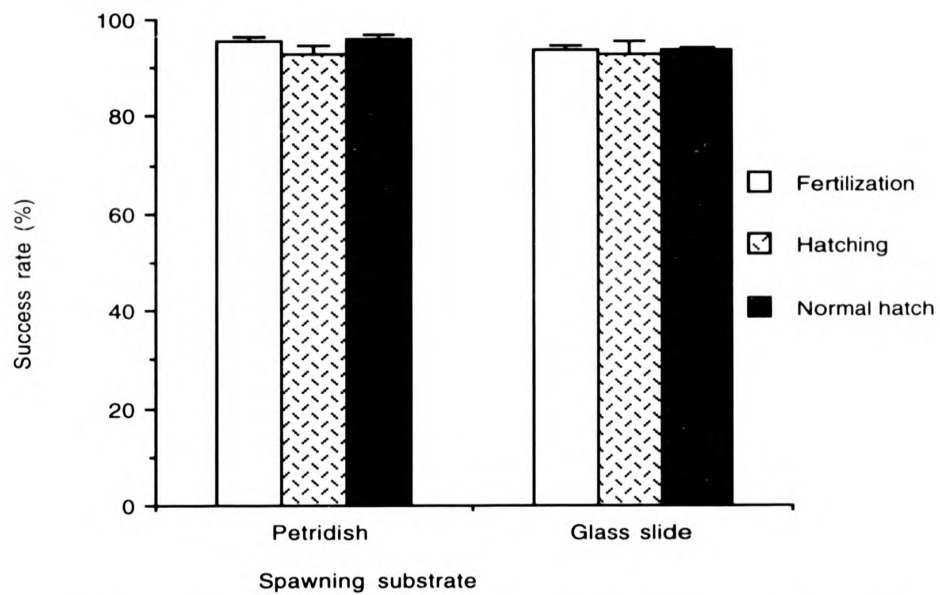


Figure 3.2 Percentage of fertilization, hatching and normal sac-fry emerged on hatching of goldfish eggs attached on two substrates (Polystyrene petridish & Glass slides) incubated into the incubation system. Bars are mean of three replicates given with + standard error of mean.

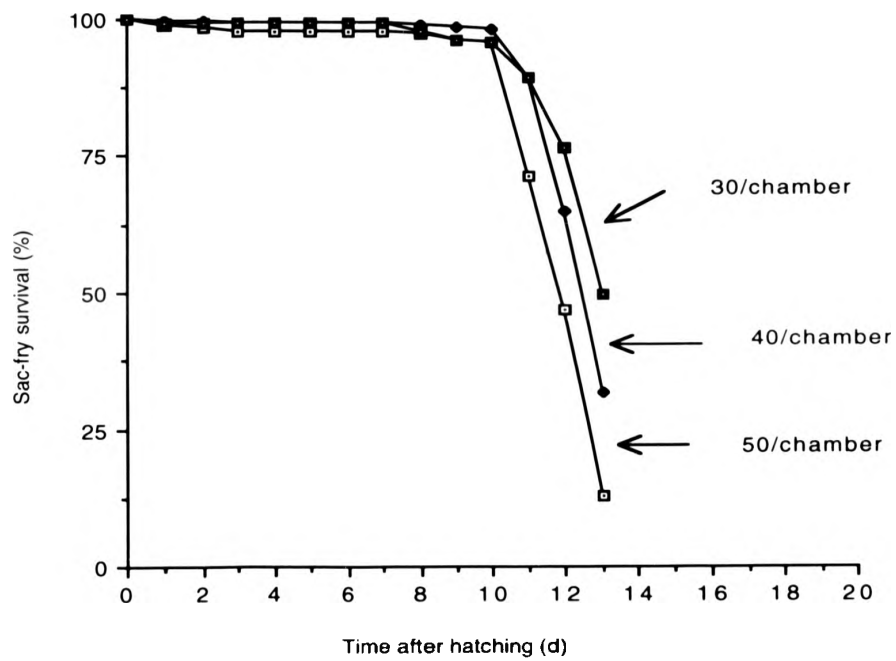


Figure 3.3 Mortality pattern of goldfish sac-fry at different densities exposed to dilution water in the exposure chambers of the toxicity testing system.

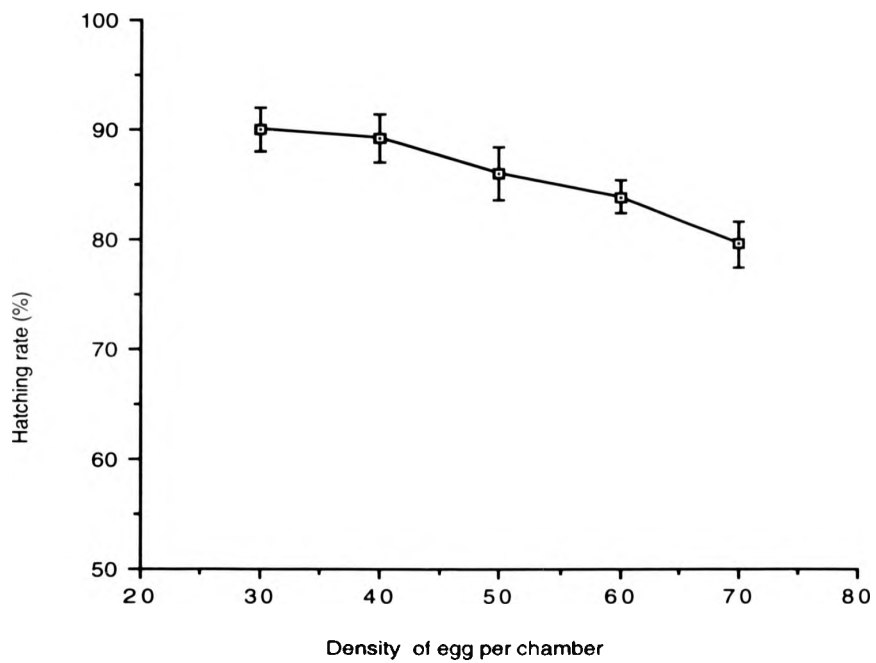


Figure 3.4 Percentage of hatching success of goldfish eggs attached on glass slides and incubated into exposure chambers of the toxicity testing system at different densities. (Bar indicates \pm standard error).

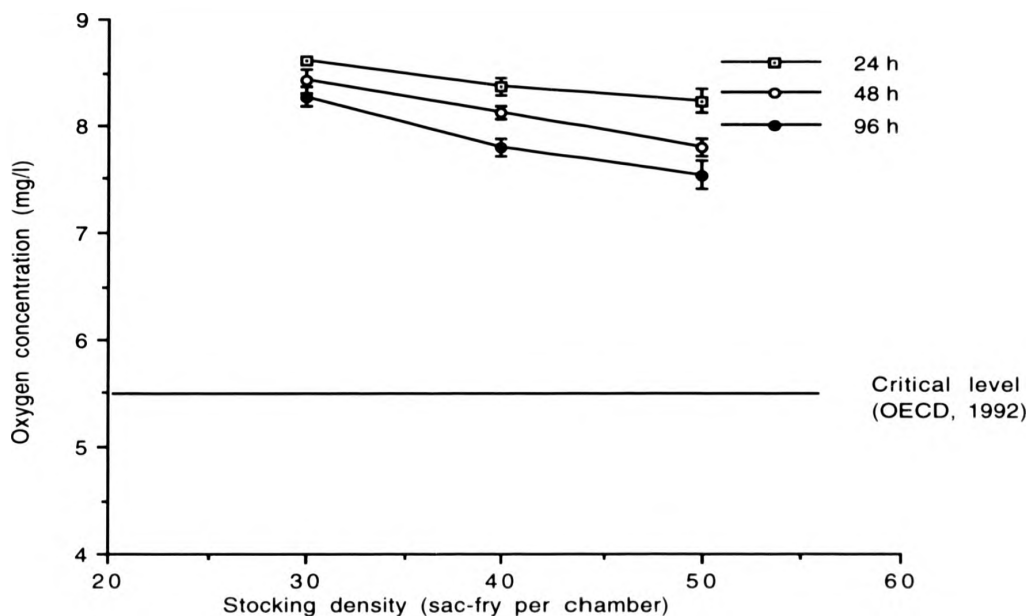


Figure 3.5 Influence of stocking density of goldfish sac-fry on the oxygen concentration in the exposure chamber of the flowthrough system. Data are means \pm standard errors. Critical level for toxicity tests is shown by a line.

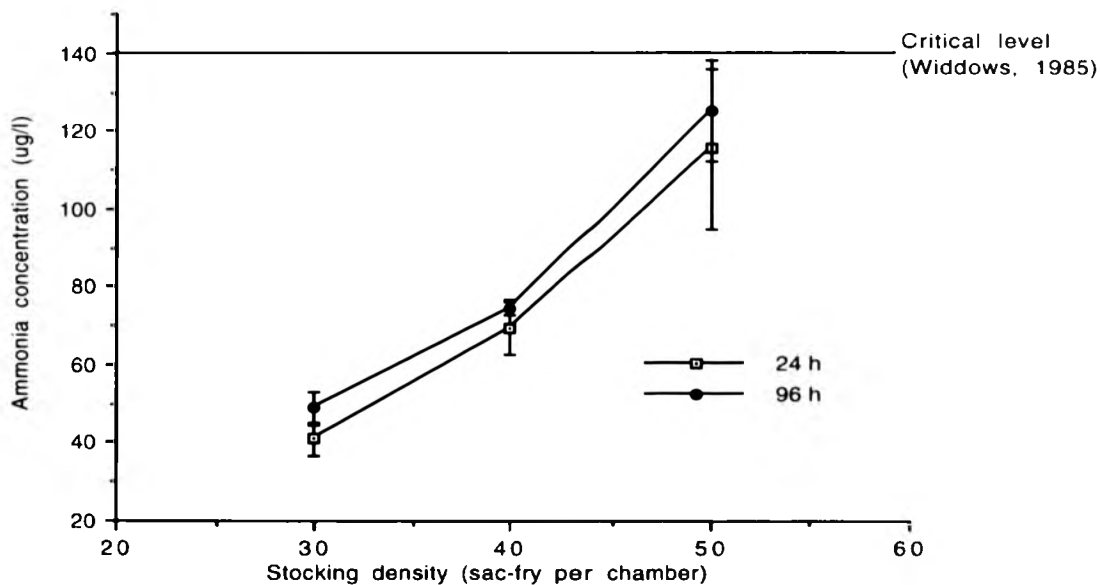


Figure 3.6 Influence of stocking density of goldfish sac-fry on the ammonia concentration in the exposure chamber of the flowthrough system. Data are means \pm standard errors. Critical level for toxicity tests is shown by a line.

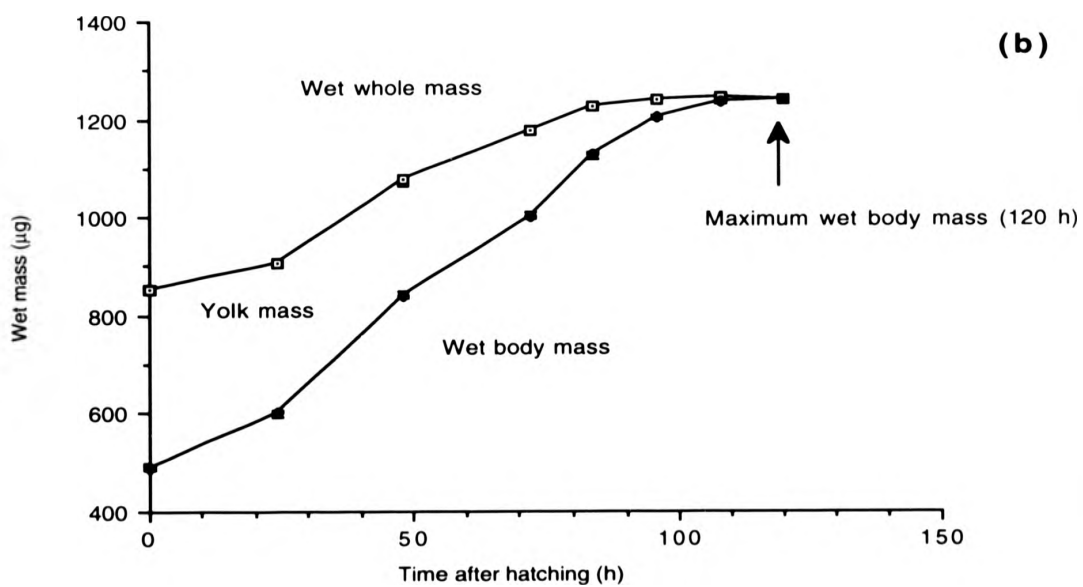
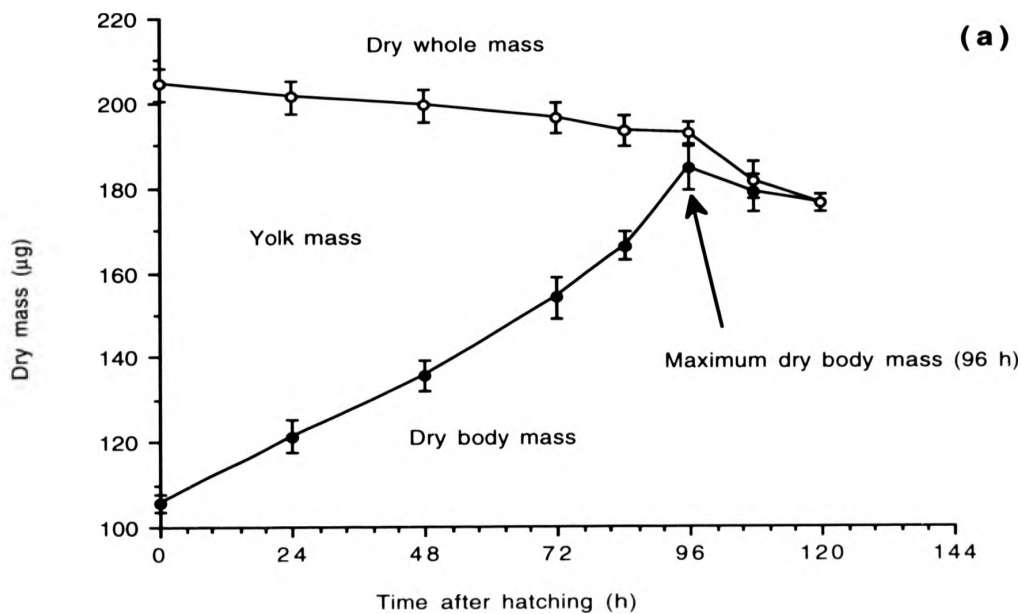


Figure 3.7 Pattern of growth and yolk utilization of goldfish sac-fry expressed as, (a) dry mass and (b) wet mass. Data are means \pm standard errors.

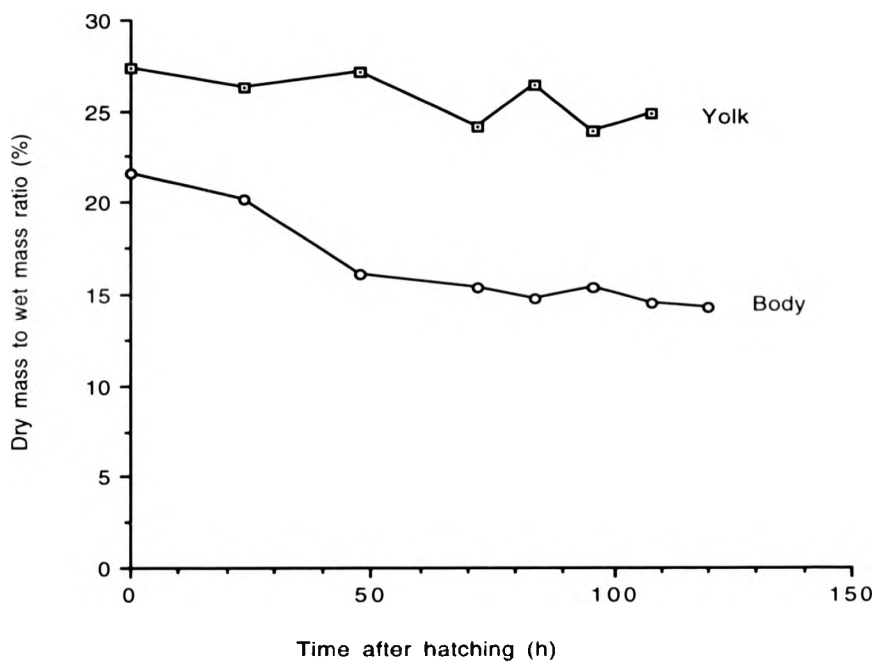


Figure 3.8 The ratio of dry mass to wet mass of goldfish sac-fry body and yolk over the yolk absorption period.

3.4 Discussion

3.4.1 Induced spawning of goldfish

Different techniques of ovulation of goldfish were investigated together with optimization of brood stock density to produce eggs for use in year-round routine toxicity tests with goldfish early life-stages.

Preliminary experiments in the present study on induced ovulation by changing temperature and photoperiod from 15°C and 12L : 12D to 20°C and 14L : 10D resulted in successful ovulation in five out of seven females (*i.e.* 71% success), a high rate of ovulation success (94%) has been previously reported by raising water temperature from 13 - 14°C to 20°C (Yamamoto *et al.* 1966). Although this technique of spawning was found to be adequate, the technique is complex and incompatible due to the requirements of two sets of laboratory conditions and the long time required for the conditioning of brood fish.

In another experiment in this study a 60% success rate of ovulation was obtained in fish treated with temperature change (from 15°C and 12L : 12D to 20°C and 14L : 10D) as well as hormonal injection at the dose of 10 µg.g⁻¹ body weight (10 µl.g⁻¹ body weight) pimozone and 0.1 µg.g⁻¹ body weight (5 µl.g⁻¹ body weight) LHRH-a. By contrast Sokolowska *et al.* (1984) reported 88% success rate in fish treated with temperature change (from 13°C to 20°C) and using the same dose of

pimozide and LHRH-a. In this study ovulation occurred within 14 - 17 h after LHRH-a injection, and this timing of ovulation was similar to the results of Peter *et al.* (1978) and Sokolowska *et al.* (1984). The technique of ovulation by injecting pimozide and LHRH-a was found nonreproducible, and required two sets of laboratory conditions and more floor space. Frequent stress due to transfer of fish and repeated use of hormone may limit the repeated spawning of the same fish.

In another experiment fish were matured and maintained at 20°C and 14L : 10D photoperiod and were induced to ovulate by injecting pimozide at the dose of 10 µg.g⁻¹ body weight (10 µl.g⁻¹ body weight) and LHRH-a at the dose of 0.1 µg.g⁻¹ body weight (5 µl.g⁻¹ body weight). Although stress, which may effect reproductive activity (Safford and Thomas, 1987) due to transfer from one set of conditions to another was reduced in this experiment, it gave a success rate of only 40%. No such technique has been found reported without temperature change for comparison of the reproducibility of the technique. Nevertheless, this technique was judged unreliable due to the poor success of ovulation.

In order to fulfil one of the aims of this study, 'the procurement of eggs and sac-fry of consistent quality from a small stock of brood fish', by repeatedly using the same brood stock, and in quest of a simple technique of maturation and ovulation, induced ovulation was investigated by inoculating ovarian fluid into the tank water. Fish held at 20°C and 14L : 10D photoperiod were stimulated to ovulation by inotroducing ovarian fluid into the water of the spawning tank at the concentration of 2 µl, 3 µl and 4 µl ovarian fluid per litre of rearing water, which gave 100%

success rate of ovulation. The repeatability of the technique of induced ovulation by inoculating ovarian fluid was confirmed by a 87% successful ovulation using 3 µl ovarian fluid per litre of tank water *i.e.* the intermediate of the used concentrations used in the earlier experiment as a stimulant. This success rate is comparable with any of the existing techniques that have been used for ovulation of goldfish and is much simpler. This technique required comparatively less laboratory space and eliminated the facilities required for temperature shock. Further advantages also exist: (1) fish can be matured and spawned under the same conditions suitable for larvae, and (2) it reduces the stress associated with repeated capture and anaesthesia.

3.4.2 Conditioning of broodfish at different stocking densities under 20°C and 14L : 10D photoperiod

Among the three stocking densities (1, 2 and 3 female of 1+ age group in each tank of 120 l capacity in a water recirculatory system at 20°C and 14L : 10D photoperiod) investigated for rematuration of brood stock in order to have a routine supply of eggs and larvae, one female stocked per tank (120 l), rematured fortnightly. There is no such published report of goldfish rematuration under the laboratory conditions specified here. However, Razani *et al.* (1989) reported a rematuration rate of only a maximum of 2.5 per female per year for goldfish stocked at a stocking density of 50 fish (25 male and 25 female) in a tank of 1750 l in a flowthrough (1 - 1.5 l.sec⁻¹) system at 16°C and 16L : 8D photoperiod.

This high rematuration rate (25 per female per year; see table 3.5) under laboratory conditions results in goldfish eggs and sac-fry being available all-year-round from a small brood stock. This makes goldfish a suitable species for routine toxicity tests by removing the constraints of material supply for fish early life-stage toxicity tests.

3.4.3 Performance of goldfish early life-stages in the incubation system and in the exposure chambers of the toxicity testing system

Here, in this study suitable control conditions for flowthrough toxicity studies using goldfish early life-stages were investigated. Dissolved oxygen is an important controlling factor in toxicity tests since exposure of fish to a toxicant may increase the rate at which they consume oxygen. A minimum level of 60% air saturation value (ASV) is recommended for test solution used in fish early life-stage toxicity studies (OECD, 1992), whereas air saturation levels of 90 - 100% and not less than 75% at any time has been recommended by USEPA (USEPA, 1982). In the present study oxygen levels (>80% ASV) in exposure chambers of the toxicity testing system stocked with different densities of sac-fry were always above the minimum recommended level. The oxygen concentration was found to decrease with density. The decrease in oxygen with density was due to more oxygen consumption by the larvae. The decrease in oxygen concentration with time was related to an increase in oxygen consumption rate of the larvae. An increase in oxygen consumption with time was found in carp sac-fry (Kamler, 1992). Ammonia concentration was always below the maximum acceptable level of 140 $\mu\text{g.l}^{-1}$ (Widdows, 1985). Ammonia

concentration was found to increase with density and over time as expected. During embryonic development carbohydrate, lipid and protein are consumed prior to hatching while protein and lipid catabolism predominate after hatching (Heming and Buddington, 1988; Kamler, 1992). During the sac-fry stage a large proportion of yolk protein is utilized for energy, resulting in high levels of ammonia excretion. In carp larvae metabolic rate has been found to increase up to the maximum body mass attained (without external food supply) and then declines during starvation (Kaushik *et al.*, 1982). In the present experiment an increase in rate of ammonia excretion was observed in goldfish sac-fry (see Figure 3.6) up to maximum body mass reached without external food supply, as observed in carp, a close relative of goldfish. Elevated ammonia concentration can inhibit the rate of normal physiological activity. An ammoniacal nitrogen level of approximately $140 \mu\text{g.l}^{-1}$ is considered inhibitory (Widdows, 1985), and although the level varies with species we can assume this level as the bench-mark.

The survival of goldfish sac-fry up to the yolk utilization stage among the three tested densities (30, 40 and 50 sac-fry per chamber) was always above 95% of the initial number in each exposure chamber of the toxicity testing system, which is above the OECD (1992) recommended minimum (80%) survival of control treatments. However, hatching success of eggs decreased significantly with density in the flowthrough system. Considering these factors a density of 30 sac-fry per chamber has been selected for future studies.

Yolk utilization and growth pattern indicated that the maximum dry mass of sac-fry was attained before the wet mass (Figure 3.7). Maximum body mass was attained before full yolk utilization, as has been reported in several studies (in herring: Heming and Buddington, 1988; Blaxter, 1969; in tilapia: Rana, 1986). The ratio of dry mass to wet mass decreased as the larvae grew, which supports the view that high density, low moisture yolk mass is transformed into low density, high moisture body mass (Blaxter, 1969; Heming and Buddington, 1988). The wet mass will be less representative for any comparison of the yolk utilization for the calculation of larval growth on account of differences in moisture content of the body and yolk and also on account of the variability of moisture content of tissues under different physiological and stressed conditions, *e.g.* starved larvae contain more moisture than non-starved larvae (Hemming and Buddington, 1988). Both wet body mass and dry body mass attained maximum values before total yolk absorption. The yolk remaining in the yolk-sac may have some adaptive value and may provide energy for emergency requirements. Starved larvae of striped bass (*Morone saxatilis*) retained oil globules for a longer time than fed ones, the rate of oil absorption increasing progressively with an increase in food concentration (Eldridge *et al.*, 1982). In the coregonid *Coregonus fera*, the energy rich oil globule remained unabsorbed at the final absorption of the remaining yolk (Loewe and Eckmann, 1988). Left over yolk may sometimes remain at the point of death from starvation in striped bass (Rogers and Westin, 1981).

CHAPTER 4

PATTERNS OF LETHAL TOXICITY IN GOLDFISH EGGS AND SAC-FRY

4.1 Introduction

A toxicant is an agent that can produce an adverse response (effect) in a biological system, seriously damaging its structure or impairing its function or resulting in death (Rand and Petrocelli, 1985). In measuring the toxicity of a chemical, the objective is to estimate as precisely as possible the range of chemical concentrations that produce selected, readily observable and quantifiable responses in a group of test organisms under controlled conditions. An acute response is defined as a response which is expressed as rapidly-occurring, irreversible harm to the organism resulting in death within a short period of time by the fastest acting toxic mechanisms of the chemical substance used. Acute toxicity tests have been designed to determine the concentration of a substance that produces a toxic effect on a specified percentage of test organisms within a relatively short period of time, normally either 48 h or 96 h so that either two or one test(s) can be completed within working days of a week (Lloyd, 1992).

In determining the relative toxicity of chemicals to aquatic organisms, lethality has been extensively used as an endpoint in acute tests. Since death is easily detectable it is widely used as an acute response. The criteria used to determine death in free-living organisms include the lack of movement and lack of reaction to gentle

prodding (OECD, 1992).

In fish, lethal toxicity is normally measured in terms of the 96 h LC50, since the 50% response is the most reproducible measure of the toxicity of test materials and 96 h usually covers the period of acute lethal action of most toxicants (Rand and Petrocelli, 1985). However, the period of acute lethal action depends on the modes of action of the toxicant and the target sites of the test organisms. Where a response other than mortality (say immobilisation, hatching success, abnormality) is used, the measured toxicity is expressed as an EC50 (effective concentration 50: concentration at which 50% of exposed group respond). Toxicity in terms of LC50 or EC50 is measured on the basis of the quantal response (all or none) by calculation, and the 95% confidence limits are reported with the value of mean lethal concentration. Macek *et al.* (1978) have stated that acute lethality tests provide means for:

1. deriving estimates of the upper limit of concentrations that produce toxic effects
2. evaluating the relative toxicity of large numbers of test materials
3. evaluating the relative sensitivity of different aquatic organisms to test materials
4. evaluating the effects of water quality (*e.g.* temperature, dissolved oxygen concentration, hardness, pH, salinity and suspended materials) on the toxicity of test materials, and
5. developing an understanding of the concentration response relationship and of the significance of duration of exposure to the test materials.

There are limitations of acute tests: they usually do not provide substantive information about the sublethal or cumulative effects of a test material (Parrish, 1985). They are not predictive of potential chronic toxicity (Macek *et al.*, 1978). In addition, the death of a proportion of a population might not affect the survival of the population rather it may stimulate the population if the stress is short lasting, *e.g.* the mortality of a fraction of a crowded population increases the survival chances of individuals in the remainder of the population (Pickering, 1981).

4.1.1 Range-finding test

The first step towards determining the acute toxicity of a substance involves conducting a range-finding test. In a range-finding test, generally a smaller group of organisms (five or more) is exposed to a series of concentrations in a geometric series (*e.g.* 1, 10, 100, 1000 $\mu\text{g.l}^{-1}$) together with a control in dilution water only, or dilution water plus carrier solvent at the maximum concentration used in the toxicant series. The duration of the range-finding test may be shorter than the planned definitive test and if this is the case then the concentration range of test substances to be selected for the definitive test has to be adjusted to allow for a greater potential toxicity on the latter part of the definitive test. Ideally the duration of the range-finding test and the number of organisms used should be the same as in the definitive test. Parrish (1985) emphasized that the range-finding test serves its function best when it most closely follows the conditions planned for the definitive test and sometimes produces results as good as a definitive test.

4.1.2 Definitive test

The results of a definitive test are used to calculate the 96 h LC50 for mortality or EC50 for other acute responses. An appropriate number of organisms (suitable for statistical analysis) should be exposed to a series of concentrations of test substance spaced at logarithmic intervals (EIFAC, 1975). Concentrations should be spaced such that in 96 h, fewer than 35% of the test animals in one treatment (other than control) are killed or affected and more than 65% of the test animals in another treatment are killed or affected (Parrish, 1985), or at least two partial mortality exposures bracketing the 50% level (USEPA, 1985). It is best to have several test concentrations with partial response (ASTM, 1980).

4.1.3 Sensitivity of fish egg and sac-fry to stress

In general, embryonic and larval stages of fish are more sensitive to environmental and chemical stress than juvenile and adult fish (Alderdice, 1985; Eaton *et al.*, 1978). Considering their sensitivities, early life stage toxicity tests have been developed not only because they are quicker and cheaper but that they are capable of estimating the chronic safe concentration of toxicant (Dave, 1993; McKim, 1985). In the development of short-term ecologically relevant toxicity tests, the originally-proposed period of early life-stage toxicity tests of one to two months (Macek and Sleight, 1977) has been reduced to one week (*e.g.* Birge *et al.*, 1985; DeGraeve *et al.*, 1991). Two different types of short-term ELS toxicity test have

emerged, (1) short-term larval survival and growth (integrated effect) test (e.g. the 7-day larval survival and growth test with fathead minnow (*Pimephales promelas*): DeGraeve *et al.*, 1991; Norberg-King, 1989) and (2) the short-term embryo-larval growth test (e.g. 8 - 10 d embryo-larval growth test with zebrafish (*Brachiodanio rerio*): Dave, 1993; Dave *et al.*, 1987; OECD, 1992). The former tests omitted the egg stage, since this stage is considered to be less sensitive, and this simplifies the test design. However, a complication remains regarding how to feed the larvae and the uneven space allowance for the groups of fish with differential survival in different treatments. The latter test places more emphasis on the use of internal food (*i.e.* yolk) to avoid problems arising from the maintenance of an external food supply but the test design remains complex.

