

PREFERENCE AVOIDANCE REACTIONS OF RAINBOW TROUT (SALMO GAIRDNERI)
FOLLOWING LONG TERM SUBLETHAL EXPOSURE TO CHROMIUM AND COPPER

by

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To my wife Valentine
to remember our last seven years together
without even an itch,
and to my daughter Irene-Melanie,
to remember the beginning of her existence.

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Abstract

A standard methodology was developed for performing avoidance-preference tests, using Rainbow Trout (Salmo gairdneri) as the test organism. Experiments were conducted in a hydraulic channel, 9.15 m long by 0.30 m wide, partly divided along its length, and at a flow depth of 0.30 m. The design combined steep and shallow gradient characteristics. The toxicants investigated included Cu(II), Cr(III) and Cr(VI). The lowest avoidance threshold values were established at 2.1 µg/l for Cu(II) and 0.0026 mg/l and 0.026 mg/l for Cr(III) and Cr(VI) respectively while avoidance reactions increased with levels of toxicant in the channel. Similar experiments were performed with rainbow trout which were pre-exposed at sublethal levels to the toxicant, in order to assess the influence of toxic pre-exposure to the subsequent fish avoidance response. The length of pre-exposure varied between 7 - 20 weeks. Avoidance threshold values were correlated with safe levels of toxicant exposure.

Pre-exposed fish exhibited decreasing avoidance reactions compared to non-exposed populations. Increased tolerance to the toxicant, was suggested by the increase in avoidance threshold values with pre-exposure levels. Fish exposed to test concentrations matching their pre-exposure levels, clearly preferred this same concentration over the adjacent lower or higher test concentration.

A two mechanism avoidance model was proposed independent of toxicant used or level of pre-exposure. The toxicant concentration where the second mechanism begins to dominate was referred

to as avoidance breakpoint, and was correlated to a MATC level for the toxicant in question. Olfactory responses were proposed to be associated with fish avoidance responses below the avoidance breakpoint, while hypoxic stress along with osmo- and iono regulatory stress appeared to be responsible for driving fish avoidance reactions beyond the avoidance breakpoint.

A clearance period of 7 days was sufficient to allow fish to recover normal avoidance behaviour following pre-exposure to Cr(VI) below the avoidance breakpoint.

Résumé

Une méthode optimisée a été développée pour l'évaluation de la réponse d'évitement et de préférence de poissons (Salmo gairdneri) à la pollution. La méthode consiste à introduire des truites arc-en-ciel dans un canal hydraulique de 9.15 m de long sur 0.3 m de large et de 0.3 m profondeur de flux. Le canal est partiellement divisé sur sa longueur, et permet d'appliquer deux gradients toxiques dans la zone divisée. La réponse des poissons est estimée par la fraction de poissons se trouvant dans la zone non polluée (courbe d'évitement).

On précisa d'abord les seuils minimums de réaction d'évitement à 2.1 $\mu\text{g/l}$ pour le Cu(II), 2.6 $\mu\text{g/l}$ pour le Cr(III) et 26 $\mu\text{g/l}$ Cr(VI). A des valeurs supérieures, les réactions d'évitement augmentaient avec la concentration en polluant.

On évalua ensuite l'influence d'une pré-exposition (7 à 20 semaines) à des niveaux toxiques sous-létaux sur la réaction d'évitement. Les poissons pré-exposés avaient des réactions d'évitement plus faibles que les poissons non-exposés. Une augmentation de tolérance envers la substance toxique se manifestait par l'augmentation des valeurs des seuils d'évitement avec le niveau de pré-exposition. Les poissons exposés à des concentrations toxiques équivalentes à celles de leur pré-exposition préféraient nettement cette même concentration plutôt que teneurs inférieurs ou supérieurs.

Un modèle d'évitement à double mécanisme est proposé lequel est indépendant de la substance toxique utilisée et du niveau de pré-exposition. La concentration toxique où le second mécanisme

devient dominant est déterminée par le point de rupture dans la courbe d'évitement et indique reliée un palier "MATC" (Maximum Allowable Toxicant Concentration) pour la substance en question. En dessous de ce palier, des réponses olfactives seraient associées avec la réaction d'évitement. Pour des concentrations toxiques supérieures au point de rupture, la réaction d'évitement serait provoquée par la combinaison d'un stress hypoxique et d'un stress de l'osmo-iono regulation.