During the process of development of fish early life-stage toxicity tests including embryo and larvae, eggs are exposed arbitrarily from immediately after fertilization (e.g. Blaylock and Frank, 1979 on *Cyprinus carpio*) to several hours after fertilization (e.g. Dave and Xiu (1991) start with 4 - 8 h old eggs of *B. rerio*). With warm water fish, *P. promelas* and *B. rerio*, generally the percentage of early embryo mortality is determined after 24 h (Dave, 1993) and a definite number of eggs from the remaining live eggs (transparent) are exposed for further period. At hatching, hatching success is determined, the proportion of deformed larvae estimated and a pre-defined number of normal larvae are exposed for the remaining period of the test (OECD, 1992). Thus test of early life-stage including embryo requires a complicated test design.

In addition to the complex design, there remains some ambiguity about the starting point of the test. The quality of the starting materials, in terms of early embryonic mortality (Dave, 1993) or fertilization rate (Nagel *et al.*, 1991) is usually determined after 24 h, when dead eggs (fertilized eggs with dead embryos and unfertilized eggs) have turned white while living embryos are transparent, is variable. There are two ways to minimise the variability of starting materials, (1) separation of fertilized and unfertilized eggs by comprehensive microscopic examination prior to the start of the experiment but this is time consuming, and (2) starting the test with embryos aged for 24 h (*e.g.* Stewart *et al.*, 1990). Other problems also remain in this sort of test, the variability of abnormality of the sac-fry emerged on hatching of eggs under controlled conditions which makes interpretation of results difficult. Above all, it is well known that once the egg is fertilized it absorbs water, the perivitelline space forms and the chorion hardens and the egg become relatively impermeable (Billard *et al.*, 1986; Heming and Buddington, 1988) resulting in reduced sensitivity to stress particularly following the blastula stage. Although the egg remains sensitive prior to the blastula stage (Sharp and Neff, 1982), it is well documented that the sac-fry stage is the most sensitive in the life-cycle (Schimmel *et al.*, 1974; van Leeuwen *et al.*, 1985).

Since eggs (embryos) are more resistant than other early life-stages, the inclusion of eggs in the early life-stage toxicity test complicates test designs and makes the results of fish ELS toxicity tests difficult to interpret, a simple short term toxicity testing method was planned to explore lethal and sublethal (physiological) stress

sensitivity of goldfish sac-fry. To support this approach, it was considered necessary to determine the relative sensitivity of goldfish sac-fry and eggs, and to investigate the differences in sensitivity of these stages in terms of acute sensitivity.

4.2 Materials and Methods

4.2.1 Lethal effects of cadmium DCA and chlorpyrifos on goldfish sac-fry

4.2.1.1 Range finding test with cadmium, DCA and chlorpyrifos

For range finding tests three replicate treatments of a control (designated as 0 $\mu\text{g.l}^{-1}$) and four exposure concentrations: 10, 100, 1000, and 10000 $\mu\text{g.l}^{-1}$ for cadmium; 10, 100, 1000, and 10000 $\mu\text{g.l}^{-1}$ for DCA and 0.1, 1.0, 10.0, and 100.0 $\mu\text{g.l}^{-1}$ for chlorpyrifos were randomly allocated among fifteen flowthrough channels (see section 2.4). The concentration ranges selected encompassed the reported lethal concentration of cadmium, DCA and chlorpyrifos to other similar test organisms (see section 1.5.1, 1.5.2, and 1.5.3 for cadmium DCA and chlorpyrifos respectively). The reservoirs were filled with freshly prepared solutions of toxicants and dilution water at the start of the experiment. Thirty sac-fry were randomly placed in each exposure chamber as indicated in section 2.1.3. From the stock solutions of 100 mg.l^{-1} of cadmium (prepared as described in 2.3.1), 50 mg.l^{-1} DCA (prepared as described in 2.3.2) and 1 mg.l^{-1} chlorpyrifos (prepared as described in 2.3.3) appropriate amounts were taken and mixed with pre-aerated dilution water to make appropriate test concentrations. Reservoirs were refilled with

freshly made test concentrations of toxicant and dilution water each day. The experiment was conducted for 96 h except if all the sac-fry in a treatment were dead when the particular channel was disconnected until the end of the experiment. Mortality was judged as noted in section 4.1 and recorded daily. Dead sac-fry were removed every day, and if possible at the same time of the day (between 10 and 12 am).

4.2.1.2 Definitive test with cadmium, DCA and chlorpyrifos on goldfish sac-fry to estimate LC50

For the definitive test, 30 sac-fry were exposed in each of 15 randomly selected exposure chambers of the four triplicate concentrations of each toxicant and a triplicate control (a carrier control for chlorpyrifos). The concentrations selected were: 1000, 2100, 4600, and 10000 $\mu\text{g.l}^{-1}$ cadmium; 1000, 2100, 4600, and 10000 $\mu\text{g.l}^{-1}$ DCA; and 12.5, 25.0, 50.0, and 100.0 $\mu\text{g.l}^{-1}$ chlorpyrifos, respectively. Test concentrations were made by mixing appropriate amount of stock solutions of 100 mg.l^{-1} cadmium, 50 mg.l^{-1} DCA and 1 mg.l^{-1} chlorpyrifos (added with 1 ml.l^{-1} of propan-2-ol) with pre-aerated dilution water. Experiments were run for 96 h by daily refilling of reservoirs with test media. The mortality was judged as noted in section 4.1 and recorded daily. Dead sac-fry were removed every day if possible at the same time of the day (between 10 to 12 am).

Similarly, a second test was carried out for each of the toxicants. Test concentrations used were: 3000, 3780, 4760, and 6000 $\mu\text{g.l}^{-1}$ cadmium; 2000, 2700,

3700, and 5000 $\mu\text{g.l}^{-1}$ DCA; and 12.5, 25.0, 50.0, and 100.0 $\mu\text{g.l}^{-1}$ chlorpyrifos with control (in case of chlorpyrifos a carrier control of propane-2-ol at the concentration of 100 $\mu\text{l.l}^{-1}$ *i.e.* the maximum of the carrier solvent used in any of the treatment).

4.2.2 Definitive test with DCA on goldfish eggs to estimate EC50

To find out the relative sensitivity of goldfish eggs compare to sac-fry, acute tests were also performed with goldfish eggs using DCA as a reference compound. Artificially fertilized eggs (section 2.1.2) were spread on each of 15 glass slides so that approximately 40 to 50 eggs were attached to one side only of each glass slide. The slides were rinsed several times with dilution water and placed on slide racks. Slide racks were placed into dilution water in hatching chamber of the incubation system by hanging (see Figure 3.1). The following day reservoirs were filled with freshly prepared triplicate concentrations of 2000, 2700, 3700, and 5000 $\mu\text{g.l}^{-1}$ of DCA and dilution water. After 30 h, dead (white) eggs were removed by gentle removing with the help of a Pasteur pipette under a magnifying glass. Thirty live eggs (transparent) were selected from the remaining surviving fertilized eggs (attached to each glass slide) for each treatment by microscopic examination. The individual glass slides were then placed randomly into each exposure chamber. A continuous flowthrough experiment was run for 96 h by refilling reservoir bottles with the toxicant solutions and dilution water on a daily basis. A total of 126 h from point of fertilization was allowed for the eggs to hatch in the system. Dead eggs, if any, were removed daily. At the end of the test, the numbers of hatched

larvae and thereafter normal and abnormal larvae (morphologically-deformed (see Figure 2.5) and/or with pooled blood in the yolk and/or heart) were counted. Abnormal larvae, together with dead and unhatched eggs were considered non-viable larvae as they are dead or will die soon after hatching (Birge *et al.*, 1985) and EC50 was calculated by probit analysis from this response nonviability. Lengths of the normal larvae were also measured to one tenth of a mm of accuracy in each treatment under a dissecting microscope by using an ocular micrometer.

4.2.3 Statistical analysis

LC50s were calculated by probit analysis (Finney, 1971) from the mortality data from sac-fry tests. EC50 was also calculated by probit analysis from the data of non-viable larvae from the egg test.

4.3 Results

The acute toxicities of cadmium, DCA and chlorpyrifos on goldfish sac-fry are presented in Table 4.1. From the definitive tests with cadmium, DCA and chlorpyrifos, the observed mortality occurred between 48 and 96 h in case of cadmium (Figure 4.1), between 72 and 96 h in case of DCA (Figure 4.2) and between 24 and 96 hours in case of chlorpyrifos (Figure 4.3).

4.3.1 Cadmium

The range finding test revealed that the 96 h LC50 of cadmium for the goldfish sac-fry lies between 1000 and 10000 $\mu\text{g.l}^{-1}$. LC50s were calculated for 24, 48, 72 and 96 h. From the first definitive test the 96 h LC50 value was calculated as 4216 $\mu\text{g.l}^{-1}$ and with 95% confidence limits was found in between 3983 and 4463 $\mu\text{g.l}^{-1}$ where as the 96 h LC50 value was found to be 3404 $\mu\text{g.l}^{-1}$ and the 95% confidence limits lay between 3352 and 3457 $\mu\text{g.l}^{-1}$ in the case of the second definitive test. The repeatability *i.e.* co-efficient of variation measured from the log transformed value of 96 h LC50 (Dave, 1993; Dave *et al.*, 1987) was 1.3%. The LC50 values at different time intervals are shown in Figure 4.4.

4.3.2 3,4-dichloroaniline

The results of the range finding test indicate that the 96 h LC50 of DCA for the goldfish sac-fry lies between 1000 and 10000 $\mu\text{g.l}^{-1}$. LC50s were calculated for 24, 48, 72 and 96 h. From the first definitive test a 96 h LC50 value was calculated as 2718 $\mu\text{g.l}^{-1}$ and with the 95% confidence limits was between 2660 and 2776 $\mu\text{g.l}^{-1}$, whereas the 96 h LC50 value was found 2359 $\mu\text{g.l}^{-1}$ and 95% confidence limit lay between 2297 and 2424 $\mu\text{g.l}^{-1}$ in case of second definitive test. The co-efficient of variation measured from the log transformed value of 96 h LC50 was found to be 1%. The LC50 values at different time intervals are shown in Figure 4.5. A comparative acute test with 30 h old egg gave a calculated 96 h EC50 value of 3332 $\mu\text{g.l}^{-1}$ and the 95% confidence limits lay between 1280 and 8676 $\mu\text{g.l}^{-1}$. In the

treatments where eggs were exposed to concentrations of 5000 $\mu\text{g.l}^{-1}$ of DCA, a small percentage (12%) of eggs hatched. Although 80% of eggs hatched in treatments of 3700 $\mu\text{g.l}^{-1}$ all the emerging sac-fry in both 3700 and 5000 $\mu\text{g.l}^{-1}$ were found to be abnormal, principally as a result of pooled blood in the yolk and heart. The length of the normal larvae which emerged from eggs exposed to 2000 and 2700 $\mu\text{g.l}^{-1}$ were significantly different from the control ($F = 999.99$; $df = 2, 6$; $P < 0.05$). Average lengths of the control treatments were found to be 4.8 mm in contrast to 4.3 and 4.1 mm in the case of sac-fry which emerged in 2000 and 2700 $\mu\text{g.l}^{-1}$ of DCA respectively.

4.3.3 Chlorpyrifos

The range finding test revealed that the 96 h LC50 of chlorpyrifos for the goldfish sac-fry lies between 10 and 100 $\mu\text{g.l}^{-1}$. LC50s were calculated for 24, 48, 72, and 96 h. From the first definitive test the 96 h LC50 was calculated as 25.0 $\mu\text{g.l}^{-1}$ and with 95% confidence limits it was in between 23.9 and 26.2 $\mu\text{g.l}^{-1}$ where as the 96 h LC50 value was found 22.6 $\mu\text{g.l}^{-1}$ and 95% confidence limit lied in between 21.4 and 23.9 $\mu\text{g.l}^{-1}$ in the case of the second definitive test. The co-efficient of variation measured from the log transformed value of the 96 h LC50 (Dave, 1993), was 1.6%. The LC50 values at different time interval are shown in Figure 4.6.

Table 4.1 Acute toxicity of cadmium, DCA and chlorpyrifos on goldfish sac-fry.

Toxicant	Acute toxicity: LC50 ($\mu\text{g.l}^{-1}$)			
	24 h	48 h	72 h	96 h
Cadmium	*	7024.4 (6935.1-7114.8)	4945.6 (4933.9-4957.4)	3403.9 (3352.2-3456.5)
DCA	*	6782.3 (6639.1-6928.7)	5752.0 (5645.7-5858.4)	2717.7 (2660.3-2776.4)
Chlorpyrifos	59.5 (57.5-61.6)	54.4 (52.3-56.5)	26.3 (25.0-27.7)	22.6 (21.4-23.9)

95% confidence limit is shown in the parenthesis.

* No LC50 was calculated because no mortality was observed.

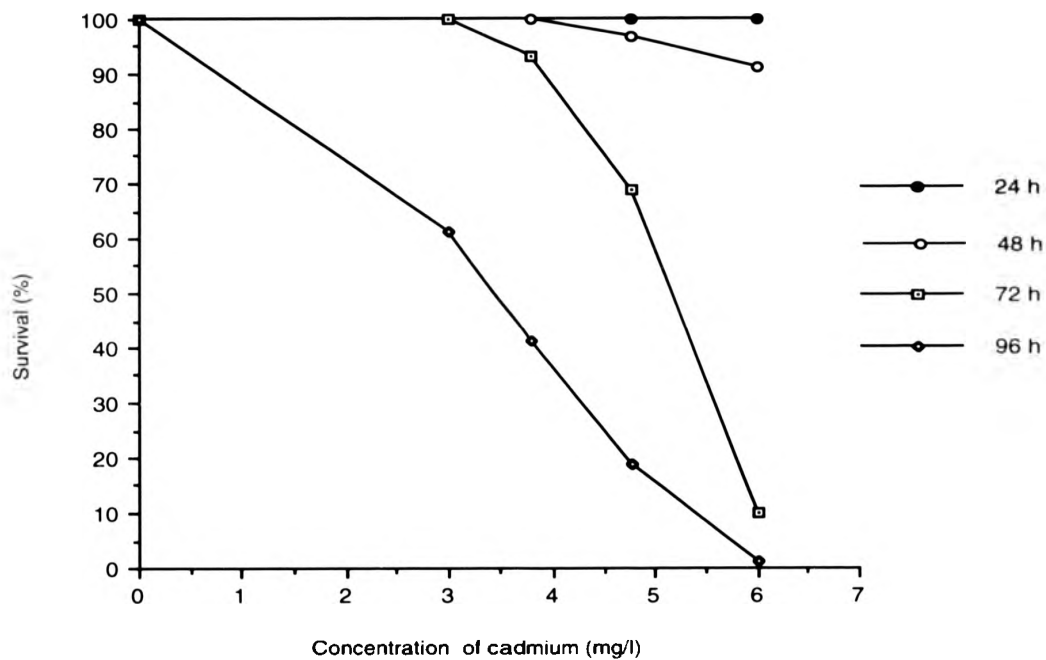


Figure 4.1 Mortality pattern of goldfish sac-fry exposed to different concentrations of cadmium with time. Group size = 90.

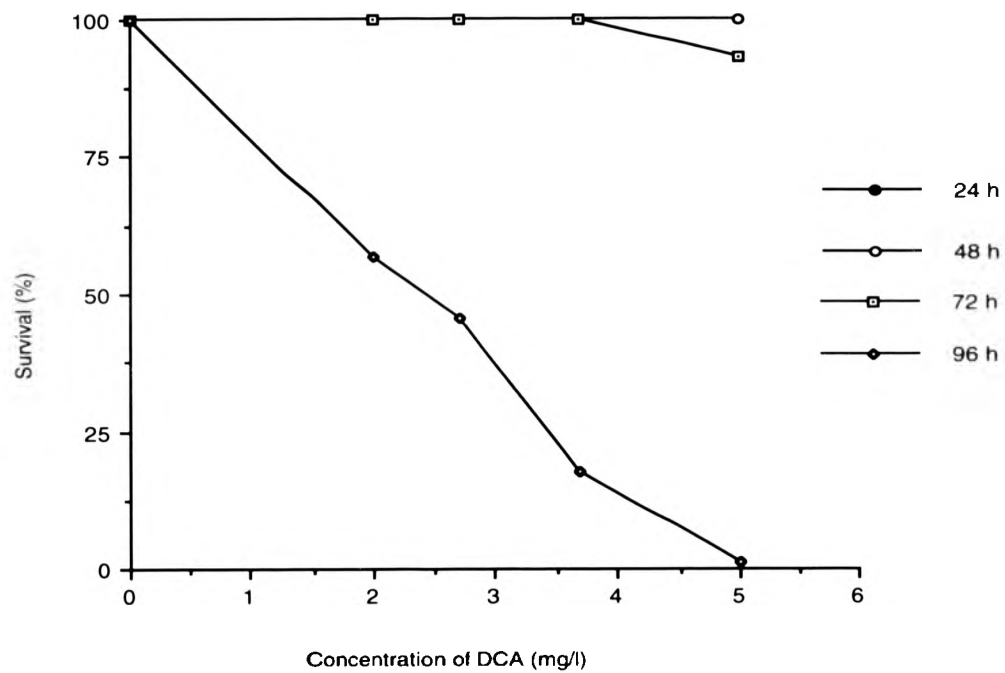


Figure 4.2 Mortality pattern of goldfish sac-fry exposed to different concentrations of DCA with time. Group size = 90.

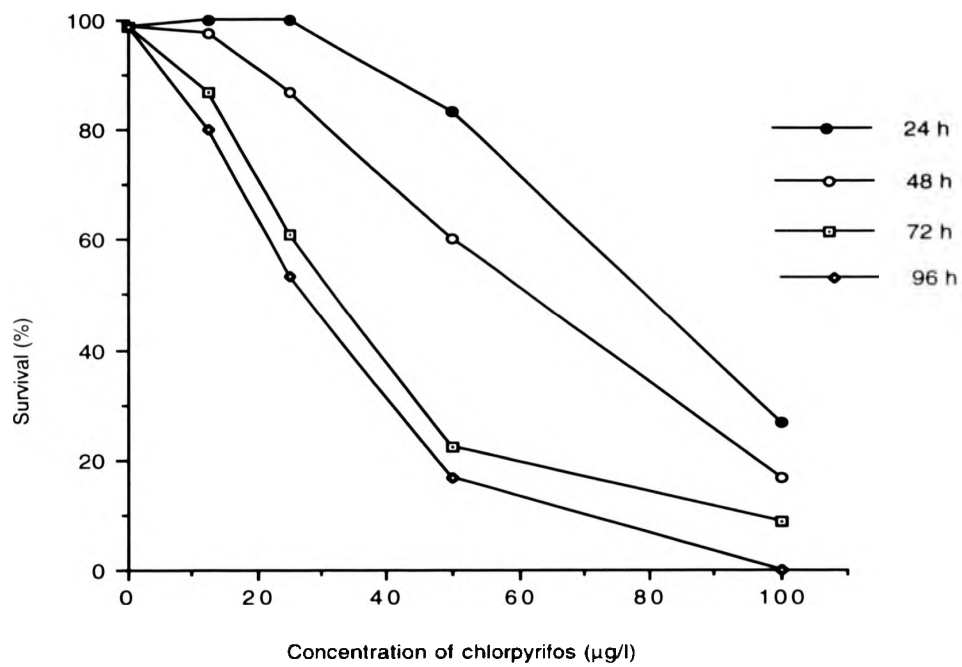


Figure 4.3 Mortality pattern of goldfish sac-fry exposed to different concentrations of chlorpyrifos with time. Group size = 90.

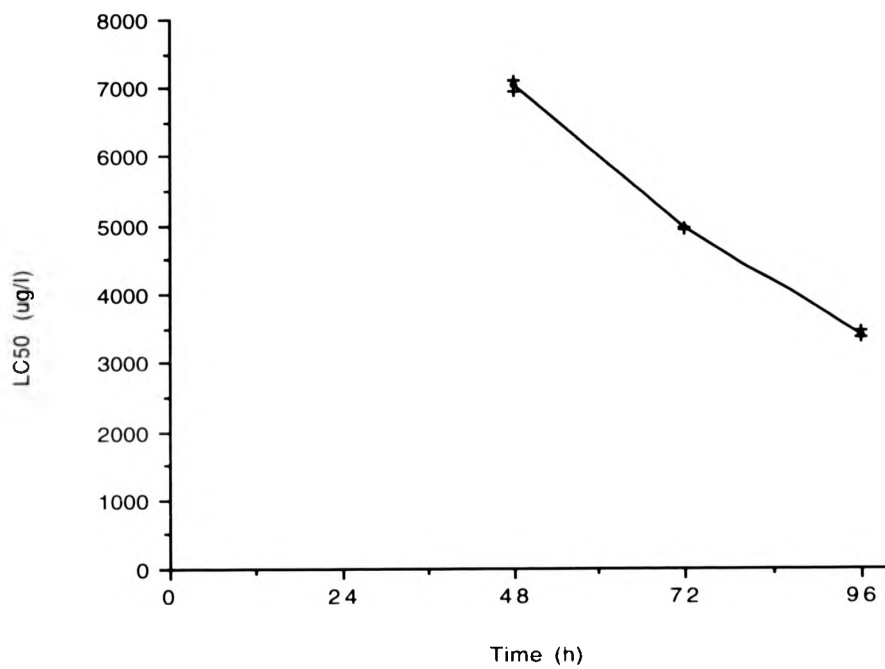


Figure 4.4 LC50 values of cadmium at 48, 72 and 96 h for goldfish sac-fry. Cross indicates 95% confidence limits.

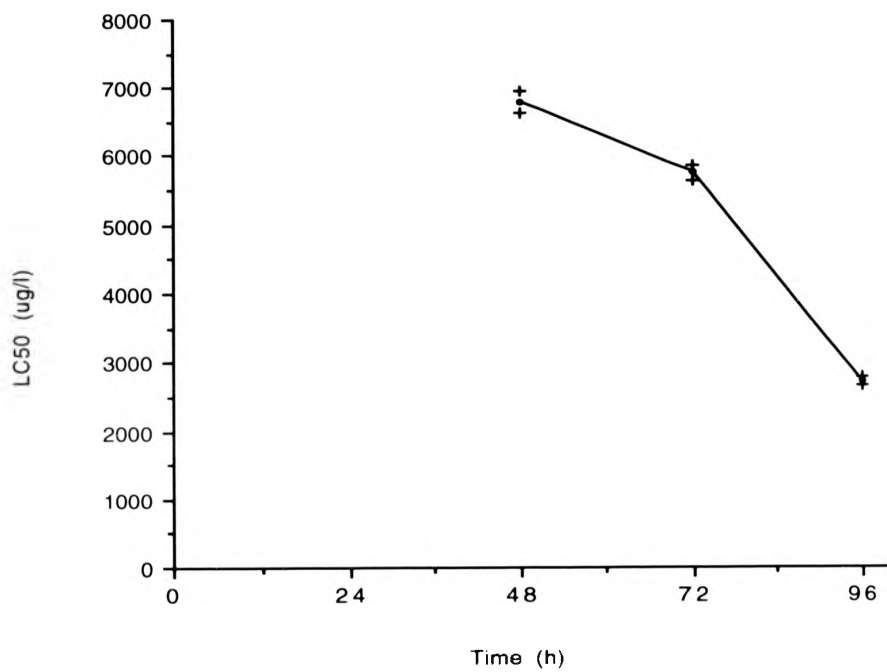


Figure 4.5 LC50 values of DCA at 48, 72 and 96 h for goldfish sac-fry. Cross indicates 95% confidence limits.

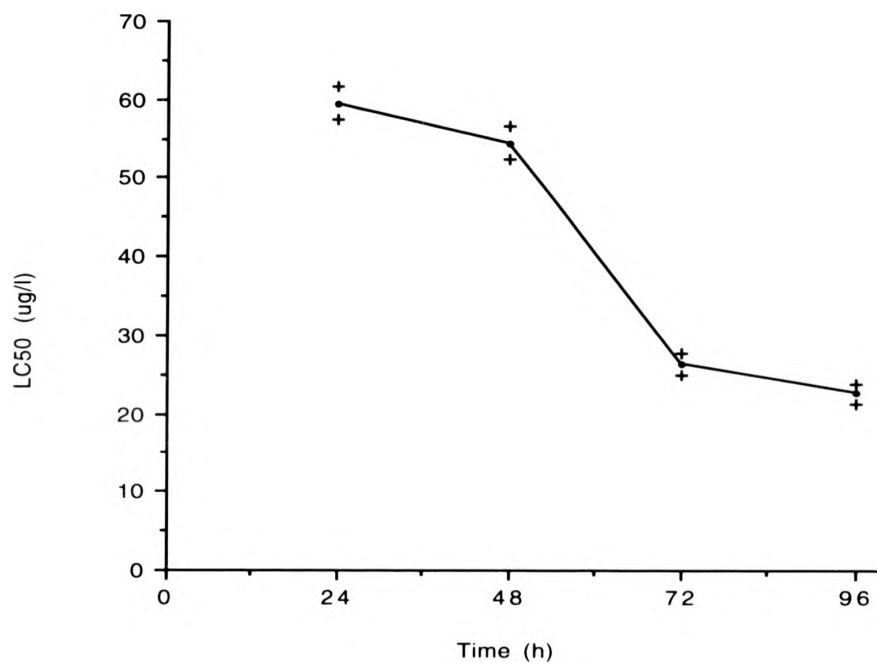


Figure 4.6 LC50 values of chlorpyrifos at 24, 48, 72 and 96 h for goldfish. Cross indicates 95% confidence limits.

4.4 Discussion

In this chapter the relative toxicity of cadmium, DCA and chlorpyrifos on goldfish sac-fry in terms of acute lethality was investigated. The relative sensitivity of eggs and sac-fry in terms of acute lethality was also investigated. The results indicate that cadmium and DCA are lethally toxic at the ppm level whereas chlorpyrifos is lethally toxic at the ppb level. From the Figure 4.4, 4.5 and 4.6, it is evident that cadmium and DCA are slow acting chemicals whereas chlorpyrifos is rapidly active. The mode of action of toxicants and also the target organ or system may be responsible for this type of differences in toxicity. It is believed that in fish, cadmium act mainly by damaging gills and skin or by influencing enzyme system or by disturbing essential metal balance (Eaton, 1974; Rombough and Garside, 1984). DCA is a nonspecific anaesthetizing agent. Chlorpyrifos inhibits acetylcholinesterase reducing acetylcholine (Rodrigues *et al.*, 1983; Jarvenen *et al.*, 1983). Cadmium toxicity showed delayed mortality observed in lethal toxicity (Alabaster and Lloyd, 1982; Haux, 1985). This type of relatively delayed activity of DCA can be justified by the assumption that anilines exert their toxic effects after metabolic activation (van Leeuwen *et al.*, 1990) and this metabolic activation may be responsible for high ACR value which has been estimated as high as 1200 (Call *et al.*, 1987).

The measured 96 h LC50 value of cadmium ($3404 \mu\text{g.l}^{-1}$) for goldfish sac-fry was found to be much higher than the 96 h LC50 values that have been reported in

previous studies by different authors for the similar life-stages *e.g.* 10 $\mu\text{g.l}^{-1}$ for rainbow trout sac-fry stage (van Leeuwen *et al.*, 1985), 21 - 148 $\mu\text{g.l}^{-1}$ for sac-fry stage of tilapias (Siriwardena, 1993), 125 $\mu\text{g.l}^{-1}$ (192 h LC50) for fathead minnow early life-stages including egg and sac-fry (Birge *et al.*, 1985), and it was even found to be much higher than the 180 h LC50 for goldfish early life-stage (egg plus sac-fry) in a previous study, reported by Birge *et al.* (1985). But the experimental conditions of those experiment were not the same as those of the present one. It has been widely documented that toxicity of chemicals depends on the conditions used in the test (Dave, 1986; Sprague, 1985). Hardness, for example, has been found to have a great deal of influence on cadmium toxicity *e.g.* estimated cadmium LC50 for brook trout fry has been found 2.4 $\mu\text{g.l}^{-1}$ and 26 $\mu\text{g.l}^{-1}$ with water hardness 44 and 340, respectively (Carrol *et al.*, 1979) and the water hardness of dilution water used in this study was very high (238-253 mg.l^{-1} CaCO_3), which may be one of the reasons behind high LC50 value of cadmium in this study. Furthermore, EDTA used in dilution water (see Table 2.1) may have contributed to the higher LC50 value of cadmium by adsorbing ionic cadmium from the exposure medium resulting in the less amount of free cadmium available to exert toxic effects. EDTA is a well known cation chelating agent (Hart and Scaife, 1977). In the present study it was not possible to find out the actual bioavailable cadmium concentrations. However, a subsidiary test was carried out to determine 96 h LC50 of cadmium for goldfish sac-fry in ASTM soft water (ASTM, 1980) with hardness 48 mg.l^{-1} CaCO_3 in absence of EDTA. The cadmium LC50 value for goldfish sac-fry was found to be 107.5 $\mu\text{g.l}^{-1}$ in ASTM soft water.

This LC50 value for goldfish sac-fry is comparable to the LC50 value obtained in ASTM soft water for a tropical fish (*Tilapia zilli*) sac-fry (see Table 1.1). Thus we must assume that M7 is not a good medium for the laboratory studies of metal toxicity. Despite difficulties M7 medium was used to study cadmium toxicity to maintain the commonality of conditions for all the chemicals used here in this study. In M7 all the necessary macro and micro nutrients have been incorporated with the understanding that test animals will be unstressed in the control treatments. However, these trials here proved that M7 is not a universal media for evaluation of chemicals.

The calculated 96 h LC50 of DCA ($2359 \mu\text{g.l}^{-1}$) for goldfish sac-fry was found to be similar to rainbow trout ($2700 \mu\text{g.l}^{-1}$; Crossland, 1990) and far less than the value reported for other commonly used species of fish which have been widely using in early life-stage toxicity studies *e.g.* zebrafish ($8500 \mu\text{g.l}^{-1}$; Nagel, 1988, reported by Nagel *et al.*, 1991), fathead minnow ($7600 \mu\text{g.l}^{-1}$; Call *et al.*, 1987). But a valid comparison of LC50 values is not possible because of the changing toxicity with alkalinity, dissolved oxygen, hardness, pH, temperature, species, developmental stages, body size, and health including nutritional status of the test animals used in the tests (Dave, 1986; Sprague, 1985). Apparently, in support of the relative sensitivity of fish early life-stage compared with other life-stages, the observed LC50 value is justified. Reported LC50s for standard species (zebrafish, fathead minnow) have not been estimated on early life-stages. It was found that the LC50 for goldfish estimated in this study lies within the range of LC50s for other

organisms (48 h LC50 of 510 $\mu\text{g.l}^{-1}$ for *Daphnia magna* and 96 h LC50 of 4400 $\mu\text{g.l}^{-1}$ for *Aedes*: Soares, pers.comm.) that have been estimated in studies under the same set of conditions used here in this study.

The 96 h LC50 value of chlorpyrifos (22.6 $\mu\text{g.l}^{-1}$) for goldfish sac-fry is well within the range of reported LC50 values for freshwater fish (2.4 for blue gill: *Lepomis macrochirus* to 280.0 $\mu\text{g.l}^{-1}$ for channel catfish: *Ictalurus punctatus* (Johnson and Finely, 1980).

Results of acute toxicity tests with DCA on goldfish sac-fry and egg stages indicate the relative lower sensitivity of the embryonic developmental stage than that of sac-fry. Lower sensitivity of rainbow trout embryo than that of sac-fry has been reported with cadmium (van Leeuwen *et al.*, 1985). The higher susceptibility of larvae in comparison to embryos has also been demonstrated by sheepshead minnow (Schimmel *et al.*, 1974) and cod (Dethlefsen, 1977) for chlorinated hydrocarbons, and by tilapia for organophosphate insecticides (Paflitschek, 1979). Inclusion of egg stage in fish early life-stage toxicity tests is an unnecessary design complication. Hence, it may be possible to develop a successful toxicity test with sac-fry only without losing the sensitivity of the embryo-larval toxicity tests, which could prove useful if integrated in ecotoxicological evaluation programmes, which are now demanding ecologically more relevant but short-term toxicity tests to cope with the necessity of evaluating a vast number of chemicals already in use and also the increasing number of newly developed chemicals.

CHAPTER 5

SUBLETHAL PHYSIOLOGICAL EFFECTS OF TOXICANTS AND VALIDATION OF A MODEL OF FISH EARLY LIFE-STAGE SENSITIVITY

5.1 Introduction

The study of sublethal effects of toxicants at ecosystem level is difficult due to the complexity of the ecosystem itself. Sublethal effects of a toxicant start as biochemical effects at the subcellular or cellular level which in turn induce a sequential change at the individual, population, community or ecosystem level and ultimately can lead to irreversible and detrimental disturbances of integrated functions of the ecosystem. Therefore, considering the difficulties of studying the sublethal effects of toxicants at an ecosystem level and also considering the sequential flow of effects from lower to higher levels of organization, most efforts have been directed towards the measurement of the biological effect concentration of a toxicant that does not cause rapid death but impairs the functioning of an organism at the biochemical, physiological, behavioural or life-cycle level (Boudou and Ribeyre, 1989) for the management of chemical impacts on natural ecosystems.

Recently, physiological energetics techniques have been employed increasingly to measure the effects of sublethal environmental stress (Koehn and Bayne, 1989) due to their low-level stress sensitivity and general expression of stress response (Widdows, 1985). In this study an effort was made to predict biological effects of

toxicants by describing the lowest exposure concentration that produces a significant response (LOEC) and at the same time the highest exposure concentration that does not produce any measurable response (NOEC) compared with control response on goldfish sac-fry physiological energetics.

To study the NOEC and LOEC of toxicants (cadmium, DCA, chlorpyrifos) on goldfish sac-fry physiological energetics, the following hypotheses were tested:

- (i) *Ho₁: Cadmium has no effect on yolk utilization and the yolk utilization efficiency in goldfish sac-fry.*
- (ii) *Ho₂: DCA has no effect on yolk utilization and the yolk utilization efficiency in goldfish sac-fry.*
- (iii) *Ho₃: Chlorpyrifos has no effect on yolk utilization and the yolk utilization efficiency in goldfish sac-fry.*

To disprove these hypotheses a preliminary conceptual model of the physiological effects of sublethal exposure to toxicants on the developing fish sac-fry is proposed (Figure 5.1). In the suggested model it is indicated that there are two basic toxic effects, (1) inhibition of energy (matter) acquisition (supply-side effects) and (2) elevation of energy (matter) consumption (demand-side effects).

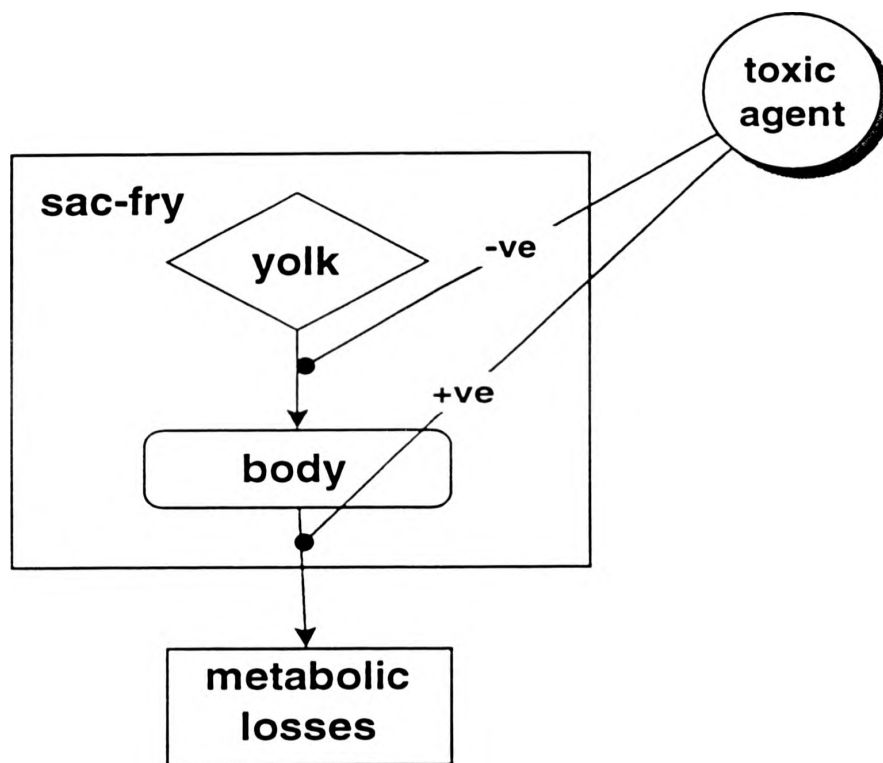


Figure 5.1 A conceptual model of the flow of energy through a goldfish sac-fry, showing that toxic effects of chemicals may result in reduced energy supply ("-ve"), and/or in increased energy demand ("+ve").

5.1.1 Yolk absorption

Fish sac-fry grow and maintain themselves by utilizing a fixed amount of energy from the yolk. Both the rate of yolk absorption and the efficiency of yolk utilization are important determinants of early development, growth and survival. Larval survival in the natural environment is dependent on the availability of food in sufficient quantity and of adequate quality after yolk reserves are exhausted. The importance of synchronizing the completion of yolk absorption, development of feeding capability and the availability of suitable food has been stressed in previous studies for better survival performance of fish larvae (Rosenthal and Alderdice, 1976; Heming and Buddington, 1988). It is also important for the sac-fry to maximise the efficiency with which yolk is converted into tissues since larger larvae have scored advantages over smaller larvae: larger larvae of a given species can be expected to be stronger swimmers (Hunter, 1972), to be less affected by competition (Hulata *et al.*, 1976), more resistant to starvation (Rana, 1985), less susceptible to predation (Ware, 1975), be able to commence feeding earlier (Wallace and Aasjord, 1984), and able to have increased survival at first feeding (Braum, 1967; Ellertsen *et al.*, 1980).

Any factor influencing the rate of yolk utilization and/or its efficiency might break the natural synchronization of the onset of exogenous feeding and availability of suitable natural food, reduce the growth of sac-fry resulting in smaller, weaker larvae at first feeding and ultimately reduce the chances of survival (see section 1.3). Yolk utilization efficiency is reduced in extreme environmental conditions

(e.g. temperature: Ehrlich and Muszynski, 1982; Kamler and Kato, 1983; Laurence, 1973) and exposure to sublethal concentrations of toxic chemicals (Crawford and Guarino, 1985; Henderson *et al.*, 1983; Rombough and Garside, 1982).

Considering the possible ecological relevance of yolk utilization patterns and the assumption of the reduced energy supply through feeding (yolk absorption is analogous to feeding, see section 5.1.7) under chemical stress (Baird *et al.*, 1990), yolk utilization and the efficiency of yolk utilization were considered as possible short-term fish early life-stage toxicity test endpoints, neither of which have been studied previously in fish early life-stage toxicity test.

5.1.2 Growth

Growth is an increase in body size, resulting from production of new tissues, and may be represented in terms of length, wet weight, dry weight or energy content with increasing validity of comparison (Blaxter, 1969). Growth, in this thesis will be assessed in terms of dry mass added to the body if not otherwise stated to avoid the possible variability of mass due to differential water content of larvae under different conditions.

Sac-fry growth occurs by the absorption of yolk, and any factor that reduces the rate of absorption of yolk will in turn reduce growth. On the other hand it is possible that yolk absorption may remain unaltered or even increase yet still result

in reduced growth, if there is an increased requirement of yolk for maintenance metabolism and/or increased costs of activity. The reason for this is simple. Increased metabolism uses yolk materials which would otherwise go to growth, therefore the rate and pattern of growth of sac-fry are functions of the following: yolk digestion by the syncytium tissue, the uptake and transport of yolk nutrient from the yolk mass to the developing tissues, activity of somatic synthetic machinery, the metabolic demands of maintenance and activity. Thus it is expected that sublethal toxicant exposure reduces somatic growth by interfering with any one or more of the processes involved. For this same reason, growth is considered a suitable endpoint for use in fish ecotoxicity tests, and has been used previously by a number of workers (Call et al., 1987; Dave, 1985; Dave and Xiu, 1991; Kristensen, 1990; Norberg-King, 1989). Growth during the endogenous nutrition (nutrition received from the yolk materials without any external food supply) period was reduced by extremes in pH (Nelson, 1982), sub- and supra-optimal temperatures (Blaxter and Hempel, 1966; Ehrlich and Muszynski, 1982; Kamler and Kato, 1983), low dissolved oxygen concentrations (Hamor and Garside, 1977), and exposure to sublethal concentrations of toxicants (Henderson *et al.*, 1983; Siriwardena, 1993; Tilseth *et al.*, 1984).

5.1.3 Oxygen consumption

Organisms receive their energy for maintenance, activity, and structural turnover by respiring different substrates: carbohydrate, protein and lipid. This respiration may take place either aerobically and/or anaerobically. It is well known that aerobic processes generate more energy than comparable anaerobic processes, and if energy is generated aerobically then oxygen consumption rate can provide a rapid method of estimating metabolic rate (Lampert, 1983). In juvenile and adult fish, respiration is aerobic and metabolic rate is usually calculated in terms of the energy value of oxygen consumption (Jobling, 1994; Tytler and Calow, 1985).

Larval anatomy facilitates aerobic respiration. The high surface area to volume ratio of larval fish facilitates the oxygen diffusion into tissues from surrounding water (Oikawa and Itazawa, 1985) and in the case of cyprinid larvae high oxygen retaining capacity of the red muscle increased the possibility of aerobic respiration (El-Fiky *et al.*, 1987), even in emergency high energy demand. Enzyme studies on coregonid larval fish have indicated that the quantity of glycolytic enzymes (enzymes responsible for glycolysis *i.e.* anaerobic respiration) in tissue is comparatively very low when compared with levels in adult muscle (Hinterleitner *et al.*, 1987), and increased amount of oxidative enzymes (enzymes responsible for aerobic respiration) have also been reported in larval fish (Dabrowski, 1989). For these reasons it has been hypothesised that in larval fish, oxygen is supplied across the whole body surface and energy is supplied mainly through aerobic processes

(Dabrowski, 1989). When organisms are exposed to toxicants, they try to escape from toxicant exposure (Lloyd, 1992), resulting in increased activity. This increase in activity together with the processes of detoxification and repair, requires energy (Sibly and Calow, 1989). To cope with the increased requirements for energy, organisms increase oxygen consumption with the result that energy available for growth will be in short supply (Bayne *et al.*, 1985) and this reduction of growth due to short supply *i.e.* energy trade off from growth to defence, may lead to the reduction of fitness of individuals in a population (Sibly and Calow, 1986). The measurement of oxygen consumption as an indicator of energy expenditure has been used in many aquatic studies (*e.g.* Brafield, 1985; Madon and Culver, 1993; Philippova and Postnov, 1988; Rombough, 1988).

5.1.4 Nitrogen excretion

A small proportion typically 2 - 7% of the total energy absorbed by an organism is excreted as metabolic waste products (Brett and Groves, 1979). Nitrogenous excretory products are derived from the catabolism of proteins and nucleic acids to amino acids. Ammonia is the most common excretory nitrogen product in freshwater teleosts (Brafield, 1985; Kaushik *et al.*, 1982). The amount of nitrogenous waste is dependent on the utilization of protein for energy and on the rate of breakdown and turnover of protein (Jobling, 1993). It is well known that stress affects the dynamic steady state of the body by increasing the rate of protein damage (Barber, 1990; Garlick *et al.*, 1975; Hawkins *et al.*, 1987; Hawkins, 1991;

Siriwardena, 1993) and this increased maintenance requirement is expensive in terms of energy which may otherwise be deposited as tissue growth (Sibly and Calow, 1989). As the stress increases non-specific protein damage, therefore consistent increases in ammonia excretion can be expected with increases in toxicant concentrations.

5.1.5 Oxygen to nitrogen ratio

A general response by an organism to stress is the utilization of nutrient reserves to meet an increased requirements for maintenance. In some cases this can be measured in terms of reduction of carbohydrate, protein and lipid stores, but generally these changes in chemical composition occur only under extreme stress (Bayne and Thompson, 1970; Widdows, 1985). An alternative but sensitive measure of stress response is the shifting of the balance between the catabolism of carbohydrate, protein and lipid substrate.

The ratio between oxygen consumed and nitrogen excreted (O : N, calculated in atomic equivalents) provides an index of the relative utilization of protein in energy metabolism (Bayne *et al.*, 1976; Widdows, 1978). The high rate of protein metabolism relative to carbohydrate and lipid results in a low O : N ratio, which is generally indicative of a stressed condition. The theoretical minimum value for O : N, with only protein catabolism is approximately 7 (Mayzaud, 1973). The O : N ratio has been studied in zooplankton (Conover, 1978), benthic molluscs (Bayne, 1975; Bayne *et al.*, 1976) and recently, in tilapia fish sac-fry (Siriwardena,

1993). There are interspecific and intraspecific differences in the O : N value for healthy animal (Widdows, 1985). Study of the oxygen consumption and nitrogen excretion together with rate of yolk utilization and efficiency of yolk utilization under sublethal exposure to three different toxicants (cadmium, DCA and chlorpyrifos) will provide a comprehensive understanding of the general effects of toxicants on the energetics of fish sac-fry.

5.1.6 Energy budget components of fish sac-fry

The bioenergetics model is a mass balance equation founded on the first law of thermodynamics regarding the conservation of matter and energy. All bioenergetics equations are based on the simple formulation that all food consumed by a fish is either lost as heat, incorporated into body tissue or excreted as waste (Winberg, 1960). The energy budget equation for endogenous feeding fish sac-fry has been described in section 1.7. Energy budget equation can be solved for any of the components, if the others are known or calculated. But the energy equation is normally used to calculate growth (scope for growth) or consumption if one or other is known and appropriate expressions for energy and waste losses are available. The energy budget can be solved on an instantaneous or cumulative basis for short or long period of time (Knight, 1985).

The appeal of bioenergetics is that the approach links basic fish physiology and behaviour with environmental conditions (Adams *et al.*, 1982; Brandt and Hartman, 1993; Ney, 1993). Energy equations have been employed to model the dynamics

of contaminants in fish (Weininger, 1978; Borgmann and Whittle, 1992). The energetics of young fish as well as older fish are well documented (*e.g.* Brett and Groves, 1979; Coutant and Edsall, 1993; Elliott, 1979; Jobling, 1994; Tytler and Calow, 1985), but little effort has been made in fish early life stages (*e.g.* Dabrowski, 1989; Rombough, 1988) due to their rapidly changing complicated process of development and their small size (Kamler, 1992).

5.1.7 Aims of the study

Toxic chemicals affect the bioenergetic performance of animals in two ways, (1) by reducing the capacity to acquire energy, through inhibition of feeding and digestion (supply-side effects), and/or (2) by increasing energy costs due to increasing maintenance energy requirements or by inducing hyperactivity (demand-side effects). Growth is a result of a combination of both supply and demand side effects. Baird *et al.* (1990) observed significant reduction in the production (growth + reproductive investment) of different clones of *Daphnia magna* under chronic exposure of cadmium and DCA, and suggested that this was due to a general reduction in the energy supply through feeding. In a linked experiment, Barber *et al.* (1990) found increased protein degradation under cadmium and DCA stress and suggested protein degradation as a general response. Although yolk sac-fry are nonfeeding, the process of yolk uptake by the developing sac-fry can be considered analogous to feeding, since yolk must be assimilated through the yolk syncytium, in an analogous fashion to food assimilation through the gut wall. In fish early life-

stage toxicity tests, 'growth' is a widely used endpoint (see section 1.6), but the physiological mechanisms underlying the response are poorly studied. Therefore, the study was designed to:

1. investigate possible sublethal physiological test endpoints for use in fish early life-stage toxicity tests.
2. validate a simple conceptual model (Figure 5.1) of fish early life-stage response to sublethal toxicant exposure.

5.2 Materials and Methods

5.2.1 Stock solution and exposure concentration of toxicants.

Cadmium stock solution of 20 mg.l⁻¹; DCA stock solution of 10 mg.l⁻¹ and chlorpyrifos stock solution of 1 mg.l⁻¹ were prepared as described in section 2.3.1, 2.3.2, and 2.3.3, respectively. Exposure concentrations were then made by serial dilutions of the stock solutions. Four exposure concentrations of each toxicant: 100, 250, 500 and 1000 µg.l⁻¹ cadmium; 10, 25, 50, and 100 µg.l⁻¹ DCA; 0.25, 0.5, 1.0, and 2.0 µg.l⁻¹ chlorpyrifos were selected on the basis of the estimated LC50 values (section 4.3) and on the basis of their reported chronic and/or fish ELS toxicity and ACR (acute to chronic ratio) for other organisms (see section 1.5.1, 1.5.2 and 1.5.3) and also on the basis of the results of some trial experiments, especially with chlorpyrifos. The cadmium stock and exposure concentrations were checked using graphite flame furnace Atomic Absorption Spectrophotometry (Golterman *et al.*, 1978). DCA stock and test concentrations were checked by HPLC (method

described in section 2.6 for each toxicant). It was not possible to estimate actual concentrations of the stock and test concentrations of chlorpyrifos. Nominal concentrations were taken into consideration.

5.2.2 Exposure of sac-fry to sublethal concentrations

In all the tests, 30 sac-fry were randomly assigned (following the section 2.1.3) to each exposure chamber. Sac-fry were exposed in triplicate of four concentrations of a toxicant and dilution water (or dilution water + maximum carrier solvent used in exposure toxicant concentrations in case of chlorpyrifos). The test medium was renewed daily throughout the experiment. The tests were carried out for 96 h (*i.e.* until sac-fry reached maximum dry body mass feeding on yolk mass, see section 3.4.3).

5.2.3 Oxygen consumption and ammonia excretion

All measurements of oxygen consumption were carried out on groups ($n = 30$) of exposed sac-fry under semi-static conditions in the flow-through system that was sealed for three to four hours starting from 92 - 93 h to 96 h exposure period of sublethal experiments. Flow-through respirometry techniques provide advantage over static respirometry but sometimes it is difficult to measure accurately the differences of oxygen content between inflow and outflow. Widdows (1985) advocated a flow-through respirometer that is sealed for a limited period of time (*i.e.* a semi-static cell) as the most suitable method. Groups of sac-fry ($n = 30$)

were used for measurements of respiratory oxygen consumption with a minimum of disturbance of experimental conditions and also to produce a detectable change in oxygen content in water. Furthermore, the use of more than a single individual organism of small size has been used in respiration and bioenergetics study, *e.g.* Madon and Culver (1993) used up to 14 larvae and juvenile walleyes for respiration measurements. After 92 - 93 h of sublethal exposure to toxicants, the oxygen content of the water was measured by taking a 500 µl sample of the water from the outflow tube of the exposure chambers with a 1 ml syringe and immediately injecting into a jacketed flow-cell micro-electrode (TC500, Strathkelvin Instruments, UK) connected to a dissolved oxygen meter (Model 781, Strathkelvin Instruments, UK). The flow had been stopped after 92 - 93 h exposure of sublethal exposure by switching off the peristaltic pump and inflow and outflow tube of the exposure chambers were clamped for 3 - 4 h to complete 96 h exposure of the sublethal test duration. After 96 h exposure, the oxygen content of the water was measured again by taking a second water sample. The amount of oxygen consumed was calculated from the differences of the two measurements, using the following equation,

$$\text{amount of oxygen consumed } (\mu\text{g}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}) = \frac{(X_o - Y_o) \times V}{T \times W} \quad (5.1)$$

Where, X_o = first measurement of oxygen ($\mu\text{g}\cdot\text{l}^{-1}$)

Y_o = second measurement of oxygen ($\mu\text{g}\cdot\text{l}^{-1}$)

V = volume of the chamber in litre

T = time (h) between two measurements

W = somatic dry weight of sac-fry (mg) in the chamber

At the same times of sampling of water for oxygen measurement, 5 ml of water from each chamber was transferred into a cleaned plastic vial, stoppered and frozen for analysis of ammonia at a later date. Ammonia was measured on an autoanalyser (Technicon Sampler IV, Gradco International Ltd., UK). The amount of ammonia excreted was calculated by the differences of two measurements by using the following equation,

$$\text{amount of ammonia excreted } (\mu\text{g}\cdot\text{mg}^{-1}\cdot\text{h}^{-1}) = \frac{(Y_a - X_a) \times V}{T \times W} \quad (5.2)$$

Where, X_a = first measurement of ammonia ($\mu\text{g}\cdot\text{l}^{-1}$)

Y_a = second measurement of ammonia ($\mu\text{g}\cdot\text{l}^{-1}$)

V = volume of the chamber in litre

T = time (h) between two measurements

W = somatic weight of sac-fry (mg) in the chamber

At the termination of the experiments after 96 h, larvae from each chamber were killed by immersion in a 1:20,000 benzocaine solution, and immediately washed with dilution water. Samples of 10 sac-fry from each exposure chamber were placed in pre-weighed aluminium foil cones stored in a microcentrifuge tube and frozen at -70°C . A further sample of 10 larvae was used to determine the remaining yolk content by dissection under a dissecting microscope.

5.2.4 Yolk utilization and larval growth

Yolk mass, if present was dissected out under a dissecting microscope. Dissecting needles were used to pierce the yolk epithelium. The yolk was collected in a capillary ended Pasteur pipette fitted with rubber bulb. The yolk-less larvae were placed in pre-weighed aluminium foil cone fitted in a microcentrifuge tube and frozen at -70°C . On a later date frozen samples were freeze dried using a freeze dryer (Modulyo 4K, Edwards, UK) and dry mass of both whole fry and yolkless body were measured using a microbalance (MT5, Mettler, UK) of $1\ \mu\text{g}$ accuracy. In each test 10 whole sac-fry and 10 yolkless sac-fry at the start of the experiment were also frozen, freeze dried and weighed.

Yolk mass was calculated indirectly by subtracting the body mass from the whole mass. The measured whole mass, body mass and yolk mass was calculated for average individual. Yolk absorbed was calculated by subtracting the average yolk remaining in each replicate from the average initial yolk mass. Growth, or increase in dry body mass, was calculated by subtracting initial body mass from each of the replicate average body mass. The efficiency with which yolk is transformed to body tissue (*i.e.* yolk utilization efficiency) was measured as a ratio of dry mass gain of body to dry mass loss of yolk (Blaxter, 1969) expressed as a percentage.

5.2.5 Oxygen to nitrogen ratio (O : N)

The rate of oxygen consumed to nitrogen excreted was calculated in atomic equivalents. First, oxygen consumed in $\text{mg}\cdot\text{h}^{-1}$ divided by the atomic weight of oxygen (16). Secondly, rate of ammoniacal nitrogen excretion in $\text{mg}\cdot\text{h}^{-1}$ was divided by the atomic weight of nitrogen (14).

$$\text{Then, O : N} = \frac{\text{mg O}_2\cdot\text{h}^{-1}}{16} : \frac{\text{mg ammoniacal nitrogen}\cdot\text{h}^{-1}}{14} \quad (5.3)$$

5.2.6 Energy budgets

Energy budgets of goldfish sac-fry, exposed to sublethal concentrations of each toxicant: cadmium, DCA and chlorpyrifos, were calculated for assimilated yolk energy using the equation 1.6. Budgets were calculated from the average absorbed yolk dry mass (C), average increased in dry body mass (P), observed R and U were used after being converted to energy value. Yolk dry mass and body dry mass was converted into energy using the value of $25.2 \text{ J}\cdot\text{mg}^{-1}$ and $19.9 \text{ J}\cdot\text{mg}^{-1}$, from studies on *Cyprinus carpio* eggs and larvae, respectively, a close relative of goldfish (Kamler, 1976). Oxygen consumed and ammonia excreted were converted to energy by using value of $13.59 \text{ J}\cdot\text{mg}^{-1}$ oxygen (Section 1.7 and Jobling, 1994) and $0.0249 \text{ J}\cdot\mu\text{g}^{-1}$ ammonia (Elliott and Davison, 1975; Widdows, 1985), respectively. Egg energy content and yolk energy content is less variable: Kamler and Kato

(1983) found the value of 27.6 J.mg⁻¹ and 27.9 J.mg⁻¹ for egg and yolk respectively in case of trout (*Salmo gairdneri*). Ideally all the factors in the energy equation should be measured independently but simultaneously over a period of time but this is not always practicable, and it is often necessary to use values for physiological processes from a well studied species or life-stages to complete the energy equation of a poorly studied species *e.g.* Boisclair and Sirois (1993) borrowed caloric density value of sockeye salmon for the calculation of energetics of brook trout.

Energy budget equations were constructed based on equation 1.6. Production (P) in the energy budget was considered in two ways 'expected growth (P₁)' and 'observed growth (P₂)' respectively. In this energy budget study observed production or growth (P₂) was calculated in terms of energy value of the measured gain in dry body mass during the experiment, whereas, expected growth, also called 'scope for growth,' was calculated as the energy value of the yolk mass remained after spending for respiration and excretion by using the balance energy equation 1.7. The balance of energy of the budget was calculated in terms of percentage as,

$$\frac{(P_2 + R + U \times 100)}{C} \quad (5.4)$$

Where, each symbol carries its meaning described in section 1.7.

5.2.7 Oxygen consumption over the period of sublethal exposure experiments

We would predict from the stress response (see section 1.1.2 and 1.1.3) that energy is required to tolerate stress and this requirement of increased energy demand will be manifested in increased respiration *i.e.* oxygen consumption in case of organisms with aerobic pathway of energy production. Therefore, oxygen consumption will depend on both intensity and duration of exposure (Figure 5.2). The basis of this is that energy is required to resist the effect of toxicants and to maintain resistance at the level of its capacity with continuous exposure in an exposure level or increased concentration and eventually the organism expends all of its energy reserve and ultimately dies. It would be important to determine the effects of toxicants in terms of oxygen consumption with concentrations and also with time to investigate the triphasic nature of GAS (Selye, 1974, 1976) as a general response of toxicants, to be certain how precisely we can use oxygen consumption as a test endpoint.

Oxygen consumption of stressed sac-fry over the experimental period exposed to different selected sublethal concentrations were studied. Sac-fry were exposed to four replicates of three concentrations of toxicant : 250, 500 and 1000 $\mu\text{g.l}^{-1}$ cadmium; 10, 25 and 50 $\mu\text{g.l}^{-1}$ DCA, and 0.25, 0.5 and 1.0 $\mu\text{g.l}^{-1}$ chlorpyrifos and four replicates in dilution water only, or dilution water + carrier solvent in case of chlorpyrifos. Concentrations were selected from the concentration series used to calculate energy budgets. Oxygen consumption by sac-fry in two randomly selected chambers from each treatment were measured following the method described in

section 5.2.3 for 3 - 4 h exposure in sealed exposure chambers of the flowthrough system. At the start of the experiment a sample of 10 larvae (yolk dissected out) was frozen at -70°C after being placed in a preweighed aluminium foil stored in a microcentrifuge tube for dry mass measurement at a latter date. After 3 - 4 h initial exposure in sealed exposure chambers of the flow-through system, the system was run for up to 48 h with sac-fry and at that point 8 channels were randomly selected (two replicate of each three concentration and control) stopped and sealed by clamping for 3 - 4 h and the oxygen content measured following the method described in section 5.2.3. A sample of 10 randomly selected sac-fry from each chamber was dissected to remove yolk and frozen at -70°C placed in a preweighed cone of aluminium foil in a microcentrifuge tube. The remaining channels were run up to 96 h, sealed for 3 - 4 h and the oxygen content of the water measured as stated in section 5.2.3. A sample of 10 larvae from each exposure chamber was dissected to remove yolk and placed in a preweighed aluminium foil cone and stored in a microcentrifuge and frozen at -70°C . On a later date dry weight was measured as described in section 5.2.3. Oxygen consumption rate was calculated in μg of oxygen consumed per mg of larval dry body mass per hour following equation 5.1. The measured rate of oxygen consumption during initial period starting from 0 to 3 - 4 h expressed as 0 h oxygen consumption, during the period starting from 48 to 51 - 52 h expressed as 48 h oxygen consumption and during the period starting from 96 to 99 - 100 h was expressed as 96 h oxygen consumption for the interpretation of results.

It was planned to observe the sac-fry activity under different exposures both qualitatively and quantitatively. However, it was not possible to quantify the behavioural activity, although it was closely observed. The activities in terms of normal swimming movement, erratic movement, moribund and thigmotaxis were observed for 2 h daily in two shifts, one during early hours of light regime (9 - 10 am) and another during the later part of the light regime (6 - 7 pm) over the experimental period.