Une période de désintoxication de 7 jours est suffisante pour permettre aux poissons pré-exposés au Cr(VI) de recouvrir des réactions d'évitement comparables à celles des poissons non-exposés, pour des concentrations inférieures au point de rupture.

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1.0 Introduction

A growing concern over environmental issues and the impact of toxic substances on the quality of human life has resulted in the application of various bioassay techniques to establish water quality standards. Lethal bioassays are routinely used to assess the toxicity of a particular pollutant. Sublethal effects, although difficult to evaluate, provide more meaningful information on the environmental impact of a toxicant, since sublethal levels are those commonly encountered in natural waters.

The objective of laboratory sublethal toxicity testing is to establish effluent threshold levels, below which fish would not be exposed to hazards and not only survive, but thrive. Further, by comparing data on sublethal threshold concentrations with expected effluent levels in receiving waters, the long term health of the fishery resource and the ecosystem in general may be assessed.

The influence of long term exposure to sublethal levels on behavioural reactions has received little attention. Resulting changes in behaviour could have significant environmental implications and might be of considerable importance in research carried out for the purpose of setting standards for water quality.

Most experimental work is designed to establish a cause - effect relationship. In environmental studies, this relationship is referred to as stress-response. The fact that an ecosystem is under stress is not necessarily of immediate concern. Within limits, ecosystems can adapt to stress; some degree of stress may even promote environmental health in the long run (Env. Canada,

1986). However, it is recently recognized that there can be hazards to humans and to the stability of ecosystems due to extended exposure of organisms to contaminants at levels that were not previously regarded as harmful. Advances in analytical chemistry provided means to detect a larger number of chemicals in extremely low concentrations (fractions of parts per trillion), and increased our level of awareness about the presence of toxicants in particular environments. Thus, information is needed on the long-term environmental effects of most chemicals in use, since those effects are not known. The only known fact is that contaminants affect ecosystems and human health to a different degree depending on age of the organism, susceptibility, previous history of the individual and combination of contaminants (Env. Canada, 1986).

In the past, most toxicity studies involved lethal bioassays, while studies on the effect of sublethal levels of toxicants invariably used fish maintained in clear water, neglecting the effects of pre-exposure and adaptation of fish to low levels of pollution (Anestis and Neufeld, 1986).

In the present study, a stress-response relationship was established under sublethal conditions of acute and long-term exposure of fish populations to toxic chemicals. Using a multidisciplinary approach, the effect of long term exposure of fish to copper and two different chromium compounds was investigated. The effect of the chemistry of the chromium compounds on chromium toxicity was also examined. The test organism was Rainbow Trout (Salmo gairdneri), and the testing involved avoidance reactions

as the criterion for toxicity.

It was demonstrated that avoidance-preference testing can be used for establishing water quality standards, and may also be applied as a tool for proposing avoidance preference mechanisms.

1.1 Objectives

The main objectives of the present study were

- 1) to establish an avoidance-preference testing methodology,
- 2) to study the avoidance reaction response of rainbow trout exposed to copper and different ionic forms of chromium,
- 3) to determine the effect of long-term exposure of fish to chromium and copper on the toxicity of these compounds, using avoidance reactions as the criterion for toxicity, and
- 4) to propose mechanisms explaining fish avoidance reactions.

2.0 Background

2.1 Literature review

2.1.1 Context and nomenclature

Industries discharge their treated or untreated effluents into the closest refuse system or receiving water. Many industrial and municipal effluents contain toxic compounds and consequently acute or long term effects on aquatic life are observed. Mobile aquatic organisms exhibit sensitive behavioural responses, such as avoidance, when exposed to a toxic environment.

Toxicity tests can provide information about lethal, sublethal or safe levels. They generally involve bioassays, which are tests to detect the presence or measure the effects of various substances, wastes or environmental factors using aquatic organisms. In this respect a bioassay is considered an analytical tool used to investigate the effect of toxicants on living organisms (Brungs, 1973).

The types of flow used in bioassays are either static, or with recirculation of test water or flow through (dynamic). Bioassays are classified according to the outcome of the test as lethal, sublethal and chronic. Depending on the duration of exposure of the organisms, bioassays can be considered as acute, subacute, subchronic, chronic or long-term. Accordingly preference -avoidance tests as performed in the present study can be characterized as dynamic, sublethal, acute bioassays.