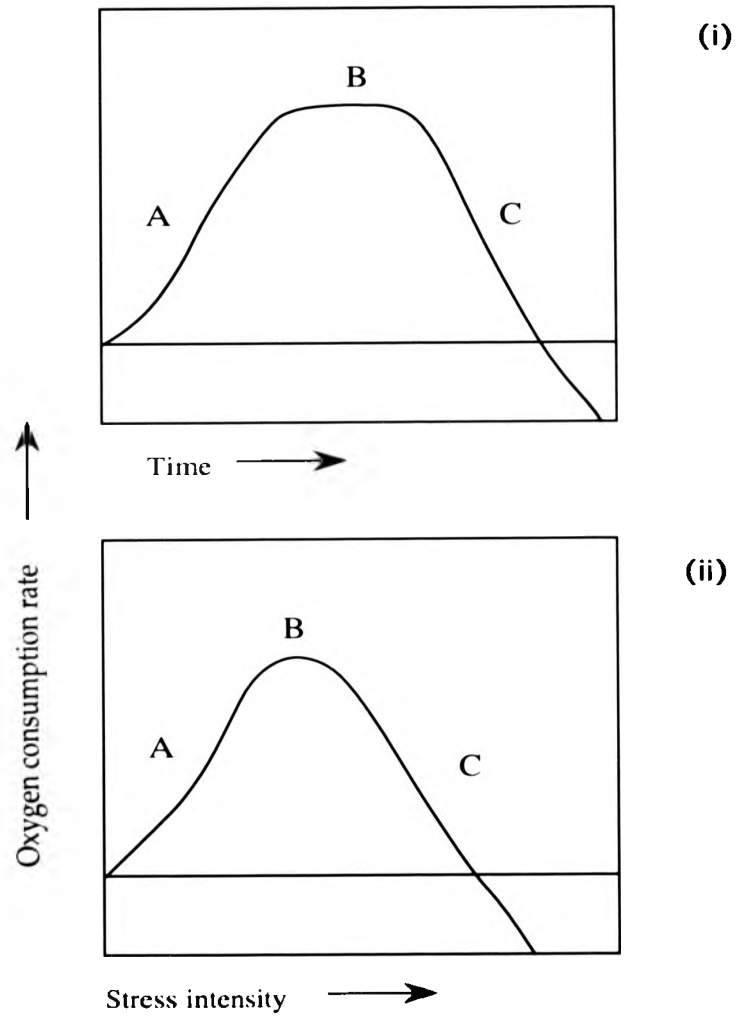


Figure 5.2 The possible effect of (i) duration of toxicant exposure and (ii) intensity of toxicant exposure on oxygen consumption, where A- alarm reaction, B- resistance, C- exhaustion .

5.3 Results

The mean actual concentration estimated for each nominal exposure concentration of cadmium and DCA are shown in Table 5.1 and 5.2, respectively.

5.3.1 Yolk utilization and somatic weight gain under sublethal stress.

The sublethal effects of cadmium, DCA and chlorpyrifos on the body weight gain, yolk utilization and yolk utilization efficiency (arcsine transformed value) are shown in Figure 5.3, 5.4 and 5.5, respectively.

(a) cadmium

There were significant differences in body weight gain, ($F = 8.149$; $df = 4, 10$; $P < 0.05$) and in yolk utilization efficiency ($F = 8.005$; $df = 4, 10$; $P < 0.05$), but no significant difference in yolk utilization ($F = 2.00$; $df = 4, 10$; $P > 0.05$) among the different cadmium concentrations tested. The lowest observed effect concentration for both body weight gain and yolk utilization efficiency was found at the highest concentration tested, *i.e.* $1000 \mu\text{g.l}^{-1}$ when compared to the corresponding control values. Although weight gain and yolk utilization efficiency were significantly reduced there was no detectable difference in the total amount of yolk consumed, even at $1000 \mu\text{g.l}^{-1}$ of cadmium compared to the control.

(b) DCA

Significant differences in body weight gain ($F = 77.839$; $df = 4, 10$; $P < 0.05$), in yolk utilization ($F = 30.293$; $df = 4, 10$; $P < 0.05$) and in yolk utilization efficiency ($F = 44.155$; $df = 4, 10$; $P < 0.05$) were found for different sublethal DCA concentrations tested. The lowest observed effect concentration was found to be $50 \mu\text{g.l}^{-1}$ for both body weight gain and yolk utilization efficiency and $100 \mu\text{g.l}^{-1}$ for yolk utilization when compared with corresponding control values. Although in both $50 \mu\text{g.l}^{-1}$ and $100 \mu\text{g.l}^{-1}$ yolk utilization efficiency were significantly different from the control value the efficiency of yolk utilization is significantly higher at $100 \mu\text{g.l}^{-1}$ than at $50 \mu\text{g.l}^{-1}$.

(c) chlorpyrifos

There were significant differences in body weight gain, ($F = 137.728$; $df = 4, 10$; $P < 0.05$), yolk utilization ($F = 78.548$; $df = 4, 10$; $P < 0.05$) and yolk utilization efficiency ($F = 38.708$; $df = 4, 10$; $P < 0.05$) for different sublethal chlorpyrifos concentrations tested. The lowest observed effect concentration was $0.50 \mu\text{g.l}^{-1}$ in all cases when compared with control values.

Table 5.1 The observed cadmium concentration of the nominal exposure concentration during the exposure period of goldfish sac-fry. Mean given with \pm standard error, n=6.

Nominal cadmium concentration ($\mu\text{g.l}^{-1}$)	Actual cadmium concentration ($\mu\text{g.l}^{-1}$)
100	107.8 \pm 19.5
250	266.7 \pm 56.1
500	546.2 \pm 68.5
1000	1099.5 \pm 121.4

Table 5.2 The observed DCA concentration of the nominal exposure concentration during the exposure period of goldfish sac-fry. Mean given with \pm standard error, n=6.

Nominal DCA concentration ($\mu\text{g.l}^{-1}$)	Actual DCA concentration ($\mu\text{g.l}^{-1}$)
10	10.64 \pm 0.33
25	26.85 \pm 0.46
50	58.35 \pm 2.88
100	112.88 \pm 3.53

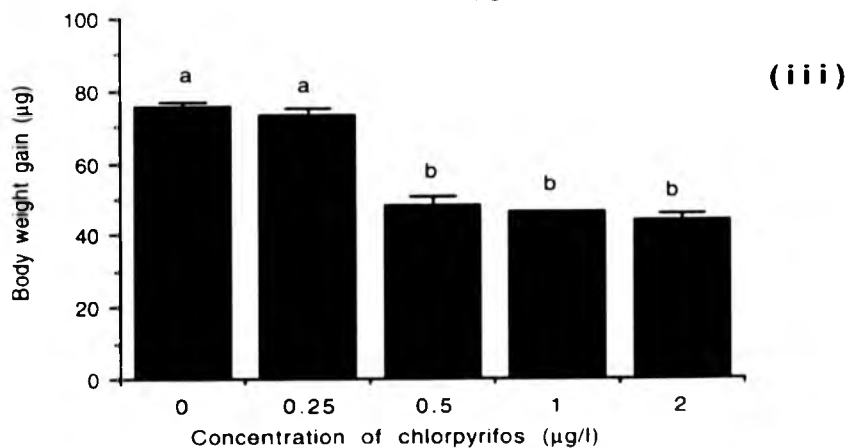
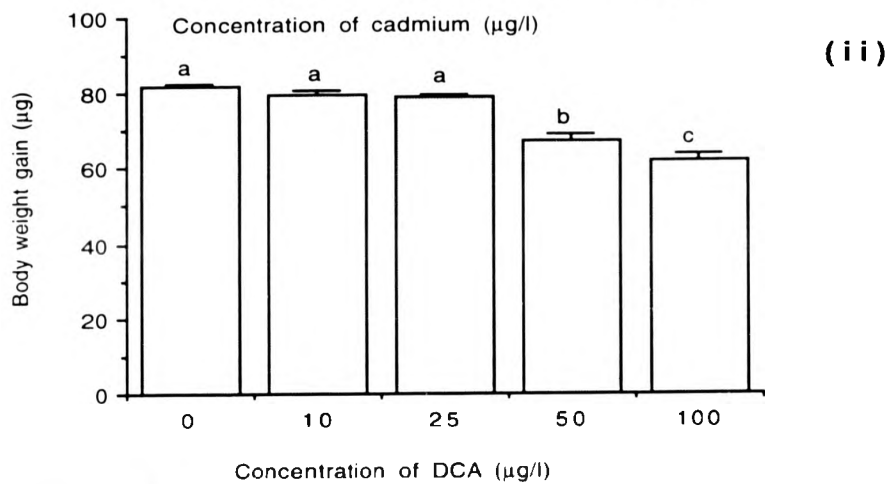
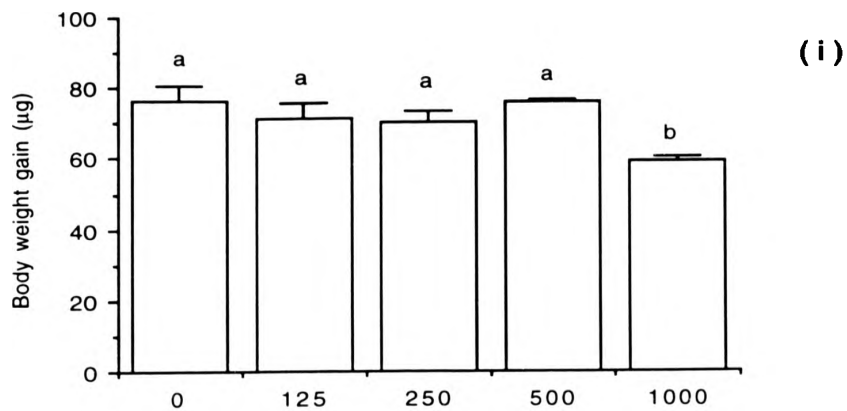


Figure 5.3 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on body weight gain of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

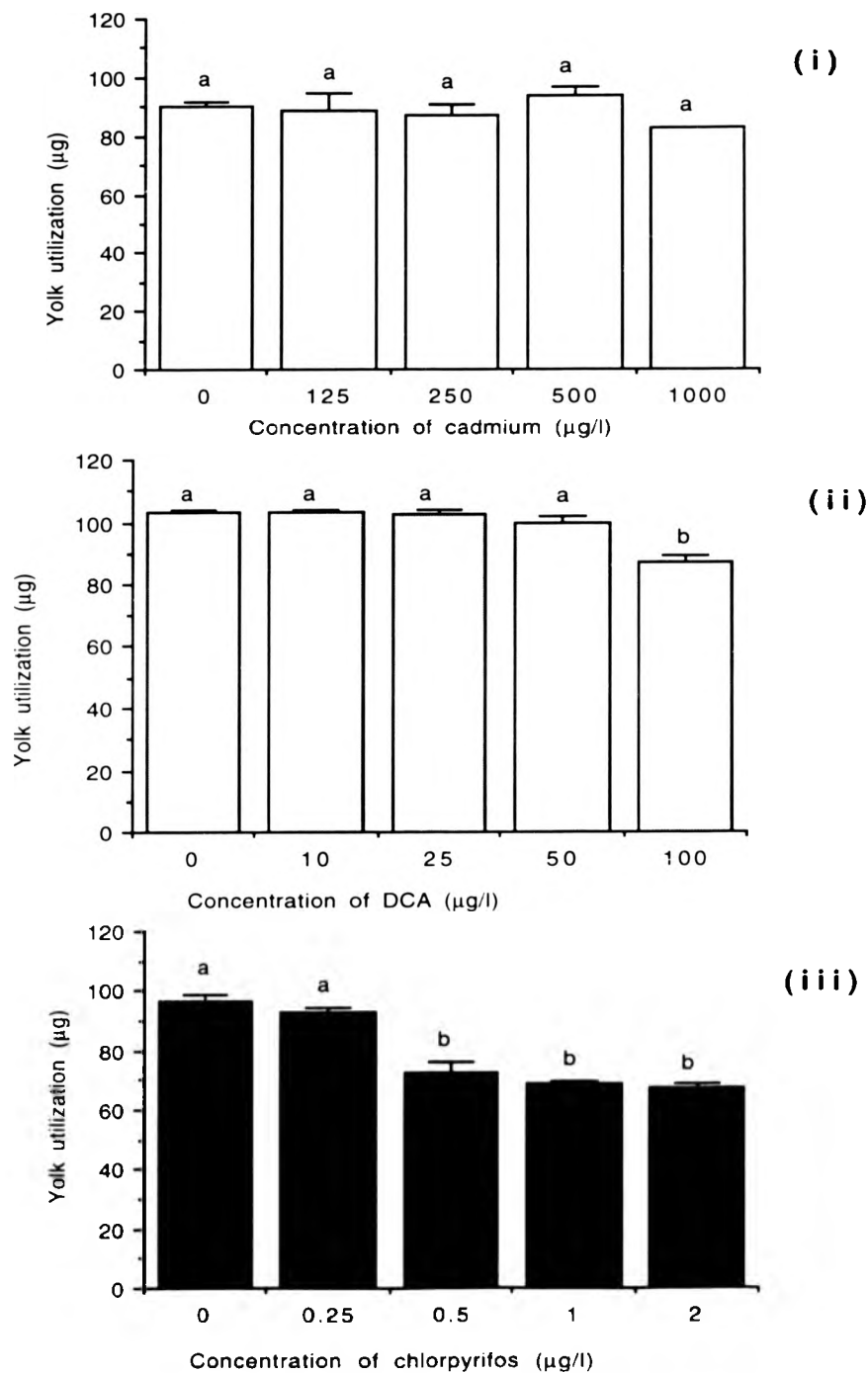


Figure 5.4 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on yolk utilization by goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

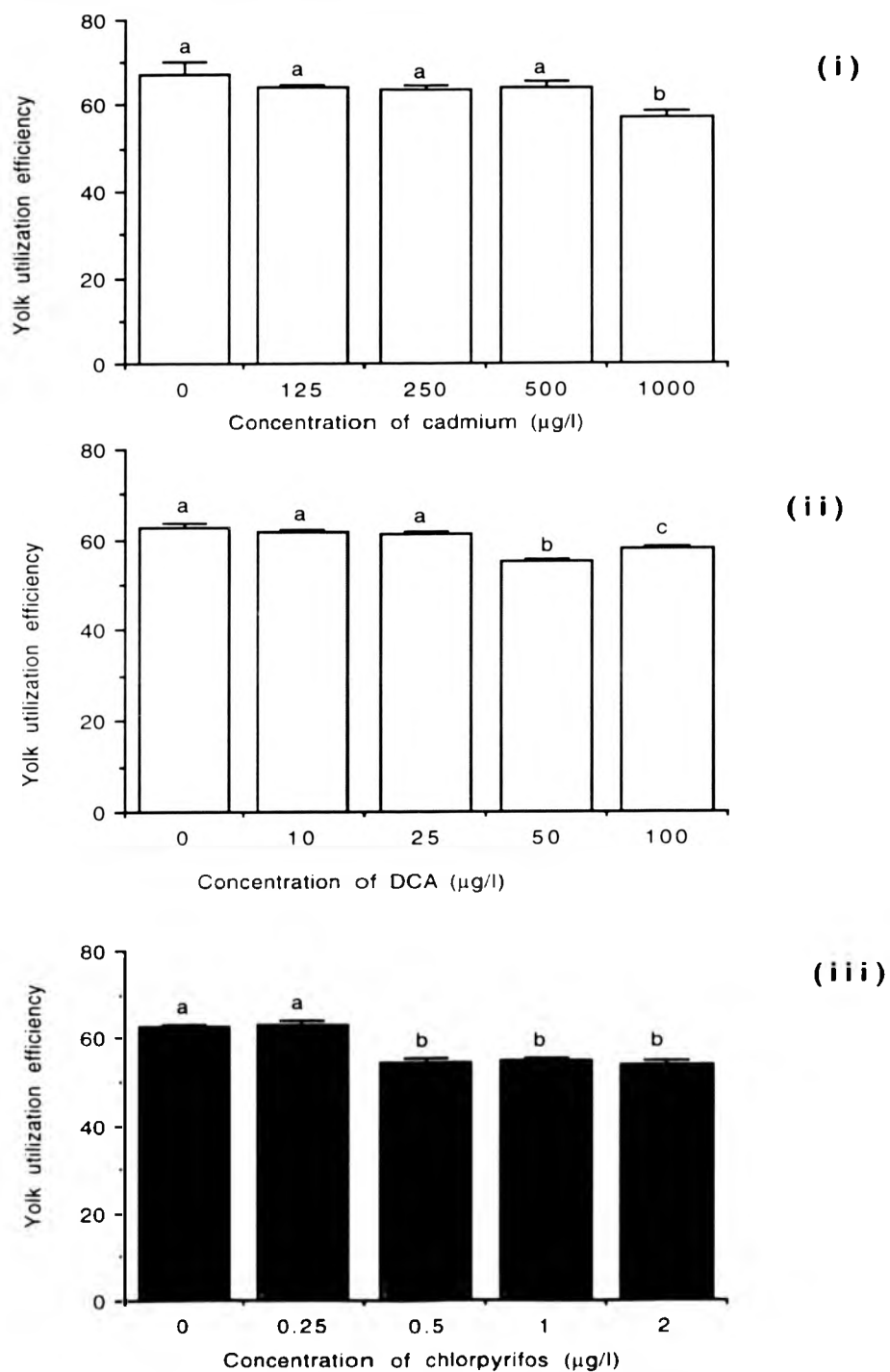


Figure 5.5 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on yolk utilization efficiency of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

5.3.2 The effects of sublethal stress of cadmium, DCA and chlorpyrifos on oxygen consumption, ammonia excretion and oxygen to nitrogen ratio (demand-side effect).

The rates of oxygen consumption, ammonia excretion and oxygen to nitrogen ratio under exposure to different sublethal concentrations are presented in Figures 5.6, 5.7 and 5.8, respectively. Cadmium induced increasing oxygen consumption with increasing concentration and significant differences ($F = 4.044$; $df = 4, 10$; $P < 0.05$) in oxygen consumption were observed. The lowest observed effect concentration was found at $1000 \mu\text{g.l}^{-1}$ when compared with control value.

Under sublethal exposure to DCA oxygen consumption increased significantly at low concentrations, and decreased at higher concentration ($F = 19.305$; $df = 4, 10$; $P < 0.05$). The lowest observed effect concentration in terms of oxygen consumption was $25 \mu\text{g.l}^{-1}$, at which oxygen consumption reached its maximum rate.

Unlike cadmium and DCA, there was no significant change in oxygen consumption by sac-fry exposed to chlorpyrifos ($F = 2.56$; $df = 4, 10$; $P > 0.05$), although there was evidence of a pattern of response similar to DCA.

Ammonia excretion consistently and significantly increased with increased exposure concentration for cadmium ($F = 15.984$; $df = 4, 10$; $P < 0.05$); DCA ($F = 36.721$; $df = 4, 10$; $P < 0.05$) and also for chlorpyrifos ($F = 87.923$; $df = 4, 10$; $P < 0.05$). The lowest observed effect concentration for cadmium was $500 \mu\text{g.l}^{-1}$, for DCA it was

50 $\mu\text{g.l}^{-1}$ and for chlorpyrifos it was 0.50 $\mu\text{g.l}^{-1}$.

The resultant oxygen to nitrogen ratios significantly decreased under increasing sublethal concentrations for cadmium ($F = 3.852$; $df = 4, 10$; $P < 0.05$), DCA ($F = 41.071$; $df = 4, 10$; $P < 0.05$) and chlorpyrifos ($F = 100.923$; $df = 4, 10$; $P < 0.05$). Lowest observed effect concentration was 1000 $\mu\text{g.l}^{-1}$, 50 $\mu\text{g.l}^{-1}$ and 0.5 $\mu\text{g.l}^{-1}$ for cadmium, DCA and chlorpyrifos respectively when compared with corresponding control values.

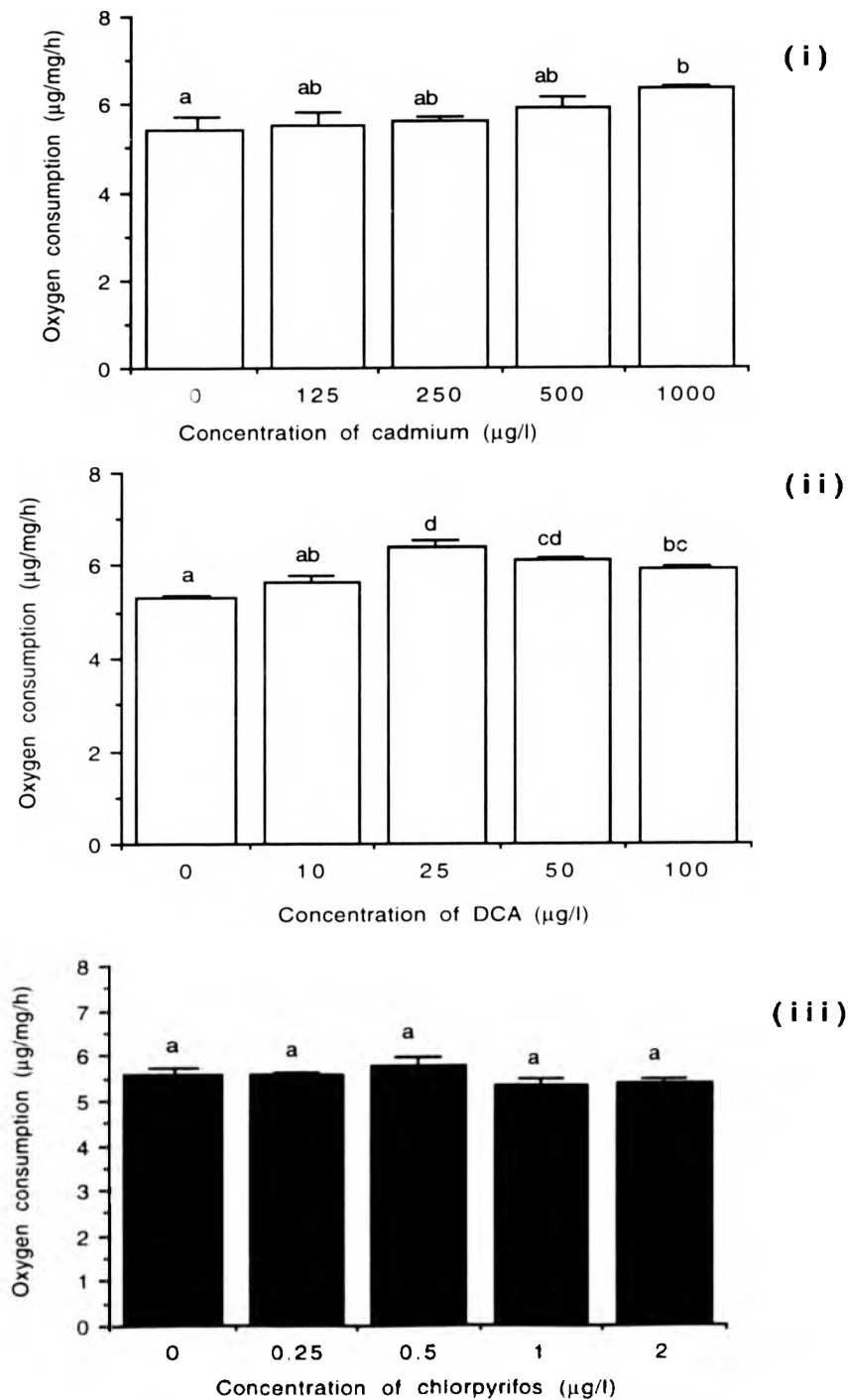


Figure 5.6 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on rate of oxygen consumption by a group of 30 goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

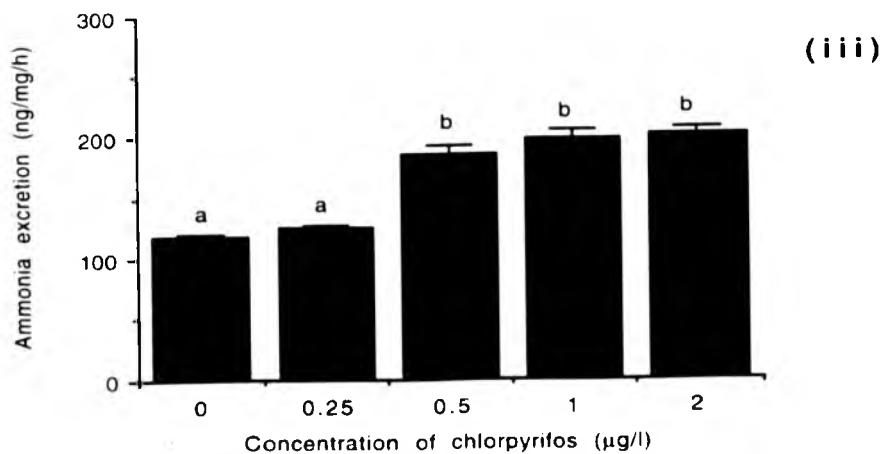
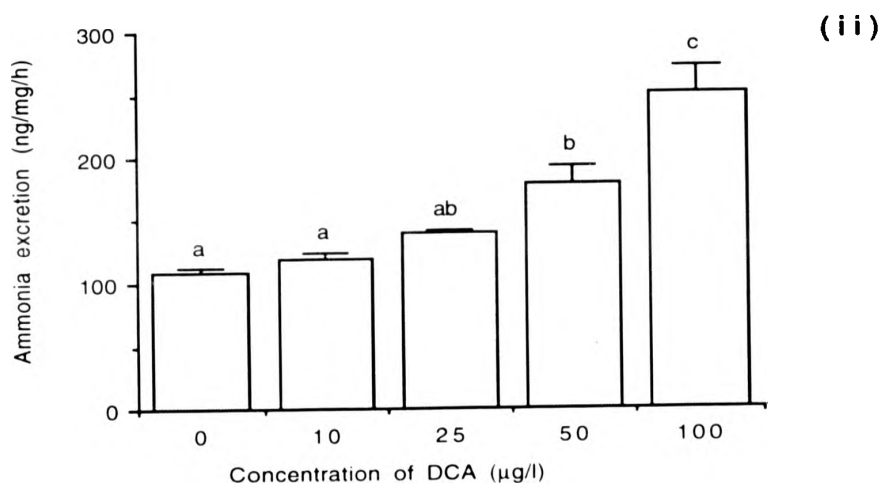
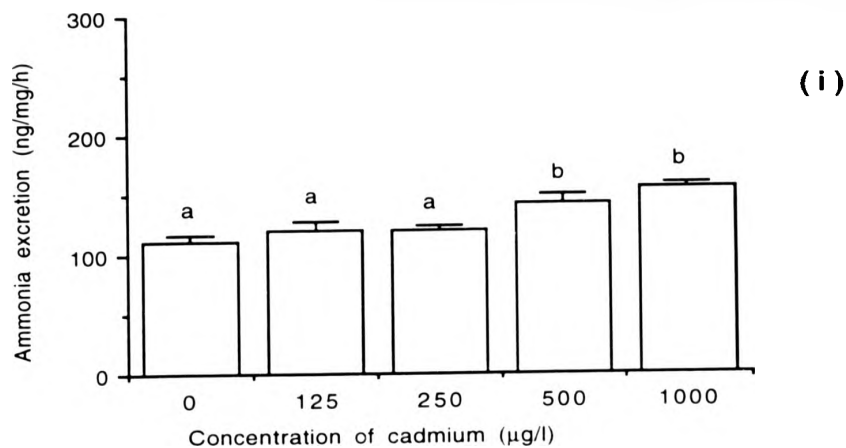


Figure 5.7 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on rate of ammonia excretion by a group of 30 goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

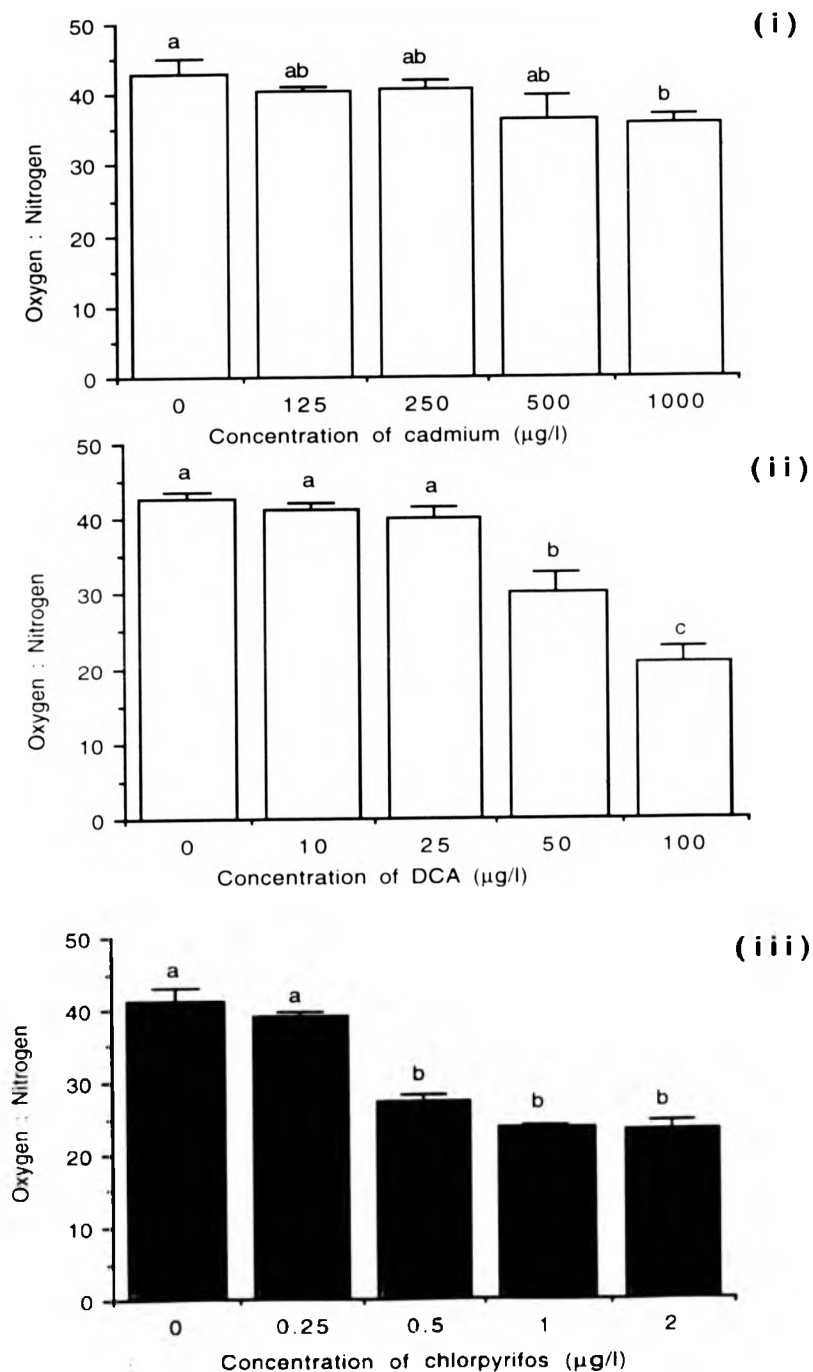


Figure 5.8 Effects of sublethal concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on oxygen to nitrogen ratio of goldfish sac-fry. Bars are mean of three replicate given with + standard error. Bars with common letter represent no significant difference.

5.3.3 Energy budget of goldfish sac-fry under sublethal stress of cadmium, DCA and chlorpyrifos.

The energy budget calculation under control and various exposure levels of sublethal cadmium, DCA and chlorpyrifos stress are shown in Table 5.3. The allocation of energy acquired from yolk into different components under various sublethal levels of cadmium, DCA and chlorpyrifos exposure are presented in Figure 5.9. The percentage contribution of 'scope for growth' calculated from the energy budget are presented with other components of the balanced energy equation in Figure 5.10. Scope for growth and observed growth were found to be highly correlated with each other (Figure 5.11). The percent balance of the energy budgets under different sublethal exposure of cadmium, DCA and chlorpyrifos remained above 95% except for two treatments of DCA.

The regression equation for scope for growth (P_1) on observed growth (P_2) was found to be,

$$P_1 = 2.65 + 0.99 P_2 \quad (5.5)$$

$$P_1 = 12.58 + 1.03 P_2 \quad (5.6)$$

$$P_1 = -9.38 + 1.13 P_2 \quad (5.7)$$

for goldfish sac-fry under the exposure of cadmium (5.5), DCA (5.6) and chlorpyrifos (5.7), respectively.

Table 5.3 The energy budget of *Carassius auratus* sac-fry under sublethal stress of cadmium, DCA and chlorpyrifos, expressed as $\text{ml} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$.

Toxicant	Concentration $\mu\text{g} \cdot \text{l}^{-1}$	Assimilation (C) (mJ)	Respiration (R) (mJ)	Excretion (U) (mJ)	Somatic growth		Balance (%) $(P_2+R+U \times 100)/C$
					Expected (P ₁) (mJ)	Observed (P ₂) (mJ)	
Cadmium	0.0	226.50	73.66	2.77	150.07	151.95	100.83
	100.0	222.14	75.15	2.98	144.01	141.70	98.96
	250.0	219.63	76.38	3.01	140.47	139.65	99.73
	500.0	235.54	80.18	3.57	151.79	145.96	97.52
	1000.0	207.82	86.30	3.88	117.64	116.44	99.42
DCA	0.0	253.84	75.29	2.72	175.83	158.77	93.28
	10.0	253.84	77.60	3.02	173.22	155.02	92.83
	25.0	252.28	82.90	3.27	166.11	153.66	95.07
	50.0	244.56	80.18	4.25	160.13	130.25	87.78
	100.0	214.44	80.72	6.26	127.46	120.91	96.95
Chlorpyrifos	0.0	236.95	75.70	2.95	158.30	146.77	95.13
	0.25	226.99	75.70	3.11	148.18	141.68	97.14
	0.50	178.26	78.55	4.62	95.09	93.34	99.01
	1.00	169.04	72.71	4.93	91.40	89.34	98.78
	2.00	165.69	73.25	5.01	87.43	85.08	98.58

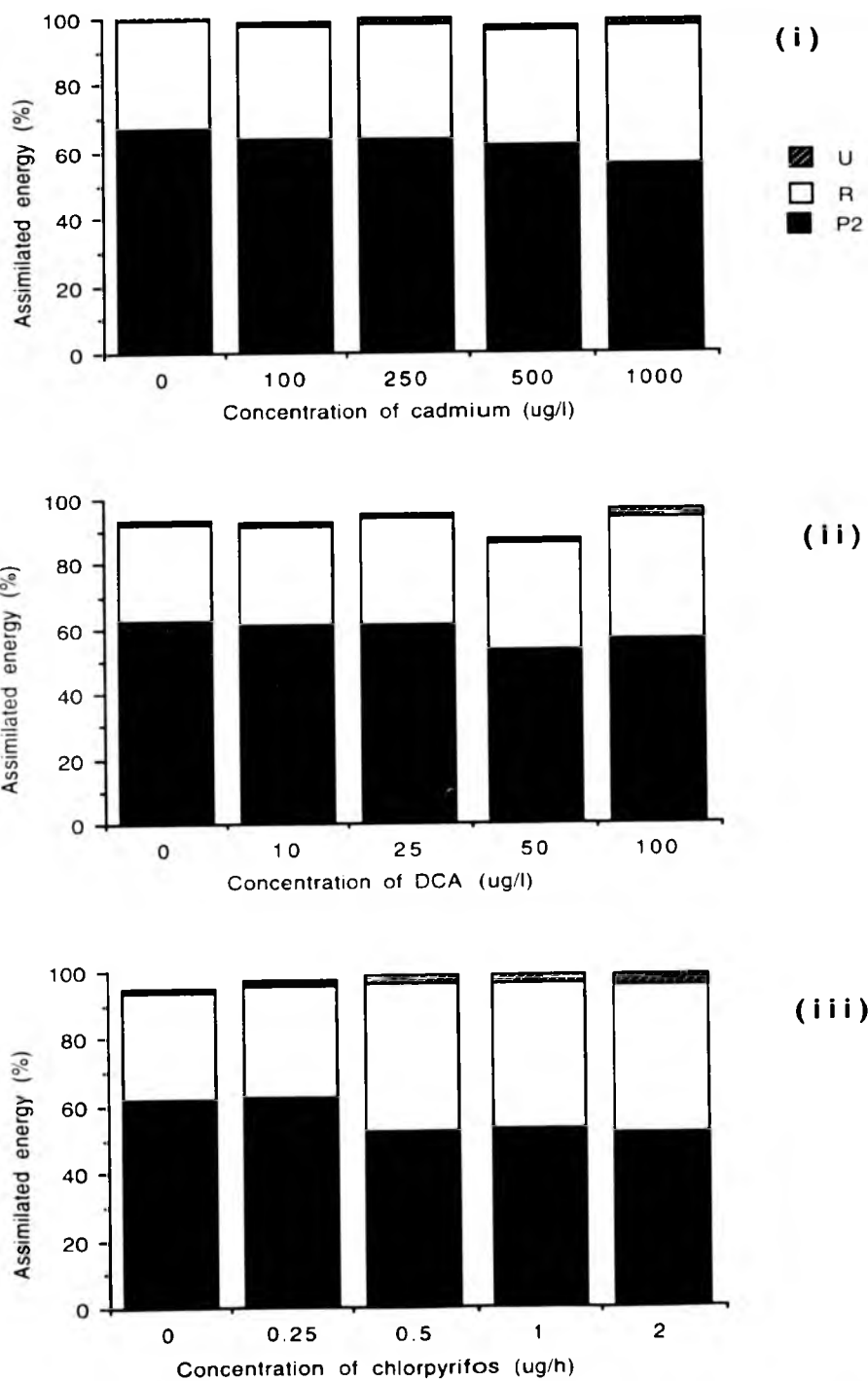


Figure 5.9 Allocation of assimilated energy to growth, respiration and excretion by the goldfish sac-fry under sublethal (i) cadmium, (ii) DCA and (iii) chlorpyrifos exposure. U - excretion, R - respiration and P₂ - observed growth.

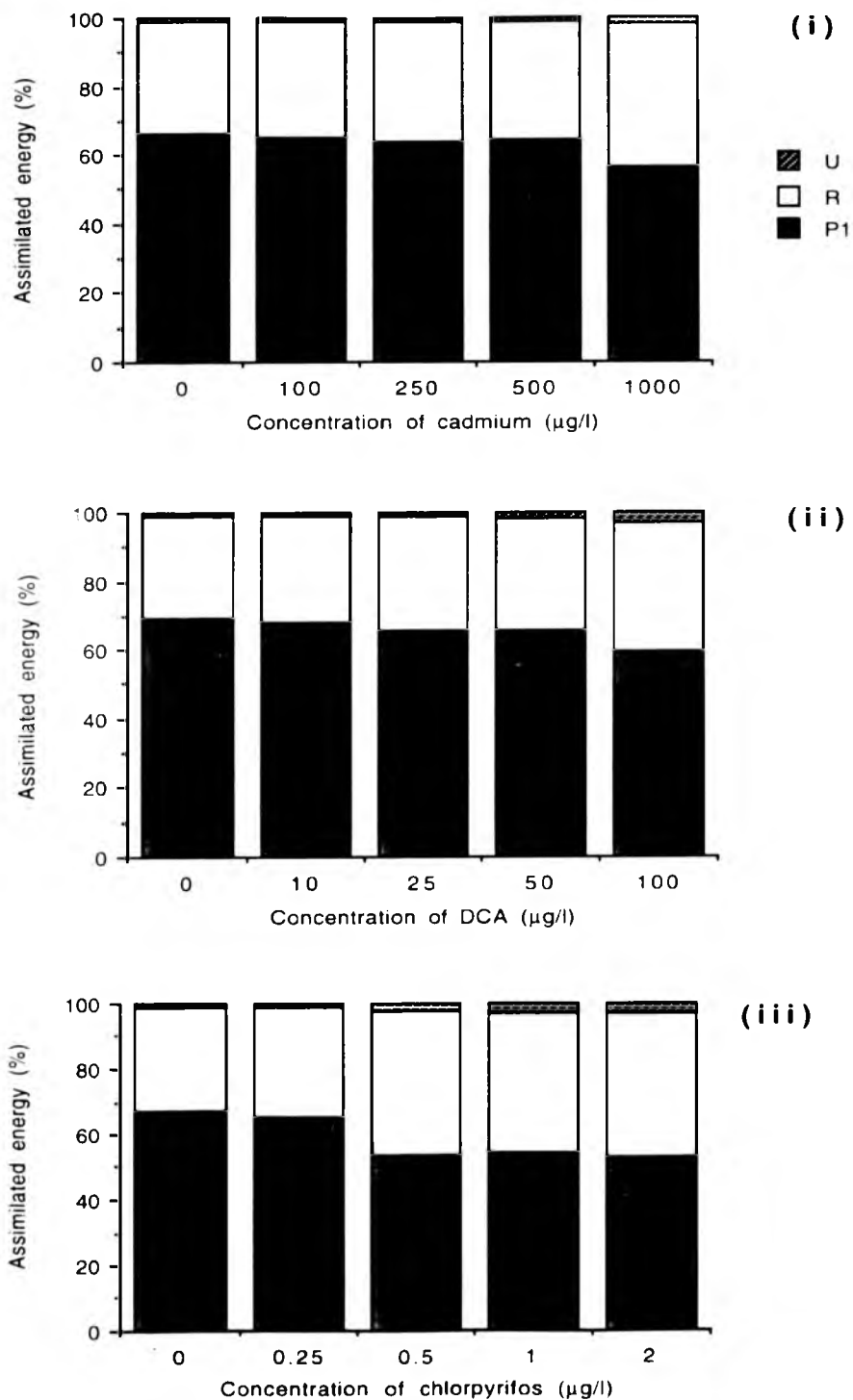


Figure 5.10 Hourly energy budget showing percentage energy distribution of expected growth, respiration and excretion under different level of sublethal (i) cadmium, (ii) DCA and (iii) chlorpyrifos exposure. U - excretion, R - respiration, P₁ - expected growth (scope for growth).

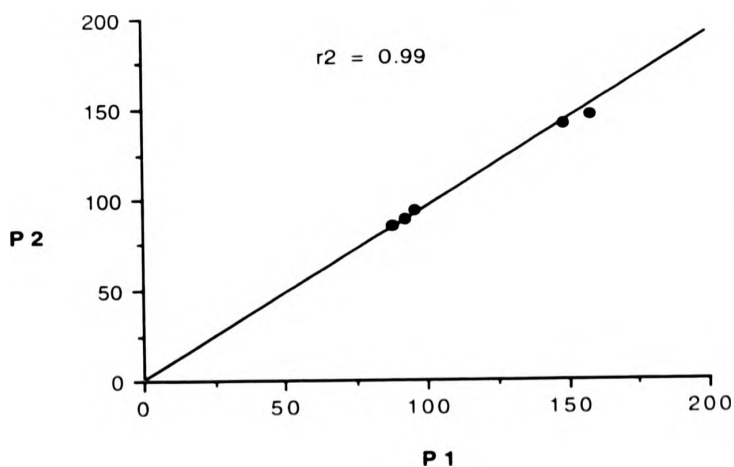
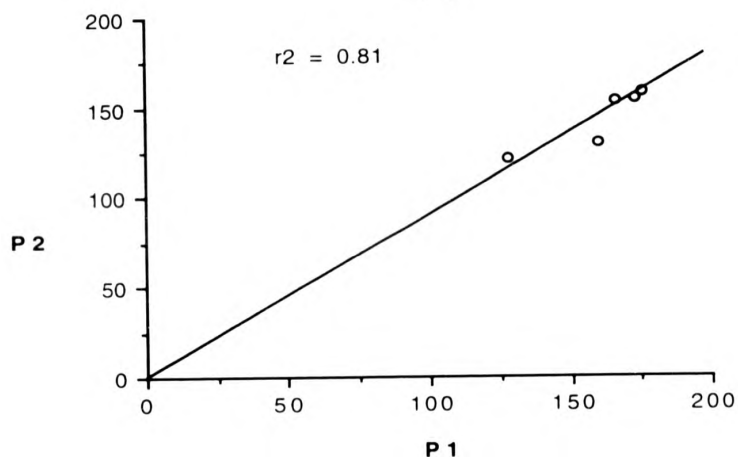
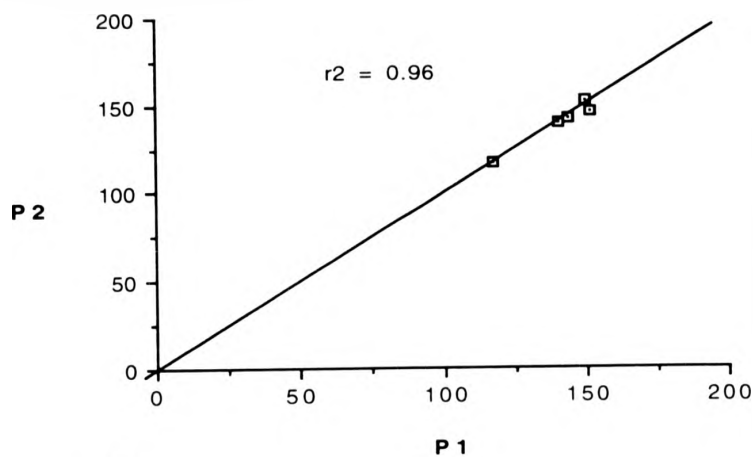


Figure 5.11 Relationship between observed growth and scope for growth of goldfish sac-fry under (i) cadmium, (ii) DCA and (iii) chlorpyrifos sublethal exposure. Growth and scope for growth are expressed as rate in energy unit ($\text{mJ} \cdot \text{mg}^{-1} \cdot \text{h}^{-1}$). P_1 - scope for growth, P_2 - observed growth.

5.3.4 Oxygen consumption over the period of sublethal exposure of cadmium, DCA and chlorpyrifos

Oxygen consumption patterns under sublethal exposure to cadmium, DCA and chlorpyrifos are shown in Figure 5.12. Initial oxygen consumption under different sublethal cadmium exposure was significantly ($F = 9.856$; $df = 3, 4$; $P < 0.05$) different from control. It was also different at 48 h ($F = 36.615$; $df = 3, 4$; $P < 0.05$) and 96 h ($F = 11.359$; $df = 3, 4$; $P < 0.05$). Oxygen consumption increased with cadmium concentration and the lowest effect concentration (see section 1.3) was found to be $500 \mu\text{g.l}^{-1}$ at 0 and 48 h but $1000 \mu\text{g.l}^{-1}$ at 96 h. Oxygen consumption rate by the goldfish sac-fry at different sublethal concentrations of cadmium over time are shown in Figure 5.13. Active swimming of sac-fry in cadmium exposure concentrations was observed during the first few hours, whereas, sac-fry in the control treatments were attached to the wall of the exposure chambers within half an hour of active swimming and showed little activities there after for up to fourth day when they increased their movement again, perhaps in search of food because of the absorption of yolk sac. Increased activity in the form of sudden erratic movement was observed in $1000 \mu\text{g.l}^{-1}$ during the later part of the experiment.

Oxygen consumption under DCA sublethal exposure concentrations showed significant ($F = 26.22$; $df = 3, 4$; $P < 0.05$) differences under initial exposure (0 h oxygen consumption) but showed no significant differences ($F = 0.488$; $df = 3, 4$; $P > 0.05$) at 48 h and again showed significant differences at 96 h ($F = 18.696$; $df = 3, 4$; $P < 0.05$). Oxygen consumption patterns under exposure to DCA are given

in Figure 5.12 and Figure 13. At the start of the experiment (0 h) significant differences in oxygen consumption were found at the lowest DCA concentration tested ($10 \mu\text{g.l}^{-1}$) when compared with control but there were no significant differences among 10, 25 and $50 \mu\text{g.l}^{-1}$ concentrations. At 48 h oxygen consumption reached the control level in all treatments. At 96 h, again oxygen consumption increased in all treatments compared with the control. Sac-fry activity under different exposures were observed by eye estimation. During the initial exposure period, sac-fry in all DCA treatments were actively swimming in the exposure chambers, whereas, sac-fry in the controls were attached to the wall of the exposure chambers and showed little activity. Then the activity of sac-fry under DCA exposures were reduced and attached to the wall of the exposure chambers. No apparent differences in movements were observed between control exposures and DCA exposures. However, during the last part of the experiment the sac-fry especially the sac-fry exposed to higher concentrations were found settled in the bottom of the exposure chambers whereas sac-fry in the control treatments were frequently moving, perhaps in search of food due to absorption of yolk.

Oxygen consumption under sublethal chlorpyrifos exposure concentrations is shown in Figure 5.12 and Figure 5.13. There were a significant differences ($F = 68.366$; $df = 3, 4$; $P < 0.05$) in oxygen consumption at 0 h (which corresponds 3 to 4 h exposure) compared with the control. At 48 h it was also significantly different ($F = 96.032$; $df = 3, 4$; $P < 0.05$) but instead of an increase in oxygen consumption compare with control it was significantly reduced at 0.5 and $1.0 \mu\text{g.l}^{-1}$. At 96 h oxygen consumption was insignificantly variable ($F = 1.333$; $df = 3, 4$; $P > 0.05$).

It was observed that larvae were less active under chlorpyrifos exposure during the later part of the experimental period but were more active during the early experimental period. Sometimes, increased activity was shown by erratic movements.

No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) of different sublethal responses on goldfish sac-fry physiological energetics under cadmium, DCA and chlorpyrifos exposure are presented in Table 5.4. The sensitivity of sublethal physiological responses (LOEC) of goldfish sac-fry in comparison to lethal response (LC50/EC50) for different test species for the equivalent exposure period is presented in Table 5.5.

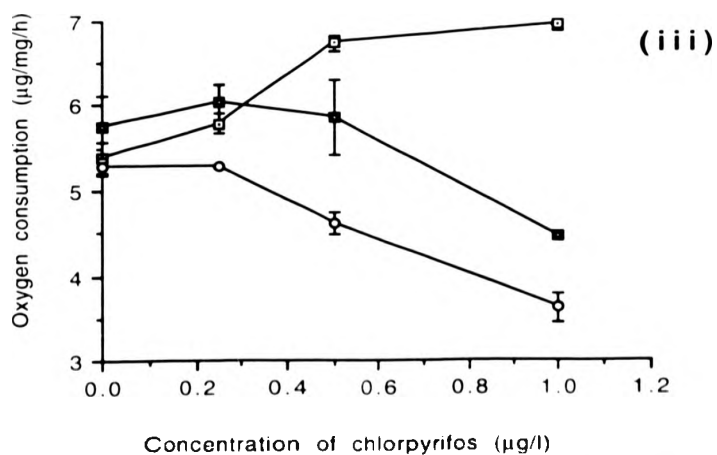
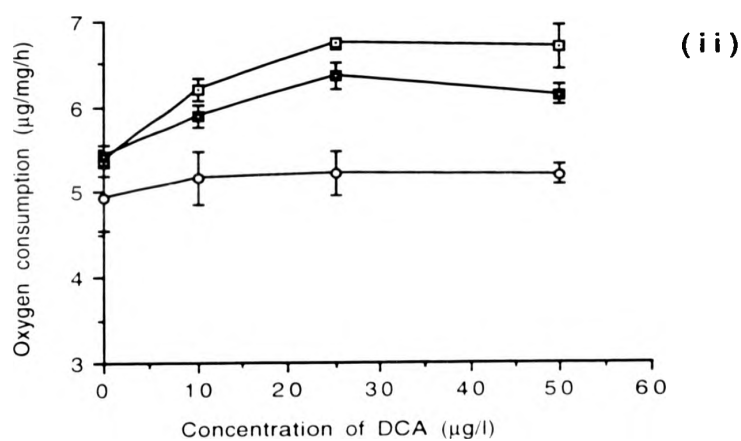
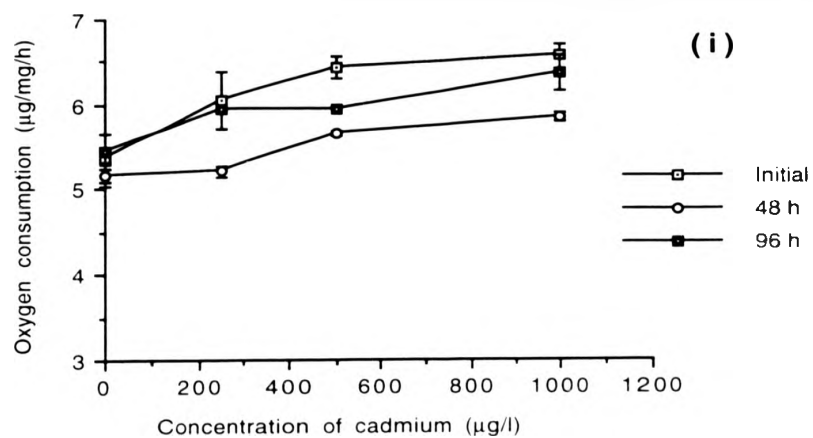


Figure 5.12 Oxygen consumption pattern of goldfish sac-fry under sublethal exposure of (i) cadmium, (ii) DCA and (iii) chlorpyrifos. Data are means \pm standard errors.

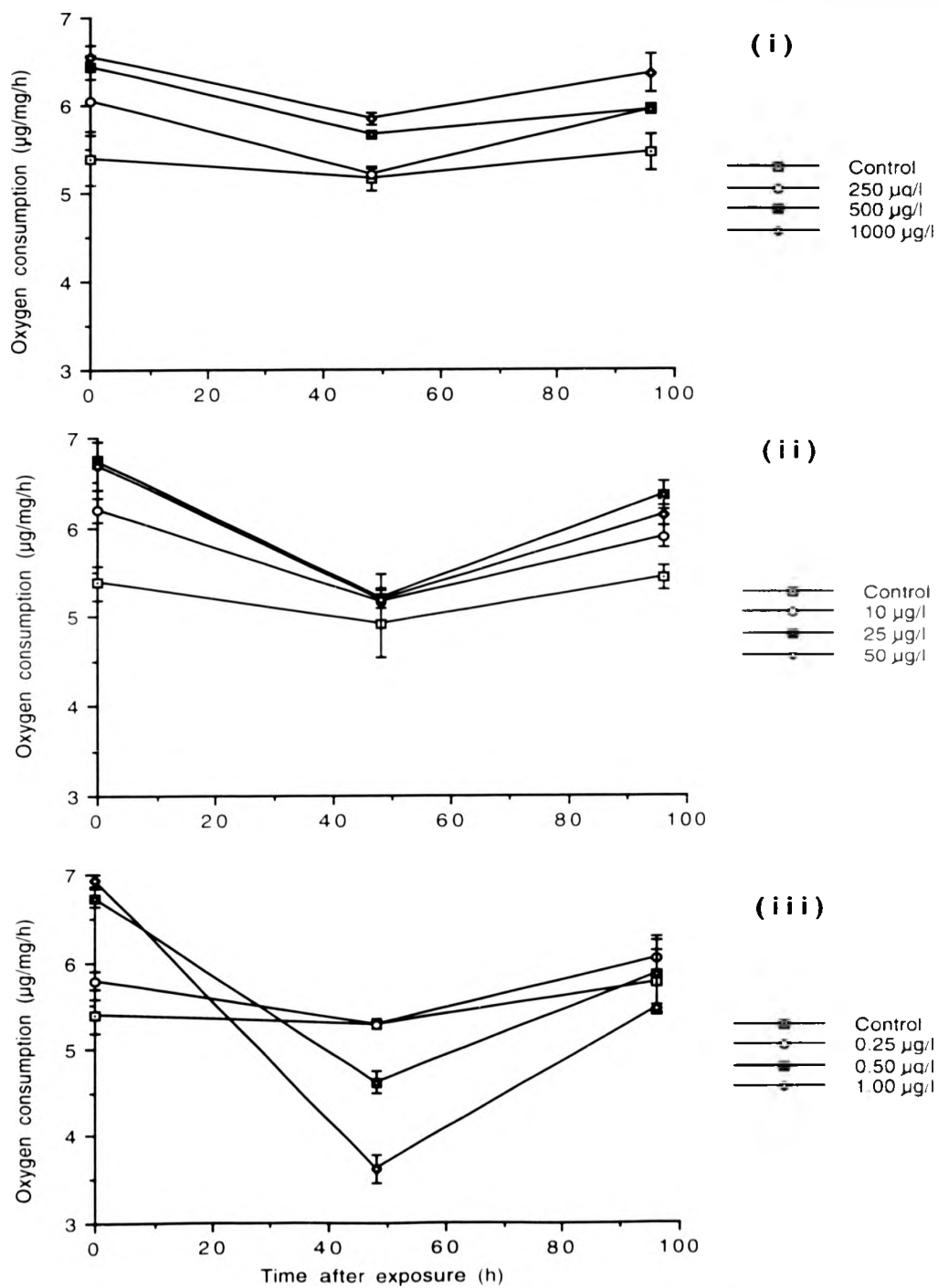


Figure 5.13 Effects of sublethal exposure concentrations of (i) cadmium, (ii) DCA and (iii) chlorpyrifos on the oxygen consumption pattern of goldfish sac-fry. Data are means \pm standard errors.

Table 5.4 Sublethal responses in goldfish sac-fry toxicity tests with cadmium, DCA and chlorpyrifos. NOEC and LOEC were calculated by ANOVA at a critical probability $P \leq 0.05$

Response	Toxicant ($\mu\text{g.l}^{-1}$)					
	Cadmium NOEC	LOEC	DCA NOEC	LOEC	Chlorpyrifos NOEC	LOEC
Body weight gain	500.0	1000.0	25.0	50.0	0.25	0.50
Yolk utilization	1000.0	>1000.0	50.0	100.0	0.25	0.50
Yolk utilization efficiency	500.0	1000.0	25.0	50.0	0.25	0.50
Oxygen consumption	250.0	500.0	<10.0	10.0	0.25	0.50
Ammonia excretion	250.0	500.0	25.0	50.0	0.25	0.50
Oxygen - Nitrogen ratio	500.0	1000.0	25.0	50.0	0.25	0.50

Table 5.5 The sensitivity of sublethal physiological responses (LOEC) of goldfish in comparison to lethal responses (LC50) for different test species for the equivalent exposure period (96 h).

Toxicant	LC50/EC50	Species	LC50 for goldfish sac-fry	LOEC in goldfish ELS	
				Concentration	Sensitive endpoint
Cd ²⁺	20.6 µg.l ⁻¹	<i>Oreochromis niloticus</i> *	3403.9 µg.l ⁻¹	500.0 µg.l ⁻¹	Ammonia excretion
	143.5 µg.l ⁻¹	<i>Tilapia zilli</i> *			
	4.1 µg.l ⁻¹	<i>Daphnia magna</i> **			
DCA	2700.0 µg.l ⁻¹	<i>Salso gardeneri</i> ***	2359.4 µg.l ⁻¹	25.0 µg.l ⁻¹	Oxygen consumption
	8500.0 µg.l ⁻¹	<i>Brachydanio rerio</i> ****			
	160.0 µg.l ⁻¹	<i>Daphnia magna</i> *****			
Chlorpyrifos	2.4 µg.l ⁻¹	<i>Lepomis macrochirus</i> *****	22.6 µg.l ⁻¹	0.5 µg.l ⁻¹	Yolk utilization, growth, ammonia excretion, O:N
	280.0 µg.l ⁻¹	<i>Ictalurus punctatus</i> *****			
	0.1 µg.l ⁻¹	<i>Daphnia magna</i> *****			

* from Siriwardena (1993)

** from Barber (1990)

*** from Crossland (1990)

**** from Nagel *et al.* (1991)

***** from Adema and Vink (1981)

***** from Johnson and Finley (1980)

***** from Soars (pers. comm.)

5.4 Discussion

The response due to sublethal stress of cadmium, DCA and chlorpyrifos on endogenous feeding goldfish sac-fry energetics was investigated in terms of NOEC and LOEC. In the present study the effect concentrations (see section 1.3) are not the same in different test endpoints studied (Table 5.4). Body weight gain, yolk utilization efficiency and oxygen to nitrogen ratio were found to be equally sensitive for all three toxicants tested. Oxygen consumption and ammonia excretion were found to be comparatively more sensitive than other endpoints in case of cadmium and DCA. In the case of chlorpyrifos all the endpoints were equally sensitive. These differences in the expression of NOEC and LOEC among the different toxicants may be due to the different mode of action (see section 1.5) of toxicants.

The body weight gain under cadmium, DCA and chlorpyrifos in terms of dry mass indicated a general reduction in energy utilization for growth from yolk. In yolk feeding sac-fry, the body weight gain is the integrated effect of the amount of yolk utilization and the efficiency by which the absorbed yolk energy is assimilated in the body. In the present study yolk utilization under DCA and chlorpyrifos exposure was reduced but under cadmium exposure, yolk utilization was found to be similar to control levels. Thus cadmium did not affect yolk utilization although body weight gain and yolk utilization efficiency was affected. Similar type of effect of sublethal cadmium exposure were found by Siriwardena (1993) in *Oreochromis niloticus* and *Tilapia zilli* sac-fry, whereas Rombough and Garside (1982) found a

reduction in growth and impaired yolk utilization. Siriwardena (1993) intended to justify this type of result with cadmium by stating that increased metabolic costs may contribute to differences in growth and yolk utilization efficiency without any significant effect in yolk utilization rate. Here that statement is justified in case of goldfish sac-fry by the balance (99.4%) of the energy equation (Table 5.3). Baird *et al.* (1990) found decreased growth in *Daphnia magna* under sublethal cadmium and DCA and suggested reduced feeding as a general response. In fish sac-fry yolk utilization is analogous to feeding (see section 5.1.7), but different results with cadmium might be due to the cadmium adsorption by the suspended daphnid-food (Taylor and Baird, pers. comm.). As sac-fry are yolk feeding, cadmium did not affect yolk-mass like suspended daphnid-food. Another reason for the no effect of cadmium in this case may be the use of EDTA in the dilution water, which is a metal chelating agent. The available cadmium was sufficient to cause increased activity resulting in the corresponding decrease in available energy for growth but not sufficient to disrupt the process of yolk absorption.

Yolk utilization efficiency was significantly reduced due to higher metabolic demand. This is supported by the increased oxygen consumption and increased ammonia excretion with increasing cadmium concentration. But this interpretation of reduced yolk utilization efficiency due to higher metabolic cost is complicated by concurrent activity change under toxicants exposure, which was experienced in the case of DCA and chlorpyrifos. In the case of DCA yolk utilization efficiency increased significantly at the highest concentration compared with intermediate concentrations. This increase in yolk utilization efficiency at higher concentrations

may be related to the concurrent reduction of activity (larval movements). There are reports that chemicals suppress activity making available more energy for growth and increasing yolk utilization efficiency. Leduc (1978), for example, found increased efficiency of yolk utilization in Atlantic salmon (*Salmo salar*) sac-fry exposed to hydrogen cyanide with increased concentration.

In the present study ammonia excretion increased with increasing concentrations of cadmium, DCA and chlorpyrifos. The ammonia excretion comes from two sources, endogenous and exogenous (Jobling, 1993). Endogenous nitrogen excretion results from tissue protein breakdown (protein degradation) and exogenous nitrogenous excretion results from direct deamination of amino acid during assimilation of ingested food. Under non-stressed conditions endogenous nitrogen excretion is generally quite low (Jobling, 1993) whereas under stressed conditions due to increased protein degradation, it is high (Barber, 1990; Hawkins *et al.*, 1987; Siriwardena, 1993). Therefore, ammonia excretion can be considered an index of environmental perturbation and can be used in environmental monitoring programme due to the reason that it showed consistent predictable relationship with the stress and it has the ecological significance regarding the transfer of effect from individual to population level. As the protein damage may induce direct mortality under long-term exposure and/or may reduce growth and/or may reduce fecundity and as a result may reduce the density of population and possibly may reduce the fitness of individuals comprising the population (Sibly and Calow, 1986).

In the present study oxygen to nitrogen ratio was found to decrease with the concentrations of cadmium, DCA, chlorpyrifos. O : N provides an index of relative utilization of protein in energy metabolism (Bayne *et al.*, 1976; Widdows, 1978). A value of 50 for O : N has been considered for the healthy *Mytilus edulis* utilizing relatively balanced proportion of nutrients and a value of 30 has been suggested for a stressful condition utilizing significantly higher proportions of protein (Widdows, 1985). Siriwardena found a value of below 30 for a tilapia species (*Tilapia zilli*). In the present study the O : N value found to be 42 for goldfish sac-fry under controlled conditions. However, the interpretation of O : N should be based on relative change rather than the absolute values as the O : N ratio is likely to vary between species and within species (Widdows, 1985). Further the measured value of O : N ratio may not translate into effect if oxygen consumption changes with the nitrogen excretion in the same direction *e.g.* if oxygen consumption increases with nitrogen excretion and if specially the oxygen consumed does not take part in energy production (respiratory uncoupling) as was found in case of DCA and chlorpyrifos in this study. Although it is one of the endpoints that showed consistency the underlying mechanisms remains complex due to the complexity of the components of oxygen consumption which are difficult to interpret.

It is well known that stress induces protein damage (Barber, 1990; Hawkins *et al.*, 1987), and that maintenance energy costs will increase due to the repair costs of protein damaged (Sibly and Calow, 1986). In this study, oxygen consumption under cadmium exposure increased with concentration but under DCA it was first

increased with concentration and then decreased. With chlorpyrifos oxygen consumption no apparent change was found. Siriwardena (1993) found an increase in oxygen consumption by tilapia sac-fry under cadmium exposure. With DCA and chlorpyrifos no such studies were found reported for comparison. But it has been reported that some stressors induce an increase in oxygen consumption rate (Barton and Barton, 1987; Siriwardena, 1993; Stearns, 1980), others cause decrease (Kamler *et al.*, 1974; Ullrich and Millemann, 1983), while others apparently have no effect (Geiger and Buikema, 1981). Similarly it has also been reported that under the effect of the same toxicant both decreased and increased respiration rate at different concentrations were observed (Kaniewska-prus, 1975).

Results of oxygen consumption study under cadmium, DCA and chlorpyrifos over the period of endogenous feeding sac-fry reflect a complicated pattern of oxygen consumption with time and intensity of stress. Response was found short lived under DCA and chlorpyrifos. The increased oxygen consumption were found during the whole period of experiment under cadmium exposure. But under DCA it showed a complex pattern, first of all it was increased then settled to controlled level after 48 h and again increased after 96 h. The increase in oxygen consumption may relate to higher maintenance costs and also to respiratory uncoupling. No comparable reports of oxygen consumption over the period of stress were found. Under chlorpyrifos there was no apparent change in oxygen consumption after 96 h but at the start it was found to increase with concentrations and after 48 h in 0.5 and 1.0 $\mu\text{g l}^{-1}$ decrease in oxygen consumption compare to control indicate exhaustion. The increase in oxygen consumption by the apparently exhausted larvae

after 96 h might be related to increased maintenance for detoxification or related to respiratory uncoupling. The increases in oxygen consumption during the starting hours under all three toxicants are likely due to the increase in swimming activity observed relative to the control, probably indicating an alarm reaction.

In the present study observed growth and scope for growth showed a high positive correlation and the balance of energy was found above 90% in all the cases except 50 $\mu\text{g l}^{-1}$ of DCA where it was found 88%. Logically the balance should be 100% but this occurred rarely due to experimental errors. Budgets were equated on the basis of the average value of body weight gain, yolk utilization and assumed energy value. Only ammonia was measured for excretion. However in energetics study some assumptions have to be made *e.g.* assessing the energy content of the same fish or group of fish both before and after the experiment is not possible, researchers must have to rely on sample of similar but another fish.

The LOEC for goldfish for physiological endpoints is sensitive by comparison with LC50 values (Table 5.5). The LOEC, for DCA, based on oxygen consumption, is the most sensitive even when compared with the lowest reported LC50 value for any species. The endpoints in terms of LOEC for chlorpyrifos are more sensitive than LC50 values for any freshwater fish. In the case of cadmium, sublethal physiological endpoints were found to be less sensitive which may be due to the confounding effects of hardness and EDTA. It can be assumed from the data that physiological endpoints are more sensitive for the equivalent exposure period of acute tests.

To summarise, there was no similarity in response with respect to oxygen consumption under sublethal cadmium, DCA and chlorpyrifos concentration. These differences may be due to their mode of action: as cadmium interferes with enzyme activity, DCA has nonspecific anaesthetizing effect and is believed to exert toxic effect after metabolic activation, whereas chlorpyrifos inhibits acetylcholinesterase. Although oxygen consumption was found to be a sensitive endpoint, the underlying mechanisms are so complex, they remain difficult to interpret.

The suggested model with the supply-side negative effect and the demand-side positive effect of the energy flow of goldfish sac-fry under sublethal exposures of toxicants was validated. Significant supply-side negative effect was found with DCA and chlorpyrifos but surprisingly no effect was found with cadmium. The demand-side positive effect was shown by DCA, chlorpyrifos and also by cadmium. Although supply-side effect was not generally shown but the combined effect of supply-side effect and of demand-side effect in the form of growth and yolk utilization efficiency were shown in all cases. Thus toxicant deprived sac-fry significantly either by the integration of decreasing ability of resource acquisition and increasing demand of metabolic costs or by increasing demand of metabolic costs only.

To reject null hypothesis H_{o2} and H_{o3} it was found that toxicants DCA and chlorpyrifos reduces yolk utilization and its efficiency under sublethal exposure. But H_{o1} can not be rejected directly as yolk utilization was not affected significantly by cadmium although in the highest sublethal concentration used yolk

utilization was reduced but not significantly. On the other hand yolk utilization efficiency was affected significantly.

To conclude, sublethal endpoints based on physiological energetics of goldfish sac-fry, such as yolk utilization rate, yolk utilization efficiency, O : N, ammonia excretion, oxygen consumption and scope for growth can be used for short-term fish early life stage toxicity tests. Oxygen consumption was the most sensitive parameter but was not equally consistent for all the toxicants. Ammonia excretion was equally or less sensitive to oxygen consumption. Ammonia excretion contributed a very little portion of the energy budget equation but showed a consistent increase with the concentration of toxicants. The underlying cause of this type of consistency may be increased protein turnover with increased toxicant concentration. In the natural environment, increased protein turnover as a result of low-level, long-term exposure may increase mortality and/or may reduce growth and/or may also reduce fecundity and/or may increase time in between life history stages and thus the effect in the individual may cause an effect at the population level with the reduction of individual fitness. Thus the endpoint 'ammonia excretion' of fish early life-stage toxicity test can be calibrated through interlaboratory studies in view of routine use for ecotoxicity studies.

CHAPTER 6

GENERAL DISCUSSION

During the process of development of toxicity tests with fish, early life stage toxicity tests emerged as substitutes for full life cycle tests to measure the 'no effect' concentration of toxicants. Recently, the period of exposure originally proposed of 1 to 2 month(s) (Macek and Sleight, 1977) for fish early life-stage toxicity tests has been reduced to 1 to 2 weeks (Dave *et al.*, 1987; Dave and Xiu, 1991; Norberg-King, 1989; OECD, 1992; USEPA, 1989) without sacrificing the sensitivity of the test. Fish toxicity tests, and in particular the fish early life-stage toxicity tests, are still at an early stage both in terms of the techniques used and in terms of development of our understanding of the physiological mechanisms behind toxicity test endpoints.

As in other aspects of toxicity testing, for pragmatic reasons only a few cultured and/or laboratory species have been used in fish early life-stage tests (Dave, 1993). Recently, efforts have been made to develop early life-stage studies with small warm water fish (*Brachydanio rerio* and *Pimephales promelas*) and two types of short term toxicity test have developed with similar sensitivities (van Leeuwen, *et al.*, 1990), (1) embryo-larval growth and survival test (Dave, 1985; Dave *et al.*, 1987; Dave and Xiu, 1991; OECD, 1992) and (2) short term larval survival and growth (integrated effect) test (Norberg-King and Mount, 1985; USEPA, 1989).

In this thesis development of a fish early life stage toxicity test with yolk feeding goldfish sac-fry has been investigated avoiding embryonic stages of the type (1) and also avoiding external feeding of the type (2) toxicity tests. Goldfish is widely distributed in different continents of the world, so any toxicity test developed on this fish can be used over a wide geographical range of Asia, Europe and America. It is a eurytolerant species in respect of temperature, salinity and handling stress, therefore, toxicity tests can be performed under a variety of laboratory conditions. It spawns under natural conditions during spring and summer. Its artificial spawning technique is also known (Sokolowska *et al.*, 1984). It can be matured under laboratory conditions all-year-round (Razani *et al.*, 1989), but the detailed techniques of procurement of eggs from a small brood-stock were not known. Here the rearing technique of goldfish for year-round supply of eggs with minimum facilities was investigated (chapter 3) and it was found that a simple recycle system is sufficient to generate an all-year-round routine supplies of experimental materials for fish ELS toxicity tests. For spawning, a simple technique has been developed by spiking ovarian fluid in the tank water as a stimulator to replace the more technically difficult and unreliable techniques of using changing water temperature and/or hormone injection. Handling stress may disrupt reproductive hormonal secretion of fish (Pickering, 1987; Safford and Thomas, 1987), reduce gamete quality and quantity (Pickering, 1993), and may even cause mortality as a result of handling and injection procedures (Thomas and Arnold, 1993). Therefore, the techniques developed here have an advantage over existing techniques of goldfish spawning by reducing handling stress yet obtaining gametes of consistent quality throughout the year. In this study a technique has also been developed to attach

eggs in a monolayer on the petridish to allow incubation prior to hatching. Between 5000 and 42,000 eggs can be collected from a single spawning depending on the size of the fish. Therefore, a test can be run with the material collected from the same individual fish, and even interlaboratory calibration studies can be performed with the material collected from a spawning by developing only incubation facilities in the individual laboratories and collecting egged Petri dishes in water from the laboratory with spawning facilities. The rate of early embryonic mortality was below 10% and the proportion of deformed larvae hatched out from eggs in the incubation chamber was also below 10%. Therefore, the use of goldfish early life-stages for toxicity testing will reduce the variability of results due to the poor quality of starting materials which is a major problem for ELS toxicity test results with zebrafish (Dave and Ziu, 1991). The technique developed for brood-fish rearing and induced spawning by exposing preovulatory fish in ovarian fluid ($3 \mu\text{g.l}^{-1}$ of tank water) facilitates the consistent spawning of goldfish at the rate of up to 25 spawnings per year.

Before carrying out toxicity tests using a new test organism and/or test exposure system it is important to assure the consistent quality of test organisms (Dave, 1993) and it is also important to demonstrate that the performance of the test organisms is consistent under controlled experimental conditions. This type of controlled testing was performed with eggs and sac-fry for the selection of proper experimental conditions such as selection of flow rate with minimum use of test media ($734 \text{ ml.day}^{-1}\text{.chamber}^{-1}$), selection of loading density ($30 \text{ eggs or sac-fry.chamber}^{-1}$) and selection of strategic age of sac-fry for the short term toxicity

studies. Growth of yolk feeding sac-fry was investigated and it was found that maximum dry body mass was attained before complete yolk absorption and before maximum wet body mass, indicating a reason for apparent disagreements in the growth response comparison made by different reviewers (Woltering, 1984; Kristensen, 1990) without proper standardization of data obtained by different authors under different conditions. The sensitivity of the growth response, especially for the embryo-larval test, depends on the precision of its determination and the way it is reported. At the termination of an experiment, fish larvae in some treatments may still be consuming yolk, while larvae in other treatments may have entered starvation. Therefore, the measured growth will invite two types of error, (1) weight measured in terms of wet body mass will over-estimate the growth for starved larvae due to high water content, and (2) weight measured as whole dry mass will over-estimate the growth of larvae still with yolk reserves. Here in this thesis weight of larvae was measured in terms of dry body weight to calculate growth to avoid the misleading pictures of growth due to the possible differential yolk remains and also differential water content of sac-fry under different degrees of stress.

For the understanding of general stress response in the environment the background laboratory study on general response of stress under different toxicants is important to provide a standard of severity and significance of a variety of environmental stress on ecosystems. Although in evaluating the effects of chemicals on organisms, populations or ecosystems the chronic effect concentration is more crucial and relevant value than LC50, the acute test has become the first step in

ecotoxicological investigations for estimation of toxicity of chemicals. In this study (chapter 4) acute toxicity tests were performed with a group of chemicals (cadmium, DCA and chlorpyrifos) varying in solubility, routes of exposure, and specific modes of action to find out the relative toxicity of chemicals on goldfish early life-stages. It was found that both cadmium and DCA are acutely toxic at the ppm level, whereas, chlorpyrifos was acutely toxic at ppb level. It was also found that chlorpyrifos exerts its acute toxicity more rapidly than cadmium and DCA, and this latter difference likely relates to differences in mode of action, as cadmium acts mainly by damaging skin, gills and disrupting enzyme function, DCA is a nonspecific anaesthetizing agent and is believed to exert toxic effect after metabolic activation (van Leeuwen *et al.*, 1990) while chlorpyrifos, like other organophosphates, exerts a toxic effect through the acetylcholinergic mode of action (Rodrigues *et al.*, 1983). Placing the toxicity data in context, it can be said in the same voice of Dave (1993) that the toxicity test results should be interpreted and compared with caution and should be standardized properly before comparing. The estimated high value of cadmium 96 h LC50 ($3404 \mu\text{g.l}^{-1}$) for goldfish sac-fry is apparently unusual but the reasoning behind this may be the high value of hardness of the dilution water used and the presence of EDTA in the dilution water. EDTA is a metal chelating agent (Hart and Scaife, 1977), which might have reduced the available free cadmium to exert toxic effect. Therefore, no valid comparison is possible for the test under variable conditions, and it is better to conduct tests with a chemical under different conditions in the laboratories, and quasi-field (enclosures of various kinds) and true field situations before prediction of effects at the ecosystem level. However, there are many constraints to make such studies

possible, such as expense to explore true effects of pollutants on ecosystem, and it is also impossible to collect all the relevant information due to the complexity of the ecosystem. Even if this could be accomplished, the generality of space and time would be small and the ecosystem or parts of an ecosystem would be exposed to toxicants (Perry and Troelstrup, 1988) that we also intend to protect. Therefore, some sort of compromise is necessary for the prediction of ecological effects of chemicals based on short-term laboratory test results.

With the demand for short-term, low cost and environmentally relevant simple test designs, ecotoxicologists have been using most sensitive life-stages of test organisms, in this study a possible short term fish early life-stage toxicity test with goldfish sac-fry was investigated by developing a new approach to embryo-larval test. In the standard embryo-larval toxicity test (*e.g.* Birge *et al.*, 1985; Dave *et al.*, 1987; OECD, 1992) embryonic period has been included by considering the great complexity of the developmental process, including a diverse array of receptor sites for toxic action that may vary qualitatively and quantitatively with the sequential stages in embryonic development so that chemicals of different modes of action and target sites could be able to express their toxic effects. However, during the period of embryogenesis the embryo remains protected by the chorion, reducing the permeability of toxic substances from the exposure media, resulting in a low sensitivity of the embryonic stage (Hemming and Buddington, 1988; von Westerhagen, 1988). To be sure how sensitive the goldfish embryos are, a comparative acute test with embryo and sac-fry were performed with DCA: which was the intermediately water-soluble of the toxicants used in this study with a

nonspecific mode of action, as a reference toxicant. It has been confirmed in this study that goldfish embryonic stage (96 h EC50 of 3332 $\mu\text{g.l}^{-1}$) is less sensitive than the sac-fry stage (96 h LC50 of 2359 $\mu\text{g.l}^{-1}$), which have been previously established in different species, *e.g.* rainbow trout with cadmium (van Leeuwen *et al.*, 1985) and tilapia with organophosphate insecticide (Paflitschek, 1979). It is clear that sac-fry are more sensitive. Therefore, in view of simplification of the fish early life stages toxicity test method, it was considered appropriate to exclude the egg stage with change in approach of toxicity test endpoint from growth to the physiological processes (yolk utilization, oxygen consumption, ammonia excretion) underlying growth.

Growth has become the choice of sublethal response in most of the fish early life-stage toxicity tests with the assumption that toxicants will negatively affect the ability of an organism to convert food into new tissue (Dave, 1986; Dave *et al.*, 1987). It is now believed that stressed organisms show impaired growth as a result of reduced ability to acquire energy (supply-side effects) and/or an increase in energy utilization (demand-side effects) (Baird *et al.*, 1990; Koehn and Bayne, 1989). In goldfish sac-fry, yolk utilization was considered analogous in energetics terms, to the dealing of ingested food of other organisms. Here (Chapter 5), short-term sublethal effects (NOEC, LOEC) with respect to physiological responses of yolk energy utilization by goldfish sac-fry were investigated with a view to validate a conceptual model indicated with the supply-side ("-ve") effect and the demand-side (" +ve") effect of the energy flow and also to determine a single fish early life-

stage toxicity test endpoint, removing the confusion of using multiple endpoints of survival, growth and hatchability. The parameters investigated were body weight gain (growth), yolk utilization, yolk utilization efficiency, oxygen consumption, ammonia excretion and oxygen to nitrogen ratio under cadmium, DCA and chlorpyrifos with a view to select a suitable test endpoint that can be consistent as a general response and at the same time sensitive to low level stress for ecotoxicity test.

The reduced body weight gain of goldfish sac-fry observed under cadmium, DCA and chlorpyrifos indicated a general reduction of available energy for growth. In sac-fry, growth is the integrated effect of the amount of yolk utilization and the yolk utilization efficiency. A general reduction in yolk utilization was expected, and although DCA and chlorpyrifos stress reduced yolk utilization while cadmium didn't, yolk utilization efficiency was reduced under sublethal exposures to all toxicants. Yolk utilization efficiency is the resultant effect of yolk energy absorbed and energy used for maintenance and activity. Thus chemical stress in general may or may not reduce yolk utilization but will always reduce yolk utilization efficiency by increasing metabolic cost. The decrease in yolk utilization efficiency was complicated by the concurrent change in activity with the increased maintenance cost. Significantly increased yolk utilization efficiency was found at higher DCA concentrations than at lower concentrations although both of these were significantly different from control.

The demand-side effect measured as oxygen consumption and ammonia excretion indicated an increase in oxygen consumption under cadmium exposure but in case of DCA exposure it was first increased then decreased with concentration and no apparent change was found under chlorpyrifos exposure *i.e.* oxygen consumption did not show any consistency under different chemicals, whereas ammonia excretion was found to consistently increase with the concentrations of all three chemicals used. This type of direct relationship may be due to the increased protein turnover with the increased concentrations of toxicants. Like ammonia excretion, O : N consistently reduced under all chemical stress which indicated increased protein utilization as respiratory substrate under stress.

Scope for growth showed a high positive correlation and the balance of energy was found above 90% except one case where it was found 88%. Although theoretically it should be 100% it has rarely been found due to the experimental errors. Scope for growth, which has been used in bivalves (Koehn and Bayne, 1989), can be addressed as a measure of test endpoint in physiological energetics of goldfish sac-fry under stress as it has been found to be predictive at concentrations below acute level and it expresses the possible future ecological effects by reducing survival and/or growth and/or reproduction, which may reduce the population density as a result and ultimately may reduce the fitness of the individuals comprising the population (Sibly and Calow, 1986).

As the oxygen consumption increased with cadmium concentration, first increased then decreased under DCA and showed no apparent change under chlorpyrifos

exposure, this may reflect the response at a particular period of time and stress intensity under GAS. To incorporate those effects into the general response, oxygen consumption study over the time of yolk feeding sac-fry at different intensities of toxicants indicated an increase in oxygen consumption with increased activity and also with decreased activity. The high oxygen consumption during low activity may be related to respiratory uncoupling. Although oxygen consumption exhibited the different phases of GAS it was complicated by the specific nature of the individual toxicant. Only the alarm response was found measurably and significantly increased oxygen consumption under all three chemicals. Oxygen consumption was found significantly increased at very low levels of toxicants compared with other endpoints.

The short term sublethal physiological test endpoints measured with cadmium, DCA and chlorpyrifos indicated that body weight gain, yolk utilization efficiency and O : N were equally sensitive endpoints. Yolk utilization was also sensitive at the same level except for cadmium, where it was not significantly different from the control. Ammonia excretion and oxygen consumption were found to be more sensitive than other endpoints with cadmium and DCA, whereas, oxygen consumption was the most sensitive with DCA. With chlorpyrifos all endpoints were equally sensitive.

Oxygen consumption as a major component of sac-fry energy budget and sensitive to stress at low level, can be considered as a toxicity test endpoint but the underlying mechanisms of oxygen consumption are so complex it is very difficult

to interpret. On the other hand ammonia excretion contributed only a very small portion of sac-fry energetics but was found to increase consistently with increases in concentration of all chemicals tested. The underlying cause of this is the increased degradation of nonspecific protein with stress (Barber *et al.*, 1990) and this increase in protein degradation reduces survival and/or growth and/or reproduction. For any factor that reduces survival and/or growth and/or fecundity at the individual level, the effect at individual can be transmitted into an effect at the population level by reducing density which may have some effect on community structure and function (Bradshaw and Hardwick, 1989; Calow, 1989; Koehn and Bayne, 1989; Sibly and Calow, 1986). Thus use of ammonia excretion of a short-term fish early life-stage toxicity test endpoint can be calibrated through interlaboratory studies and recommended for regulatory ecotoxicity test.

REFERENCES

- Adams, S.M., McLean, R.B. and Parotta, J.A., 1982. Energy partitioning in largemouth bass under condition of seasonally functioning prey availability. *Trans. Am. Fish. Soc.*, **111**: 549-558.
- Adema, Ms.D.M.M. and Vink, Jr.G.J., 1981. A comparative study of the toxicity of 1,1,2-trichloroethane, dieldrin, pentachlorophenol and 3,4-dichloroaniline for marine and freshwater organisms. *Chemosphere*, **6**: 533-554.
- Agarwal, Y.L., Raj, K.P.S., Panchal, R., 1978. Metal contents in the drinking water in cambay. *Internat. J. Environ. Stud.*, **12**: 250-259
- Alabaster, J.S. and Lloyd, R., 1982. *Water quality criteria for freshwater fish. 2nd edition.* 351 pp. Butterworth Scientific, London.
- Alderdice, D.F., 1985. A pragmatic view of early life history studies of fishes. *Trans. Am. Fish. Soc.*, **114**: 445-451.
- Alderdice, D.F., Rao, T.R. and Rosenthal, H., 1979. Osmotic response of eggs and larvae of the Pacific herring to salinity and cadmium. *Helgol. Wiss. Meeresunters.*, **32**: 508-538.
- Allen, S.E., Grimshaw, H.M., Parkinson, J.A. and Quarmby, C., 1974. *Chemical analysis of ecological materials.* 565 pp. Blackwell Scientific Publications, Oxford, London.
- Anonymous, 1991. *Sources of variation in ecotoxicological tests with Daphnia magna.* Report from the second workshop held at the University of Sheffield, UK, 11-12 April, 1991.
- APHA, 1989. *Standard methods for the examination of water and waste water. 17th edition.* American Public Health Association, Washington DC 20005.
- ASTM, 1980. *Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates and amphibians.* 25 pp. E-729-80. American Standard for Testing and Materials. Philadelphia, Pa.
- Atkinson, D.E., 1977. *Cellular energy metabolism and its regulation.* 293 pp. Academic press, New York.

- Baird, D.J., 1994. Pest control in tropical aquaculture: an ecological hazard assessment of natural and synthetic control agents. *Mitt. Internat. Verein. Limnol.*, **24**: 285-292.
- Baird, D.J., Barber, I. and Calow, P., 1990. Clonal variation in general response of *Daphnia magna* Straus to toxic stress. I. Chronic life-history effects. *Func. Ecol.*, **4**: 399-407.
- Baird, D.J., Barber, I., Bradeley, M., Soares, A.M.V.M., and Calow, P., 1991. A comparative study of genotype sensitivity to acute toxic stress using clones of *Daphnia magna* Straus. *Ecotoxicol. Environ. Safety*, **21**: 257-265
- Barber, I., 1990. *Clonal variation in general responses of Daphnia magna Straus (Crustacea: Cladocera) to toxic stress: physiological and life history effects*. Ph.D. thesis. 233 pp. Department of Animal and Plant Sciences, University of Sheffield, UK.
- Barber, I., Baird, D.J., and Calow, P., 1990. Clonal variation in general response of *Daphnia magna* Straus to toxic stress. II. Chronic life-history effects. *Func. Ecol.*, **4**: 409-414.
- Barton, M. and Barton, A.C., 1987. Effects of salinity on oxygen consumption of *Cyprinodon variegatus*. *Copeia*, **1**: 230-232.
- Bayne, B.L., 1975. Aspects of physiological condition in *Mytilus edulis* (L.) with special reference to the effects of oxygen tension and salinity. In: H. Barnes (Editor), *Proceedings of the 9th european marine biology symposium*. pp. 213-238. Aberdeen University Press, Aberdeen.
- Bayne, B.L. and Thompson, R.J., 1970. Some physiological consequences of keeping *Mytilus edulis* in the laboratory. *Helgol. Wiss. Meeresunters.*, **20**: 526-552.
- Bayne, B.L. and Widdows, J., 1978. The physiological ecology of two populations of *Mytilus edulis* L. *Oecologia Berl.*, **37**: 137-162.
- Bayne, B.L., Widdows, J. and Thompson, R.J., 1976. Physiological integrations. In: B.L. Bayne (Editor), *Marine mussels: their physiology and ecology*. pp. 261-191. Cambridge University Press, Cambridge.
- Bayne, B.L., Clarke, K.R., Dixon, D.R., Ivanovici, A., Livingstone, D.R., Lowe, D.M., Moore, M.N., Stebbing, A.R.D. and Widdow, J. (Editors), 1985. *The effects of stress and pollution on marine animals*. 384 pp. Praeger Scientific, New York.

- Benoit, D.A., Puglisi, F.A. and Olson, D.L., 1982. A fathead minnow, *Pimephales promelas* early life stage toxicity test method evaluation and exposure to four organic chemicals. *Environ. Pollut.*, **28**(A): 189-197.
- Berg, G.L. (Editor), 1983. *Farm chemicals handbook*. Meister, Willoughby, Ohio.
- Billard, R., Gatty, J.L., Hollebeca, M.G., Marcel, J. and Saadet, A., 1986. Biology of gametes, eggs and embryos. In: Billard, R. and Marcel, J. (Editors), *Aquaculture of cyprinids*. pp. 151-161. Institut National De la Recherche Agronomique, Paris.
- Birge, W.J., Black, J.A. and Westerman, A.G., 1985. Short-term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and complex effluents. *Environ. Toxicol. Chem.*, **4**: 807-821.
- Blaxter, J.H.S., 1969. Development: Eggs and larvae. In: W.S. Haor, and D.J. Randal (Editors), *Fish physiology. Vol. III*. pp. 177-252. Academic Press, London.
- Blaxter, J.H.S., 1988. Patterns and variety in development. In: W.S. Haor, and D.J. Randal, (Editors), *Fish physiology. Vol. XI. The physiology of developing fish. Part A. Eggs and larvae*. pp. 1-58. Academic Press, San Diego.
- Blaxter, J.H.S. and Hempel, G., 1966. Utilization of yolk by herring larvae (*Clupea harengus* L.) *J. Mar. Biol. Assoc. U.K.*, **46**: 219-234.
- Blaylock, B.G. and Frank, M.L., 1979. A comparison of the toxicity of nickel to the developing eggs and larvae of carp (*Cyprinus carpio*). *Bull. Environ. Contam. Toxicol.*, **21**: 604-611.
- Boisclair, D. and Sirois, P., 1993. Testing assumptions of fish energetics model by direct estimation of growth, consumption and activity rates. *Trans. Am. Fish. Soc.*, **122**: 784-796.
- Borgmann, V. and Whittle, D.M., 1992. Bioenergetics and PCB, DDE and mercury dynamics in lake ontario lake trout (*Salvelinus namaycush*): a model based on surveillance data. *Can. J. Fish. Aquat. Sci.*, **49**: 1086-1096.
- Boudou, A. and Ribeyre, F. (Editors), 1989. *Aquatic ecotoxicology: fundamental concepts and methodologies. Vol. II*. 272 pp. CRC Press Inc. Florida.

- Bourdeau, P., 1984. Environmental research programme of the european community. *In: G. Persoone, E. Jaspers and C. Claus (Editors), Marine pollution aspects.* pp. 3-12. State University Ghent and Institute of Marine Scientific Research, Bredene, Belgium.
- Bradshaw, A.D. and Hardwick, K., 1989. Evolution and stress - genotypic and phenotypic components. *In: P. Calow and R.J. Berry, (Editors), Evolution ecology and environmental stress.* pp. 137-155. Academic press, London.
- Brafield, A.E., 1985. Laboratory studies of energy budgets. *In: P. Tytler and P. Calow (Editors), Fish energetics: new perspectives.* pp. 257-281. Croom Helm, London.
- Brandt, S.B. and Hartman, K.J., 1993. Innovative approaches with bioenergetics models: future applications to fish ecology and management. *Trans. Am. Fish. Soc.*, **122**: 731-735.
- Braum, E., 1967. The survival of fish larvae with reference to their feeding behaviour and the food supply. *In: S.D. Gerking (Editor), The biological basis of freshwater fish production.* pp. 113-131. Blackwell Scientific Publications, Oxford.
- Brett, J.R., 1958. Implications and assessments of environmental stress. *In: P.A. Larkin (Editor), The investigation of fish power problems. H.R. MacMillan lectures in fisheries.* pp. 69-83. Univ. British Columbia, Vancouver, Canada.
- Brett, J.R. and Groves, T.D.D., 1979. Physiological energetics. *In: W.S. Hoar, D.J. Randal and J.R. Brett (Editors), Fish physiology: bioenergetics and growth. Vol VIII.* pp. 279-352. Academic press, New York.
- Brody, S., 1945. *Bioenergetics and growth.* 1023 pp. Reinhold Pub. Corp., New York.
- Burnstock, G., 1958. Saline for fresh water fish. *J. Physiol.*, **141**: 35-44.
- Butler, G.C., 1984. Developments of ecotoxicology. *Ecol.Bull.*, **36**: 9-12.
- Caduto, M. J., 1990. *Pond and brook: a guide to nature in freshwater environments.* 276 pp. University Press of New England, London.
- Cairns, J.Jr. 1989. *Functional testing of aquatic biota for estimating hazards of chemicals. STP 988.* 242 pp. ASTM, Philadelphia, Pa.

- Call, D.J., Poirier, S.H., Knuth, M.L., Harting, S.L. and Lindberg, C.A., 1987. Toxicity of 3,4-dichloroaniline to fathead minnows, *Pimephales promelas*, in acute and early life stage exposures. *Bull. Environ. Contam. Toxicol.*, **38**: 352-358.
- Call, D.J., Brooke, L.T., Kent, R.J., Knuth, M.L., Anderson, C. and Moriarity, C., 1983. Toxicity, bioconcentration, and metabolism of the herbicide propanil (3,4-dichloropropioanilide) in freshwater fish. *Arch. Environ. Contam. Toxicol.*, **12**: 175-182.
- Calow, P., 1989. Proximate and ultimate response to stress in biological systems. In: P. Calow and R.J. Berry (Editors), *Evolution ecology and environmental stress*. pp. 173-181. Academic Press, London.
- Calow, P. and Townsend C.R., 1981. Energetics, ecology and evolution. In: C.R. Townsend and P. Calow (Editors), *Physiological ecology: an evolutionary approach to resource use*. pp. 1-19. Blackwell Scientific Publications, London.
- Cardwell, J.R., Dulka, J.G. and Stacey, N.E., 1991. Species specificity of olfactory responsiveness to potential sex pheromones. In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), *Proceedings of the fourth international symposium on the reproductive physiology of fish*. p. 201. University of East Anglia, Norwich, UK. Published by Fish-Symp. 91, Sheffield.
- Carrol, J.J., Stephen, J.E. and Watter, S.D., 1979. Influence of hardness constituents on the toxicity of cadmium to brook trout (*Salvelinus fontinalis*). *Bull. Environ. Contam. Toxicol.*, **22**: 575-581.
- Cathcart, E.P., 1953. The early development of the science of nutrition. In: G.F. Bourne and G.W. Kidder (Editors), *Biochemistry and physiology of Nutrition. Vol. I*. pp. 1-16. Academic Press, New York.
- Chow, A.Y.K. and Murphy, S.D., 1975. Production of a methaemoglobin forming metabolite of 3,4-dichloroaniline by liver *in vitro*. *Bull. Environ. Contam. Toxicol.*, **13**: 9-13.
- Clark, J.R., Patrick, J.M.Jr., Diddaugh, D.P. and Moore, J.C., 1985. Relative sensitivity of six estuarine fishes to carbophenothion, chlorpyrifos, and fenvalerate. *Ecotoxicol. Environ. Safety*, **10**: 382-390.
- Conover, R.J., 1978. Transformation of organic matter. In: Kinne, O. (Editor), *Marine ecology. Vol. IV. Dynamics*. pp. 221-500. Wiley-Interscience, Chichester.

- Cossin, A.R., Fiedlander, M.J. and Prosser, C.L., 1977. Correlation between behavioral temperature adaptations of goldfish and the viscosity and fatty acid composition of their synaptic membranes. *J. Comp. Physiol.*, **120**: 109-121.
- Coutant, C.C. and Edsall, T.A. (Editors), 1993. Innovative approaches with bioenergetics model. *Trans. Am. Fish. Soc.*, **122**: 731-1030.
- Crawford, R.B. and Guarino, A.M., 1985. Effects of environmental toxicants on development of a teleost embryo. *J. Environ. Pathol. Toxicol.*, **6**: 185-194.
- Crossland, N.O., 1990. A review of the fate and toxicity of 3,4-dichloroaniline in aquatic environments. *Chemosphere*, **21**: 1489-1497.
- Crossland, N.O. and Hillaby, J.M., 1985. Fate and effects of 3,4-dichloroaniline in the laboratory and in outdoor ponds: II. Chronic toxicity to *Daphnia* spp. and other invertebrates. *Environ. Toxicol. Chem.*, **4**: 489-499.
- Dabrwski, K., 1989. Formulation of a bioenergetic model for coregonine early life history. *Trans. Am. Fish. Soc.*, **118**: 138-150.
- Das, M., Islam, M.A. and Mughal, G.U., 1992. Induced breeding and fry rearing of catfish, *Clarias batrachus* (Linn.). *Bangladesh J. Zool.*, **20**(1): 87-95
- Dave, G., 1985. The influence of pH on the toxicity of aluminium, cadmium and iron to eggs and larvae of the zebrafish, *Brachydanio rerio*. *Ecotoxicol. Environ. Safety*, **10**: 253-267.
- Dave, G., 1986. Toxicity testing procedure. In: S. Nilsson and S. Holmgren (Editors), *Fish physiology: recent advances*. pp. 170-195. Croom, Helm, London.
- Dave, G., 1993. Replicability, repeatability and reproducibility of embryo-larval toxicity tests with fish: A critical review. In: A. Soares and P. Calow (Editors), *Progress in standardization of aquatic toxicity tests*. pp. 129-157. Lewis Publishers, CRC Press Inc., Florida.
- Dave, G. and Xiu, R., 1991. Toxicity of mercury, copper, nickel, lead and cobalt to embryos and larvae of zebrafish, *Brachydanio rerio*. *Arch. Environ. Contam. Toxicol.*, **21**: 126-134.

- Dave, G., Damgaard, B., Grande, M., Martelin, J.E., Rosander, B. and Victor, T., 1987. Ring test of an embryo-larval toxicity test with zebrafish (*Brachydanio rerio*) using chromium and zinc as toxicants. *Environ. Toxicol. Chem.*, **6**: 61-71.
- DeGraeve, G.M., Cooney, J.D., McIntyre, D.O., Pollock, T.L., Reichenbach, N.G., Dean, J.H., and Marcus, M.D., 1991. Variability in the performance of the seven day fathead minnow (*Pimephales promelas*) larval survival and growth test. An intra and interlaboratory study. *Environ. Toxicol. Chem.*, **10**: 1189-1203.
- Dethlefsen, V., 1977. The influence of DDT and DDE on the embryogenesis and the mortality of larvae of cod (*Gadus morhua* L.). *Ber. Dtsch. Wiss. Komm. Meeresforsch.*, **25**: 135-148.
- Deuel, L.E., Brown, K.W.Jr., Turner, F.C., Westfall, D.G. and Price, J.D., 1977. Persistence of propanil, DCA, and TCAB in soil and water under flooded rice culture. *J. Environ. Qual.*, **6**: 127-132.
- DiMuccio, A., Camoni, I. and Dommarco, R., 1984. 3,3',4,4'-tetrachloroazobenzene and 3,3',4,4'-tetrachloroazoxybenzene in technical grade herbicides: propanil, diuron, linuron, and neburon. *Ecotoxicol. Environ. Safety*, **8**: 511-515.
- Donaldson, E.M. and Hunter, G.A., 1983. Induced final maturation, ovulation and spermiation in cultured fish. *In*: W.S. Hoar, D.G. Randal and E.M. Donaldson (Editors), *Fish physiology. Vol. IX B.* pp. 351-403. Academic Press, New York.
- Duffus, J.H., 1983. *Environmental toxicology*. 164 pp. Edward Arnold (Pub.) Ltd., London.
- Eaton, J.G., 1970. Chronic malathion toxicity of the blue gill (*Lepomis macrochirus* Rafinesque). *Water Res.*, **4**: 673-684.
- Eaton, J.G., 1973. Chronic toxicity of copper, cadmium, and zinc mixture to the fathead minnow (*Pimephales promelas*). *Water Res.*, **7**: 1723-1736.
- Eaton, J.G., 1974. Chronic cadmium toxicity of the blue gill (*Lepomis macrochirus* Rafinesque). *Trans. Am. Fish. Soc.*, **103**: 729-735.
- Eaton, J.G., McKim, J.M. and Holcombe, G.W., 1978. Metal toxicity to embryos and larvae of seven freshwater fish species. I: Cadmium. *Bull. Environ. Contam. Toxicol.*, **19**: 95-103.

- Ehrlich, K.F. and Muszynski, G., 1982. Effects of temperature on interactions of physiological and behavioral capacities of larval california grunion: adaptation to the planktonic environment. *J. Exp. Mar. Biol. Ecol.*, **60**: 223-244.
- EIFAC, 1975. *Working party on toxicity testing procedures. Report on fish toxicity testing procedures.* 25 pp. EIFAC Tech. Paper, **24**. EIFAC.
- El-Dib, M.A., Aly, O.A., 1976. Persistence of some phenylamide pesticides in the aquatic environment III. Biological degradation. *Water Res.*, **10**: 1055-1059.
- Eldridge, M.B., Whipple, J.A. and Bowers, M.J., 1982. Bioenergetics and growth of striped bass, *Morone saxatilis*, embryos and larvae. *Fish. Bull.*, **80**: 461-474.
- El-Fiky, N., Hinterleitner, S. and Wieser, W., 1987. Differentiation of swimming muscle and gills and development of aerobic power in the larvae of cyprinid fish (Pisces, Teleostei). *Zoomorphology (Berlin)*, **107**: 126-132.
- Ellertsen, B., Moksness, E., Solemdal, P., Stromme, T., Tilseth, S. Westgard, T. and Oiestad, V., 1980. Some biological aspects of cod larvae (*Gadus morhua* L.). *Fiskeridir. Skr. Ser. Havunders.*, **17**: 29-47.
- Elliott, J.M., 1979. Energetics of freshwater teleosts. *Symp. Zool. Soc. London*, **44**: 29-61.
- Elliott, J.M. and Davison, W., 1975. Energy equivalents of oxygen consumption in animal energetics. *Oecologia*, **19**: 195-201.
- Escaffre, A.M. and Bergot, P., 1984. Utilization of the yolk in rainbow trout alevins (*Salmo gairdneri* Richardson): effects of egg size. *Reprod. Nutr. Dev.*, **24**: 449-460.
- Esch, G.W. and Hazen, T.C., 1978. Thermal ecology and stress: a case study for red-sore disease in large-mouth bass. In: J.H. Thorp and J.W. Gibbons (Editors), *Energy and environmental stress in aquatic ecosystems.* pp. 331-363. US DOE. CONF-771114.
- Finney, D.J., 1971. *Probit analysis. 3rd edn.* 333 pp. Cambridge University Press, Cambridge.
- Forbes, V.E. and Forbes, T.L., 1994. *Ecotoxicology in theory and practice.* 247 pp. Chapman & Hall, London.

- Forlin, L., Haux, C., Anderson, P.E. and Larsson, A., 1986. Physiological methods in fish toxicology: laboratory and field studies. In: S. Nilsson and S. Holmgren (Editors), *Fish physiology: recent advances*. pp. 158-169. Croom Helm, London.
- Fukien-Kiangsu-Chekiang-Shanghai Cooperative Group for artificial production of freshwater economic fishes, 1977. A further investigation on the stimulatory effect of a synthetic analogue of hypothalamic luteinizing hormones releasing hormone (LRH-a) on spawning in "domestic" fishes. *Acta Biochim. Biophys. Sinica*, **9**: 15-24.
- Games, L.M. and Hites, R.H., 1977. Composition, treatment efficiency and environmental significance of dye manufacturing plant effluents. *Anal. Chem.*, **49**: 1433-1440.
- Garlick, P.J., Milward, D.J., James, W.P.T. and Waterlow, J.C., 1975. The effect of protein deprivation and starvation on the rate of protein synthesis in tissues of rat. *Acta. Biochem. Biophys.*, **414**: 71-84.
- Geiger, J.G. and Buikema, A.L., 1981. Oxygen consumption and filtering rate of *Daphnia pulex* after exposure to water soluble fractions of naphthalene, phenanthrene, no.2 fuel oil, and coal tar creosote. *Bull. Environ. Contam. Toxicol.*, **27**: 783-789.
- Giesy, J.P. and Graney, R.L., 1989. Recent development in and inter comparison of acute and chronic bioassays and bioindicators. *Hydrobiologia*, **188/189**: 21-60.
- Gillet, C., Breton, B. and Billard, R., 1978. Seasonal effects of exposure to temperature and photoperiod regimes on gonad growth and plasma gonadotropin in goldfish (*Carassius auratus*). *Ann. Biol. Anim. Biochim. Biophys.*, **18**: 1045-1049.
- Golterman, H.L., Clymo, R.S. and Ohnstad, M.A.M., 1978. *Methods for the physical and chemical analysis of fresh waters*. 213 pp. Blackwell Scientific Publications, Oxford.
- Goodman, L.R., Hansen, D.J., Middaugh, D.P., Cripe, G.M. and Moore, J.C., 1985. Methods for early life stage toxicity test using three atherinid fishes and results with chlorpyrifos. *Aquatic toxicology and hazard assessment*. pp. 145-154. ASTM STP 854. ASTM, Philadelphia.
- Grime, J.P., 1979. *Plant strategies and vegetation processes*. 222 pp. John Wiley, Chichester.

- Grime, J.P., 1989. The stress debate: symptom of impending synthesis? In: P. Calow and R.J. Berry (Editors), *Evolution ecology and environmental stress*. pp. 3-7. Academic press, London.
- Grzimek, B. and Ladiges, W. (Editors), 1973. *Grzimek's animal life encyclopedia: the goldfish*. pp. 346-350. Van Nostrand Reinhold Company.
- Halder, S., Sen. S., Bhattacharya, S., Ray, A.K., Ghosh, A. and Jhingran, V.G., 1991. Induced spawning of Indian major carps and maturation of a perch and a catfish by murrel gonadotropin and calcium. *Aquaculture*, **97**: 373-383.
- Hamor, T. and Garside, E.T., 1977. Size relations and yolk utilization in embryonated ova and alevins of Atlantic salmon (*Salmo salar* L.) in various combination of temperature and dissolved oxygen. *Can. J. Zool.*, **55**: 1892-1898.
- Hansen, D.J. and Parrish, P.R., 1977. Suitability of sheepshead minnows (*Cyprinodon variegatus*) for life cycle toxicity tests. In: F.L. Mayer, J.L. Hamelink (Editors), *Aquatic toxicology and hazard evaluation*. pp. 117-126. ASTM STP 634. ASTM, Philadelphia.
- Hart, B.A. and Scaife, B.D., 1977. Toxicity and bioaccumulation of cadmium in *Chlorella pyrenoidosa*. *Environ. Res.*, **14**: 401-413.
- Haux, C., 1985. Ion balance and metabolism during cadmium exposure. In: C. Haux (Editor), *Aspects of ion balance and metabolism in teleost fish in relation to vitellogenin synthesis and during cadmium exposure*. pp. 11-29. Dept. Zoophysiology. Univ. Goteborg. Sweden.
- Haux, C. and Larsson, A., 1984. Long-term sublethal physiological effects on rainbow trout, *Salmo gairdneri*, during exposure to cadmium and after subsequent recovery. *Aquat. Toxicol.*, **5**: 129-142.
- Hawkins, A.J.S., 1991. Protein turnover: a functional appraisal. *Func. Ecol.*, **5**: 222-233.
- Hawkins, A.J.S., Wilson, I.A. and Bayne, B.L., 1987. Thermal responses reflect protein turnover in *Mytilus edulis*. *Func. Ecol.*, **1**: 339-351.
- Heming, T.A., and Buddington, R.K., 1988. Yolk absorption in embryonic and larval fishes. In: W.S. Haor and D.J. Randal (Editors), *Fish physiology. Vol. XI. The physiology of developing fish. Part A. Eggs and larvae*. pp. 408-446. Academic Press, San Diego.

- Henderson, V., Fisher, J.W., D'Allessandris, R. and Livingston, J.M., 1983. Effect of hydrazine on functional morphology of rainbow trout embryos and larvae. *Trans. Am. Fish. Soc.*, **112**: 100-104.
- Hervey, G.F. and Hems, J., 1968. *The goldfish*. 232 pp. The Batchworth Press Ltd., London.
- Hinterleitner, S., Platzer, V. and Wieser, W., 1987. Development of the activities of oxidative, glycolytic and muscle enzymes during early larval life in three families of freshwater fish. *J. Fish Biol.*, **30**: 315-326.
- Hochachka, P.W. and Somero, G.N., 1984. *Biochemical adaptation*. Princeton University Press, Princeton.
- Horvath, L., Horvath, G.T. and Seagrave, G., 1992. *Carp and pond fish culture*. 158 pp. Fishing News Books, Oxford.
- Hulata, G., Moav, R. and Wohlfarth, G., 1976. The effects of maternal age, relative hatching time and density of stocking on growth rate of fry in the european and chinese races of common carp. *J. Fish Biol.*, **19**: 499-513.
- Hunter, J.R., 1972. Swimming and feeding behaviour of larval anchovy *Engraulis mordax*. *Fish. Bull.*, **70**: 821-838.
- Hutson, D.H. and Roberts, T.R. (Editors), 1987. *Progress in pesticide biochemistry and toxicology*. Vol. 6. *Herbicides*. John Wiley & Sons, New York.
- Hynes, H.B.N., 1960. *The biology of polluted waters*. 202 pp. Liverpool University Press.
- Idler, D.R., Crimand, L.W. and Sumpter J.M. (Editors), 1987. *Proceedings of the third international symposium on the reproductive physiology of fish*. 321 pp. Memorial University of Newfoundland Press, St John's.
- ISO, 1984. *Water quality: determination of the acute lethal toxicity of substances to a freshwater fish 'Brachydanio rerio' Hamilton Buchanan (Teleostei, Cyprinidae)*. Part 1: *Static method, ISO 7346/1 - 1984(E)*, Part 2: *Semistatic method, ISO 7346/2 - 1984(E)*, Part 3: *Flowthrough method, ISO 7346/3 - 1984(E)*. ISO.
- Ivanovici, A.M. and Wiebe, W.J., 1981. For working definition of "stress": a review and critique. In: G.W. Barrett and R. Rosenberg (Editors), *Stress effects on natural ecosystems*. pp. 13-27. John Wiley & Sons, New York.

- Jarvinen, A.W. and Tanner, D.K., 1982. Toxicity of selected controlled release and corresponding unformulated technical grade pesticides to the fathead minnow *Pimephales promelas*. *Environ. Pollut.*, **27**: 179-195.
- Jarvinen, A.W., Nordling, B.R. and Henry, M.E., 1983. Chronic toxicity of dursban (chlorpyrifos) to the fathead minnow (*Pimephales promelas*) and the resultant acetylcholinesterase inhibition. *Ecotoxicol. Environ. Safety*, **7**: 423-434.
- Jobling, M., 1993. Bioenergetics: feed intake and energy partitioning. In: J.C. Rankin and F.B. Jensen (Editors), *Fish ecophysiology*. pp. 1-44. Chapman & Hall, London.
- Jobling, M., 1994. *Fish energetics*. 309 pp. Chapman & Hall, London.
- Johnson, W.W. and Finley, M.T., 1980. *Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. Resource publication 137*. US Department of the Interior, Fish and Wildlife Service, Washington, DC.
- Kalsch, W., Nagel, R. and Urich, K., 1991. Uptake estimation and bioconcentration of two anilines in zebrafish (*Brachydanio rerio*). *Chemosphere*, **22**: 351-363.
- Kamler, E., 1976. Variability of respiration and body composition during early developmental stages of carp. *Pol. Arch. Hydrobiol.*, **23**: 431-485.
- Kamler, E., 1992. *Early life history of fish: an energetic approach*. 267 pp. Chapman & Hall, London.
- Kamler, E. and Kato, T., 1983. Efficiency of yolk utilization by *Salmo gairdneri* in relation to incubation temperature and egg size. *Pol. Arch. Hydrobiol.*, **30**: 271-306.
- Kamler, E., Matlak, O. and Srokosz, K., 1974. Further observation on the effect of sodium salt of 2,4-D on early developmental stages of carp (*Cyprinus carpio* L.). *Pol. Arch. Hydrobiol.*, **21**: 481-502.
- Kaniewska-prus, M., 1975. The effect of 2,4-D-Na herbicide on oxygen consumption and survival of *Simocephalus veyulus* (O.F. Muller) (Cladocera). *Pol. Arch. Hydrobiol.*, **22**: 593-599.
- Kaushik, S.J., Dabrowski, K. and Luquet, P., 1982. Pattern of nitrogen excretion and oxygen consumption during ontogenesis of common carp (*Cyprinus carpio*). *Can. J. Fish. Aquat. Sci.*, **39**: 1095-1105.

- Kersting, K. and van Wijngaarden, R., 1992. Effects of chlorpyrifos on a microecosystem. *Environ. Toxicol. Chem.*, **11**: 365-372.
- Klaassen, C.D. and Eaton, D.L., 1991. Principles of toxicology. In: M.O. Amdur, J. Doull and C.D. Klassen (Editors), *Casarett and doull's toxicology: The basic science of poisons. 4th edn.* pp. 12-49. Pergamon Press, New York.
- Klekowski, R.Z., 1970. Bioenergetic budget and their application for estimation of production efficiency. *Pol. Arch. Hydrobiol.*, **17**: 55-80.
- Klekowski, R.Z. and Duncan, A., 1975. Physiological approach to ecological energetics. In: W. Grodzinski, R.Z. Klekowski and A. Duncan (Editors), *Methods for ecological bioenergetics (IBP handbook no.24).* pp. 15-64. Blackwell Scientific Publications, Oxford.
- Klekowski, R.Z., Prus, T. and Zyromska-Rudzka, H., 1967. Elements of energy budget of *Tribolium castaneum* (Hbst.) in its developmental cycle. In: K. Petruswicz (Editor), *Secondary productivity of terrestrial ecosystems.* pp. 859-879. PWN Warszawa.
- Knight, B., 1985. Energetics and fish farming. In: P. Tytler and P. Calow (Editors), *Fish energetics: new perspectives.* pp. 309-340. Croom Helm, London.
- Koehn, R.K. and Bayne, B.L., 1989. Towards a physiological and genetical understanding of the energetics of the stress response. In: P. Calow and R.J. Berry, (Editors), *Evolution ecology and environmental stress.* pp. 3-7. Academic press, London.
- Kristensen, P., 1990. *Evaluation of the sensitivity of short term fish early life-stage tests in relation to other FELS test methods.* Final report to the commissions of the European Communities. 60 pp. Water Quality Institute, Horsholm, Denmark.
- Kuhnhold, W.W., 1972. The influence of crude oil on fish fry. In: M. Ruivo (Editor), *Marine pollution and sea life.* pp. 315-318. Fishing News Books, London.
- Kuiper, J. and Hanstveit, A.O., 1984. Fate and effects of 3,4-dichloroaniline (DCA) in marine plankton communities in experimental enclosures. *Ecotoxicol. Environ. Safety*, **8**: 334-54.
- Kulshrestha, S.K., Arora, N. and Sharma, S., 1986. Toxicity of four pesticides on the fingerlings of Indian major carps *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*. *Ecotoxicol. Environ. Safety*, **12**: 114-119.

- Lampert, W., 1983. The measurement of respiration. In: J.A. Downing, and F.H. Rigler, (Editors), *A manual of methods for the assessment of secondary productivity in freshwaters. IBP handbook no.17. 2nd edition.* pp. 413-468. Blackwell Scientific Publications, Oxford.
- Larsson, A., Andersson, T., Forlin, L. and Hardig, J., 1988. Physiological disturbances in fish exposed to bleached kraft mill effluents. *Water Sci. Technol.*, **29**: 67-76.
- Lasker, R., 1962. Efficiency and rate of yolk utilization by developing embryos and larvae of the Pacific sardine *Sardinops caerulea* (Girard). *J. Fish. Res. Board Can.*, **19**: 867-875.
- Laurence, G.C., 1973. Influence of temperature on energy utilization of embryonic and prolarval tautog *Tautoga onitis*. *J. Fish. Res. Board Can.*, **30**: 435-442.
- Leatherland, J.F. and Sonstegard, R.A., 1984. Pathobiological responses of feral teleosts to environmental stressors: interlake studies of the physiology of Great lake's salmon. In: V.W. Cairns, P.V. Hodson and J.O. Nriagu (Editors), *Contaminant effects on fisheries.* pp. 116-139. John Wiley & Sons, New York.
- Leduc, G., 1978. Deleterious effects of cyanide on early life stages of Atlantic salmon (*Salmo salar*). *J. Fish. Res. Board Can.*, **35**: 166-174.
- Lindeman, R.L., 1942. The trophic-dynamic aspects of ecology. *Ecology*, **23**: 399-418.
- Linden, O., 1974. Effects of oil spill dispersants on the early development of Baltic herring. *Ann. Zool. Fenn.*, **11**: 141-148.
- Livingston, A.G. and Willacy, A., 1991. Degradation of 3,4-dichloroaniline in synthetic and industrially produced wastewaters by mixed cultures freely suspended and immobilized in a packed-bed reactor. *Appl. Microb. Biotech.*, **35**: 551-557.
- Lloyd, R., 1992. *Pollution and freshwater fish.* 176 pp. Fishing News Books, Oxford.
- Loewe, H. and Eckmann, R., 1988. The ontogeny of the alimentary tract of coregonid larvae: normal development. *J. Fish Biol.*, **33**: 841-850.
- Lugo, A.E., 1978. Stress and ecosystems. In: J.H. Thorp and J.W. Gibbons (Editors), *Energy and environmental stress in aquatic ecosystems.* pp. 62-101. US DOE symposium series, 48. Conf-771114.

- Macek, A.E. and Sleight, B.H., 1977. Utility of toxicity tests with embryo and fry of fish in evaluating hazards associated with the chronic toxicity of chemicals to fishes. *In*: F.L. Mayer, J.L. Hamelink (Editors), *Aquatic toxicology and hazard evaluation*. pp. 137-146. ASTM STP 634. ASTM, Philadelphia.
- Macek, K., Birge, W., Mayer, F.L., Buikema, A.L.Jr., Maki, A.W., 1978. Toxicological effects. *In*: J. Cairns Jr., K.L. Dickson and A.W. Maki (Editors), *Estimating the hazard of chemical substances to aquatic life*. pp. 27-32. ASTM STP 657. American Society for Testing and Materials, Philadelphia.
- Madon, S.P. and Culver, D.A., 1993. Bioenergetics model for larval and juvenile walleyes: an in situ approach with experimental ponds. *Trans. Am. Fish. Soc.*, **122**: 797-813.
- Mance, G., 1987. *Pollution monitoring series: pollution threat of metals in aquatic environment*. 372 pp. Elsevier Applied Science, London and New York.
- May, R.C., 1974. Larval mortality in marine fishes and the critical period concept. *In*: J.H.S. Blaxter (Editor), *The early life history of fish*. pp. 3-19. Springer-Verlag, Berlin and New York.
- Mayzaud, P., 1973. Respiration and nitrogen excretion of zooplankton. II. Studies of the metabolic characteristics of starved animals. *Mar. Biol.*, **21**: 19-28.
- McKim, J.M., 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. *J. Fish. Res. Board Can.*, **34**: 1148-1154.
- McKim, J.M., 1985. Early life stage toxicity tests. *In*: G.M. Rand and S.R. Petrocelli (Editors), *Fundamentals of aquatic toxicology*. pp. 58-95. Hemisphere Publishers, Washington, DC.
- McKim, J.M. and Benoit, D.A., 1971. Effects of longterm exposure to copper on the survival, growth and reproduction of brook trout. *J. Fish. Res. Board Can.*, **26**: 655-662.
- McKim, J.M. and Benoit, D.A., 1974. Duration of toxicity tests for establishing 'no effect' concentrations for copper with brook trout. *J. Fish. Res. Board Can.*, **31**: 449-452.
- McKim, J.M., Arther, J.W. and Thorsland, T.W., 1975. Toxicity of a linear alkylate sulphonate detergent to larvae of four species of freshwater fish. *Bull. Contam. Toxicol.*, **14**: 1-7.

- McKim, J.M., Eaton, J.G. and Holcombe, G.W., 1978. Metal toxicity to embryos and larvae of eight species of freshwater fish. II copper. *Bull. Environ. Contam. Toxicol.*, **19**: 608-616.
- Meer, C. van der, Teunissen, C. and Boog, Th.F.M., 1988. Toxicity of sodium chromate and 3,4-dichloroaniline to crustaceans. *Bull. Environ. Contam. Toxicol.*, **40**: 204-211.
- Melotti, P., 1986. Goldfish (*Carassius auratus* L.) farming in Italy. In: R. Billard, and J. Marcel (Editors), *Aquaculture of cyprinids*. pp. 369-376. Institut National De la Recherche Agronomique, Paris.
- Miller, G.C., Zissok, R. and Zeep, R., 1980. Photolysis of 3,4-dichloroaniline in natural water. *J. Agric. Food Chem.*, **28**: 1053-1056.
- Moriarty, T., 1991. *Ecotoxicology: the study of pollutants in ecosystems. Second edition*. 289 pp. Academic Press, London.
- Moriwaki, T., Kobayashi, M., Aida, K. and Hanyu, I., 1991. Changes in plasma gonadotropin and steroid hormone levels during ovulation induced by HCG treatment in female goldfish. *Nippon Suisan Gakkaishi*, **57**: 41-43.
- Mount, D.I. and Stephan, C.E., 1967. A method for establishing acceptable toxicant limits for fish: malathion and the butoxyethanol ester of 2,4-D. *Trans. Am. Fish. Soc.*, **96**: 185-193.
- Mount, D.I. and Stephan, C.E., 1969. Chronic toxicity of copper to the fathead minnow (*Pimephales promelas*) in soft water. *J. Fish. Res. Board Can.*, **26**: 2449-2457.
- Nagel, R., Bresh, H., Caspers, N., Hansen, P.D., Market, M., Munk, R., Scholz, N. and Hofte, B.B., 1991. Effect of 3,4-dichloroaniline on the early life stages of zebrafish (*Brachydanio rerio*): results of a comparative laboratory study. *Ecotoxicol. Environ. Safety*, **21**: 157-164.
- Naylor, C., Maltby, L. and Calow, P., 1989. Scope for growth in *Gammarus pulex*, a freshwater benthic detritivore. *Hydrobiologia*, 188/189: 517-523.
- Nelson, J.A., 1982. Physiological observations on developing rainbow trout, *Salmo gairdneri* (Richardson), exposed to low pH and varied calcium ion concentrations. *J. Fish Biol.*, **20**: 359-372.
- Ney, J.J., 1993. Bioenergetic modelling today: growing pains on the cutting edge. *Trans. Am. Fish. Soc.*, **122**: 736-748.

- Norberg-King, T.J., 1989. An evaluation of the fathead minnow seven day subchronic test for estimating chronic toxicity. *Environ. Toxicol. Chem.*, **8**: 1075-1089.
- Norberg-King, T.J. and Mount, D.I., 1985. A new fathead minnow (*Pimephales promelas*) subchronic toxicity test. *Environ. Toxicol. Chem.*, **4**: 711-718.
- Odum, H.T., 1967. Work circuits and systems stress. In: H.E. Young (Editor), *Symposium on primary productivity and mineral cycling in natural ecosystems*. pp. 81-138. University of Maine Press Orono, Maine, USA.
- OECD, 1992. *Draft OECD guideline for testing of chemicals. Fish toxicity tests on eggs and sac-fry stages. TGP/92.75*. 18 pp. OECD, Paris.
- Oikawa, S. and Itazawa, Y., 1985. Gill and body surface areas of the carp in relation to body mass with special reference to the metabolism-size relationship. *J. Exp. Biol.*, **117**: 1-14.
- Ojaveer, E., Annist, J., Jankowski, H., Palm, T. and Raid, T., 1980. On effect of copper, cadmium and zinc on the embryonic development of Baltic spring spawning herring. *Finn. Mar. Res.*, **247**: 135-140.
- Olson, P.A. and Foster, R.F. 1956. *Effects of chronic exposure to sodium dichromate on young chinok salmon and rainbow trout*. pp. 35-47. Battelle Pacific H.W. Lab., Richland, W.A. Report H.W. 41500.
- Paflitschek, R., 1979. Untersuchungen über die toxische Wirkung von Bayluscid und Lebaycid auf Ei-, Jugend und Adultstadien der Buntbarsche *Tilapia leucosticta* (Trewawas, 1933) and *Heterotilapia multispinosa* (Guenther, 1898). *Z. Angew. Zool.*, **66**: 143-172.
- Parrish, P.R., 1985. Acute toxicity tests. In: G.M. Rand and S.R. Petrocelli (Editors), *Fundamentals of aquatic toxicology*. pp. 31-57. Hemisphere Publishers, Washington DC.
- Pascoe, D. and Matthey, D.L., 1977. Studies on the toxicity of cadmium to the three-spined stickleback, *Gasterosteus aculeatus* L. *J. Fish Biol.*, **11**: 207-215.
- Perry, J.A. and Troelstrup, N.H.Jr., 1988. Whole ecosystem manipulation: a productive avenue for test system research. *Environ. Toxicol. Chem.*, **7**: 941-951.

- Peter, R.E., Crim, L.W., Goos, H.J. and Crim, J.W., 1978. Lesioning studies on the gravid female goldfish: neuroendocrine regulation of ovulation. *Gen. Comp. Endocrinol.*, **35**: 391-401.
- Peter, R.E., Sokolowska, M., Nahorniak, C.S., Rivier, J.E., Vale, W.W., 1987. Comparison of [D-Ala⁶, Trip⁷, Leu⁸, Pro⁹ NEt]-luteinizing hormone releasing hormone (sGnRH-A), and [D-Ala⁶, Pro⁹ NEt]-luteinizing hormone releasing hormone (LHRH-A), in combination with pimozide, in stimulating gonadotropin release and ovulation in the goldfish, *Carassius auratus*. *Can. J. Zool.*, **65**: 987-991.
- Peter, R.E., Lin, H.R. and VanDer Kraak, G., 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. *Aquaculture*, **74**: 1-10.
- Peter, R.E., Vaughan, W.Ge., Rivier, J. and Vale, W., 1991. Inhibin/activin like protein mediated feed back between the pituitary and ovary in goldfish. In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), *Proceedings of the fourth international symposium on the reproductive physiology of fish*. p. 25. University of East Anglia, Norwich, U.K. Published by Fish-Symp. 91, Sheffield.
- Petrocelli, S.R., 1985. Chronic toxicity test. In: G.M. Rand and S.R. Petrocelli (Editors), *Fundamentals of aquatic toxicology*. pp. 96-109. Hemisphere Publishers, Washington, DC.
- Petrusewicz, K., 1967. Concept in studies on the secondary productivity of terrestrial ecosystem. In: K. Petruswicz (Editor), *Secondary productivity of terrestrial ecosystems*. pp. 17-49. Panstwowe Wyd. Naukowe, Warszawa-Krakow.
- Philippova, T.G. and Postnov, A.L., 1988. The effect of food quantity on feeding and metabolic expenditure in cladocera. *Int. Rev. Gesamten Hydrobiol.*, **73**(6): 601-615.
- Phillipson, J., 1966. *Ecological energetics*. 57 pp. Edward Arnold, London.
- Pickering, A.D., 1981. The concept of biological stress. In: A.D. Pickering (Editor), *Stress and fish*. pp. 1-7. Academic Press, London.
- Pickering, A.D., 1993. Husbandry and stress: stress and adaptation. In: J.F. Muir and R.J. Roberts (Editors), *Recent advances in aquaculture IV*. pp. 155-169. Blackwell Scientific Publications, London.

- Pickering, A.D., Pottinger, T.G., Carragher, J. and Sumpter, J.P., 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature brown trout, *Salmo trutta*. *Gen. Comp. Endocrinol.*, **68**: 249-259.
- Pickering, Q.H. and Gast, M.A., 1972. Acute and chronic toxicity of cadmium to the fathead minnow *Pimephales promelas*. *J. Fish. Res. Board Can.*, **29**: 1099-1106.
- Pickering, Q.H. and Thatcher, T.O., 1970. The chronic toxicity of linear alkylate sulfonate (LAS) to *Pimephales promelas*, Rafinesque. *J. Water Pollut. Control Fed.*, **42**: 243-254.
- Powers, E.B., 1917. *The goldfish (Carassius auratus) as a test animal in the study of toxicity*. Illinois Biological Monographs. IV. 2. 73 pp. University of Illinois, Illinois.
- Quantz, G., 1985. Use of endogenous energy sources by larval turbot, *Scophthalmus maximus*. *Trans. Am. Fish. Soc.*, **114**: 558-563.
- Raciborski, K., 1987. Energy and protein transformation in sea trout (*Salmo salar* L.) larvae during transition from yolk to external food. *Pol. Arch. Hydrobiol.*, **34**: 437-502.
- Rana, K.J., 1985. Influence of egg size on the growth, onset of feeding, point-of-no-return, and survival of unfed *Oreochromis mossambicus* fry. *Aquaculture*, **46**: 119-131.
- Rana, K.J., 1986. *Parental influence on egg quality, fry production and fry performance in Oreochromis niloticus (Linnaeus) and Oreochromis mossambicus (Peters)*. Ph.D. thesis. 295 pp. Institute of Aquaculture, University of Stirling, Scotland, UK.
- Rand, G.M. and Petrocelli, S.R. (Editors), 1985. *Fundamentals of aquatic toxicology*. 666 pp. Hemisphere Publishers, Washington, DC.
- Razani, H. and Hanyu, I., 1986a. Annual reproductive cycle of 2-3 years old female goldfish and its artificial modification by manipulations of water temperature and photoperiod. *Bull. Jap. Soc. Sci. Fish.*, **52**: 965-969.
- Razani, H. and Hanyu, I., 1986b. Effects of continued short photoperiod at warm temperature and following changes of regimes on gonadal maturation of goldfish. *Bull. Jap. Soc. Sci. Fish.*, **52**: 2061-2068.

- Razani, H., Hanyu, I. and Aida, K., 1987. Critical day length and temperature level for photoperiodism in gonadal maturation of goldfish. *Environ. Biol.*, **47**: 89-94.
- Razani, H., Hanyu, I. and Aida, K., 1988a. Environmental influences on testicular activity and related hormones in yearling goldfish. *Nippon Suisan Gakkaishi*, **54**: 1513-1520.
- Razani, H., Hanyu, I. and Aida, K., 1988b. Environmental influences on ovarian activity and related hormones in yearling goldfish. *Nippon Suisan Gakkaishi*, **54**: 1505-1511.
- Razani, H., Hanyu, I., Aida, K. and Furukaza, K., 1989. Rematuration of female goldfish under continued warm or cool temperature in combination with short or long photoperiod. *Nippon Suisan Gakkaishi*, **55**: 1499-1504.
- Ricker, W.E., 1968. *Methods for assessment of fish production in freshwaters (IBP handbook no.3)*. 313 pp. Blackwell Scientific Publications, Oxford.
- Rodrigues, C.S., Malloy, D. and Kaushik, N.K., 1983. Laboratory evaluation of microcapsulated formulations of chlorpyrifos-methyl against black-fly larvae (Diptera: Simuliidae) and selected nontarget invertebrates. *Can. Entomol.*, **115**: 1189-1201.
- Rogers, B.A. and Westin, D.T., 1981. Laboratory studies on effects of temperature and delayed initial feeding on development of striped bass larvae. *Trans. Am. Fish. Soc.*, **110**: 100-110.
- Rombough, P.J., 1988. Respiratory gas exchange, aerobic metabolism and effects of hypoxia during early life. In: W.S. Haor and D.J. Randal (Editors), *Fish physiology. Vol. XI. The physiology of developing fish. Part A. Eggs and larvae*. pp. 59-161. Academic Press, San Diego.
- Rombough, P.J. and Garside, E.T., 1982. Cadmium toxicity and accumulation in eggs and alevins of Atlantic salmon *Salmo salar*. *Can. J. Zool.*, **60**: 2006-2014.
- Rombough, P.J. and Garside, E.T., 1984. Disturbed ion balance in alevins of Atlantic salmon *Salmo salar*, chronically exposed to sublethal concentrations of cadmium. *Can. J. Zool.*, **62**: 1443-1450.
- Rosenthal, H., and Alderdice, D.F., 1976. Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. *J. Fish. Res. Board Can.*, **33**: 2047-2065.

- Safford, S.E. and Thomas, P., 1987. Effects of capture and handling on circulation levels of gonadal steroids and cortisol in the spotted seatrout, *Cynoscion nebulosus*. In: D.R. Idler, L.W. Crim and J.M. Sumpter (Editors), *Proceedings of the third international symposium on the reproductive physiology of fish*. 321 pp. Memorial University of Newfoundland Press, St John's.
- Sauter, S., Buxton, K.S., Macek, K.J. and Petrocelli, S.R., 1976. *Effects of exposure to heavy metals on selected freshwater fish: toxicity of copper, cadmium, chromium and lead to eggs and sac-fry on seven fish species*. 75 pp. EPA-600/03-76-105. US EPA, Cincinnati, OH.
- Schimmel, S.C., Hansen, D.J. and Forester, J., 1974. Effects of Aroclor 1254 on laboratory reared embryos and fry of sheepshead minnows (*Cyprinodon variegatus*). *Trans. Am. Fish. Soc.*, **103**: 582-586.
- Scott, A.P., Sumpter, J.P., Kime, D.E. and Rolfe, M.S. (Editors), 1991. *Proceedings of the fourth international symposium on the reproductive physiology of fish*. 338 pp. University of East Anglia, Norwich, UK. Published by Fish-Symp. 91, Sheffield.
- Selye, H., 1946. The general adaptation syndrome and the disease of adaptation. *J. Clinical Endocrinol.*, **6**: 117-230.
- Selye, H., 1950. *Stress*. 822 pp. Montreal Acta. Inc. B-40,000/50.
- Selye, H., 1974. *Stress without distress*. 177 pp. J. B. Lippincott Company, Philadelphia and New York.
- Selye, H., 1976. *Stress in health and disease*. 1256 pp. Butterworths, London.
- Sharp, J.R. and Neff, J.M., 1980. Effects of duration of exposure to mercuric chloride on the embryogenesis of the estuarine teleost, *Fundulus heteroclitus*. *Mar. Environ. Res.*, **3**: 195-213.
- Sharp, J.R. and Neff, J.M., 1982. The toxicity of mercuric chloride and methylmercuric chloride to *Fundulus heteroclitus* embryos in relation to exposure conditions. *Environ. Biol. Fishes*, **7**: 277-284.
- Sibly, R.M. and Calow, P., 1986. *Physiological ecology of animals: an evolutionary approach*. 179 pp. Blackwell Scientific Publications, Oxford.
- Sibly, R.M. and Calow, P., 1989. A life cycle theory of responses to stress. In: P. Calow and R.J. Berry, (Editors), *Evolution ecology and environmental stress*. pp. 101-116. Academic press, London.

- Siriwardena, P.P.G.S.N., 1993. *Tolerance of early life stages of tilapia (Cichlidae: Tilapiini) to metal stress*. Ph.D. thesis. 248 pp. Institute of Aquaculture, University of Stirling, Scotland, UK.
- Smith, W.E., 1973. A cyprinodontid fish, *Jordanella floridae*, as reference animals for rapid chronic bioassays. *J. Fish. Res. Board Can.*, **39**: 329-330.
- Sokolowska, M., Peter, R.E. and Nahorniak, C.S., 1985. The effects of different doses of pimozide and [D-Ala⁶, Pro⁹ N ethylamide] -LHRH(LHRH-A) on gonadotropin release and ovulation in female goldfish. *Can. J. Zool.*, **63**: 1252-1256.
- Sokolowska, M., Peter, R.E., Nahorniak, C.S., Pan, C.H., Chang, J.P., Crim, L.W. and Weil, C., 1984. Induction of ovulation in goldfish, *Carassius auratus*, by pimozide and analogues of LHRH. *Aquaculture*, **36**: 71-83.
- Solbe, J.F.De L.G., 1993. Fresh water fish. In: P. Calow (Editor), *Handbook of ecotoxicology*. pp. 66-82. Blackwell Scientific Publications, London.
- Somasundaram, B., King, P.E. and Shackley, S.E., 1984. The effect of zinc on the ultrastructure of the brain cell of the larva of *Clupea harangus* L. *Aquat. Toxicol.*, **5**: 323-330.
- Sorensen, P.W., 1991. Hormones, pheromones and chemoreception. In: T.J. Hara (Editor), *Fish chemoreception*. Chapman & Hall, London.
- Sorensen, P.W. and Stacey, N.E., 1987. 17 α , 20 β -dihydroxy-4-pregnen-3-one functions as a bisexual priming pheromone in goldfish. *Amer. Zool.*, **27**: 412.
- Sorensen, P.W. and Stacey, N.E., 1990. Identified hormonal pheromones in the goldfish: the basis for a model of sex pheromone function in teleost fish. In: D. MacDonald, D. Muller-Schwarze and S.E. Natynczuk (Editors), *Chemicals signals in vertebrates. Vol. V*. pp. 302-311. Oxford University Press, Oxford.
- Sorensen, P.W., Goetz, F.W., Scott, A.P. and Stacey, N.E., 1991. Recent studies indicate that goldfish use mixtures of unmodified hormones and hormonal metabolites as sex pheromones. In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), *Proceedings of the fourth international symposium on the reproductive physiology of fish*. pp. 191-193. University of East Anglia, Norwich, UK. Published by Fish-Symp. 91, Sheffield.

- Sorensen, P.W., Irvine, I.A.S., Scott, A.P. and Stacey, N.E., 1991. Electrophysiological measures of olfactory sensitivity suggest that goldfish and other fish use species-specific mixtures of hormones and their metabolites as sex pheromones. *In: R. Doty and D. Muller Schwarze (Editors), Chemical signals in vertebrates, Vol. VI.* Plenum, New York.
- Sprague, J.B., 1969. Measurement of pollutant toxicity to fish. 1. Bioassay method for acute toxicity. *Water Res.*, **3**: 793-821.
- Sprague, J.B., 1985. Factors that modify toxicity. *In: G.M. Rand and S.R. Petrocelli (Editors), Fundamentals of aquatic toxicology.* pp. 96-109. Hemisphere Publishers, Washington, DC.
- Stacey, N., 1991. Hormonal pheromones in fish: status and prospect. *In: A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe (Editors), Proceedings of the fourth international symposium on the reproductive physiology of fish.* pp. 177-181. University of East Anglia, Norwich, UK. Published by Fish-Symp. 91, Sheffield.
- Stacey, N.E. and Sorensen, P.W., 1991. Function and evolution of fish hormonal pheromones. *In: P.W. Hochachka and T.P. Mommsen (Editors), The biochemistry and molecular biology of fishes. Vol. 1.* 361 pp. Elsevier, London.
- Stacey, N.E., Cook, A.F. and Peter, R.E., 1979. Spontaneous and gonadotropin - induced ovulation in the goldfish, *Carassius auratus* L: effects of external factors. *J. Fish Biol.*, **15**: 349-361.
- Stacey, N.E., Sorensen, P.W., Dulka, J.G. and VanDer Kraak, G.J., 1987. Teleost sex pheromones: recent studies on identity and function. *In: D.R. Idler, L.W. Crim and J.M. Walse (Editors), Proceedings of the third international symposium on the reproductive physiology of fish.* pp. 150-154. Memorial University Press, St. John's.
- Stearns, S.C., 1980. A new version of life-history evolution. *Oikos*, **35**: 266-281.
- Stewart, A.J. Kszos, L.A., Harvey, B.C., Wicker, L.F., Haynes, G.J. and Bailey, R.D., 1990. Ambient toxicity dynamics: assessments, using *Ceriodaphnia dubia* and fathead minnow (*Pimephales promelas*) larvae in short-term tests. *Environ. Toxicol. Chem.*, **9**: 367-379.
- Still, G.G., and Herrett, R.A., 1976. Methylcarbamates, carbanilates and acylanilides. *In: P.C. Kearney and D.D. Kaufmann (Editors), Herbicides chemistry: degradation and mode of action. Vol. 2.* pp. 610-664. Dekker, New York.

- Suzuki, Y., Kobayashi, M., Nakamura, O., Aida, K. and Hanyu, I., 1988. Induced ovulation of the goldfish by oral administration of salmon pituitary extract. *Aquaculture*, **74**: 379-384.
- Tarzwel, C.M., 1967. Water quality requirements for aquatic life. *National symposium on quality standards for natural waters. Proceedings Ann. Arbor. MI.* pp. 185-197. University of Michigan, School of Public Health, Continued Education Series No. 161.
- Tatsumi, K., Liu, S.Y. and Bollag, J.M., 1992. Enzyme-catalysed complex formation of chlorinated anilines with humic substances. *Water Sci. Technol.*, **25**(11): 57-60.
- Thomas, P. and Arnold, C.R., 1993. Environmental and hormone control of reproduction in sciaenid fish: controlled maturation and induced spawning. In: J.F. Muir and R.J. Roberts (Editors), *Recent advances in aquaculture IV.* pp. 31-42. Blackwell Scientific Publications, London.
- Tilseth, S., Solberg, T.S. and Westheim, K., 1984. Sublethal effects of the water soluble fractions of Ekofisk cruid oil on the early larval stages of cod (*Gadus morhua* L). *Mar. Environ. Res.*, **11**: 1-16.
- Truhaut, R., 1977. Ecotoxicology: objectives, principles and perspectives. *Ecotoxicol. Environ. Safety*, **1**: 151-173.
- Tytler, P. and Calow, P. (Editors), 1985. *Fish energetics: new perspectives.* 349 pp. Croom Helm, London.
- Ullrich, S.O. and Millemann, R.E., 1983. Survival, respiration and food assimilation of *D. magna* exposed to petroleum and coal derived oils at 3 temperatures. *Can. J. Fish. Aquat. Sci.*, **40**: 17-26.
- US EPA, 1982. *Fish early life stage toxicity test. EG-11.* Office of pesticides and toxic substances. 35 pp. EPA, Washington, DC. 20460.
- US EPA, 1985. *Standard evaluation procedure: acute toxicity test for freshwater fish.* Office of pesticide program. 12 pp. EPA/540/9-85-006. EPA, Washington, DC.
- US EPA, 1989. *Shortterm methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 2nd edition.* 260 pp. EPA/600/4-89/001. EPA, Cincinnati, OH.

- VanDer Kraak, G., Pankhurst, N.W., Peter, R.E. and Lin, H.R., 1989. Lack of antigenity of human chorionic gonadotropin in silver carp (*Hypophthalmichthys molitrix*) and goldfish (*Carassius auratus*). *Aquaculture*, **78**: 81-85.
- van Leeuwen, C.J., Griffioen, P.S., Vergouw, W.H.A. and Maas-Diepeveen, J.I., 1985. Differences in susceptibility of early life stages of rainbow trout (*Salmo gairdneri*) to environmental pollutants. *Aquat. Toxicol.*, **7**: 59-78.
- van Leeuwen, C.J., Adema, D.M.M. and Hermens, J., 1990. Quantitative structure-activity relationships for fish early life stage toxicity. *Aquat. Toxicol.*, **16**: 321-334.
- van Wijngaarden, R., Leeuwaugh, P., Lucassen, W.G.H., Romiju, K., Ronday, R., vander Velde, R. and Willigenburg, W., 1993. Acute toxicity of chlorpyrifos to fish, newt and aquatic invertebrates. *Bull. Environ. Contam. Toxicol.*, **5**: 716-723.
- Vaughan, D.S., Yoshiyama, R.M., Breck, J.E. and DeAngelis, D.L., 1984. Modelling approaches for assessing the effects of stress on fish population. In: V.W. Cairns, H.V. Hodson, and J.O. Nriagu, (Editors), *Contaminants effects on fisheries*. pp. 259-278. John Wiley & Sons, Newyork.
- Viswanathan, R., Schewmert, I, Kohli, J., Klein, W. and Korte, F., 1978. Longterm studies on the fate of 3,4-dichloroaniline ¹⁴C in a plant-soil system under outdoor conditions. *J. Environ. Sci. Health*, **13**(B): 243-259.
- von Westernhagen, H., 1988. Sublethal effects of pollutants on fish eggs and larvae. In: S.W. Hoar, and D.J. Randal (Editors), *Fish physiology. Vol.XI. The physiology of developing fish. Part A. Eggs and larvae*. pp. 253-345. Academic Press, San Diego.
- von Westernhagen, H., Dethlefsen, V. and Rosenthal, H., 1975. Combined effects of cadmium and salinity on development and survival of garpike eggs. *Helgol. Wiss. Meeresunters*, **27**: 268-282.
- von Westernhagen, H., Rosenthal, H. and Sperling, K.R., 1974. Combined effects of cadmium and salinity on development and survival of herring eggs. *Helgol. Wiss. Meeresunters*, **26**: 416-433.
- Wallace, J.C. and Aasjord, D., 1984. An investigation of the consequences of egg size for the culture of Arctic char, *Salvelinus alpinus* (L.). *J. Fish Biol.*, **24**: 427-435.

- Ward, G.S. and Parrish, P.R., 1980. Evaluation of early life stage toxicity tests with embryos and juveniles of sheepshead minnows (*Cyprinodon variegatus*). In: J.G. Eaton, P.R. Parrish and A.C. Hendricks (Editors), *Aquatic toxicology*. pp. 243-247. ASTM STP 707. ASTM, Philadelphia.
- Ware, D.M., 1975. Relation between egg size, growth and natural mortality of larval fish. *J. Fish. Res. Board Can.*, **32**: 2503-2512.
- Warren, G.E. and Davis, G.E., 1967. Laboratory studies on the feeding, bioenergetics and growth of fish. In: S.D. Gerking (Editor), *The biological basis of freshwater fish production*. pp. 175-214. Blackwell Scientific Publications, Oxford.
- Wedemeyer, G.A. and McLeay, D.J., 1981. Methods for determining the tolerance of fishes to environmental stressors. In: A.D. Pickering (Editor), *Stress and fish*. pp. 247-275. Academic Press, London.
- Wegman, R.C.C. and deKorte, G.A.L., 1981. Aromatic amines in surface waters of the Netherlands. *Water Res.*, **15**: 391-394.
- Weininger, D., 1978. *Accumulation of PCBs by lake trout in lake Michigan*. Doctoral dissertation. University of Wisconsin, Madison.
- Welcomme, R.L., 1988. *International introduction of inland aquatic species*. 318 pp. FAO Fish. Tech. Pap., **294**.
- Widdows, J. 1978. Physiological indices of stress in *Mytilus edulis*. *J. Mar. Biol. Assoc. U.K.*, **58**: 125-142.
- Widdows, J., 1985. Physiological measurements. In: B.L. Bayne, D.A. Brown, K. Burns, D.R. Dixon, A. Ivanovici, D.R. Livingstone, D.M. Lowe, M.N. Moore, A.R.D. Stebbing and J. Widdows (Editors), *The effects of stress and pollution on marine animals*. pp. 3-45. Praeger Scientific, New York.
- Widdows, J. and Bayne, B.L., 1971. Temperature acclimation of *Mytilus edulis* with reference to its energy budget. *J. Mar. Biol. Assoc. U.K.*, **51**: 827-843.
- Widdows, J., Bayne, B.L., Donkin, P., Livingstone, D.R., Lowe, D.M., Moore, M.N. and Salkeld, P.N., 1981. Measurement of the responses of mussels to environmental stress and pollution in Sullom Voe: a baseline study. *Proc. R. Soc. Edinb. Sec. B*, **80**: 323-338.

- Wiegand, M.D., Buchanan, L.G., Loewen, J.M. and Hewitt, C.M., 1988. Effects of rearing temperature on development and survival of embryonic and larval goldfish. *Aquaculture*, **71**: 209-222.
- Wiegand, M.D., Hataley, J.M., Kitchen, C.L. and Buchanan, L.G., 1989. Induction of developmental abnormalities in larval goldfish, *Carassius auratus* L., under cool incubation conditions. *J. Fish Biol.*, **35**: 85-95.
- Winberg, G. G., 1960. Rate of metabolism and food requirements of fishes. *Fish. Res. Board Can. Transl. Ser.*, **194**: 1-253.
- Wolff, C.J.M. and Crossland, N.O., 1985. Fate and effects of 3,4-dichloroaniline in the laboratory and in outdoor ponds: 1. Fate. *Environ. Toxicol. Chem.*, **4**: 481-487.
- Woltering, D.M., 1984. The growth response in fish chronic and early life stage toxicity tests: a critical review. *Aquat. Toxicol.*, **5**: 1-21.
- Worthing, C.R. and Walker, S.B., 1983. *The pesticide manual: a world compendium. 7th edition.* 3050 pp. British Crop Protection Council, London.
- Yamamoto, K., Nagahama, Y. and Yamazaki, F., 1966. A method to induce artificial spawning of goldfish all through the year. *Bull. Jap. Soc. Sci. Fish.*, **32**: 977-983.
- Yamazaki, F., 1965. Endocrinological studies of the reproduction of the female goldfish *Carassius auratus* L. with special reference to the function of the pituitary gland. *Mem. Fac. Fish. Hokkaido Univ.*, **13**: 1-64.
- Ying, S.Y., 1988. Inhibin, activin and follistins: gonadal proteins modulating the secretion of follicle stimulating hormone. *Endocr. Rev.*, **9**: 267.
- Zar, J.H., 1984. *Biostatistical analysis. 2nd edition.* 718 pp. Prentice Hall Inc., Engle Wood Cliffs, N.J.