Lethal bioassays represent the bulk of the toxicity testing. They provide information on the lethal concentration of the toxicant, which is the only presently accepted evidence of environ-

mental impact in the courts of law. The most frequently used terms are:

Lethal Concentration (L.C.) is the result of bioassays using lethality as a criterion of toxicity.

L.C.50 is the concentration of a substance for which 50% of test organisms are killed following exposure.

Incipient Lethal Concentration or Incipient Lethal Level (I.L.C.50 or I.L.L.) is the concentration at which acute toxicity ceases and 50% of the aquatic population can live indefinitely. It is also referred to as Lethal threshold concentration.

Sublethal bioassays focus on sublethal or safe toxicant levels and are used in establishing water quality standards. Some of the terms used in sublethal tests are:

Effective or Inhibitory Concentration (E.C. or I.C.) is the concentration for which effects or inhibitions other than lethality are manifested (e.g. avoidance).

Safe Concentration (S.C.) is the maximum concentration for which no harmful effects are observed after chronic exposure (one or more generations).

Maximum Allowable Toxicant Concentration (M.A.T.C.) is the concentration of a toxic agent that does not restrict any water usage. It provides the best prediction for safe levels (Mount, 1977) and is interpolated as the geometric mean of the lowest concentration having an effect and the highest concentration having no effect.

Application Factor (A.F.) is defined as:

$$A.F. = \frac{M.A.T.C.}{I.L.C.50} \quad \text{or} \quad A.F. = \frac{S.C.}{I.L.C.50}$$

2.1.2 Toxicity tests

The main reason for performing toxicity tests, is to predict levels of toxicants that will be safe for the general well-being of the receiving ecosystem.

Toxicity bioassays can be used to predict the environmental impact of a toxicant discharge, providing information on relative toxicities of various pollutants on different species under a variety of conditions, and are also used to establish regulatory restrictions on effluent discharges (Buikema et al., 1982). Toxicity tests at the organism level or lower, can answer questions best about locus and mode of action of the toxicant in question (Buikema et al., 1982).

The objective in the design and use of toxicity tests in bio-monitoring is the ability to predict with known accuracy a concentration that will not harm an entire ecosystem and its elements, and make the prediction in a responsible and cost effective manner.

2.1.3 Preference-avoidance tests

This study can be classified according to its outcome, as a sublethal toxicity bioassay. Reaction tests are not deterministic (Sprague, 1971). Nevertheless avoidance-preference studies are essential in establishing the range of concentrations that fish avoid, in order to maintain fishing grounds intact. If fish avoid a certain chemical, they will eventually move to another ground with more favourable conditions, and the previous fishing ground will lose its economic value (Ishio, 1964). Complexity in

behavioural responses increase by variables such as territoriality, schooling and level of activity. Therefore it is necessary to minimize the influence of these variables on avoidance results by choosing appropriate species, apparatus and conditions. Avoidance is exhibited as a characteristic of toxicity in combination with level of detection, conditioning and acclimation (Bogardus et al., 1976).

Scherer (1975) proposed avoidance reactions as a criterion of toxicity since detection and avoidance of sublethal concentrations will help fish escape lethal levels, and altered spatial distribution affects the general ecosystem in addition to economic considerations. The study of avoidance responses is essential since concentrations causing behavioural responses are different and lower from those causing physiological damage (Westlake et al., 1977).

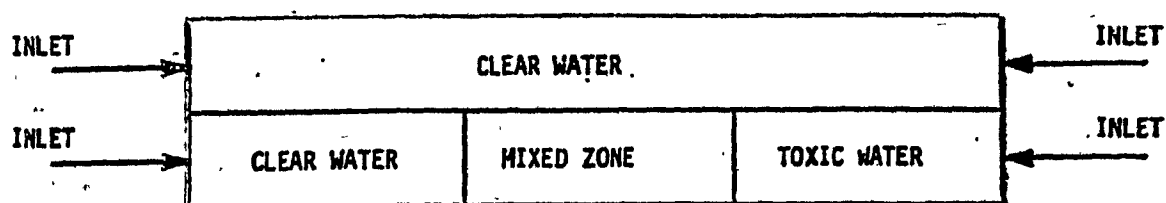
Fish seem to spontaneously avoid a compound of a specific concentration, or they can develop the ability to distinguish and avoid a pollutant given time. They can also detect changes in concentration (Ishio, 1964; Sprague & Drury, 1969). Among the different toxicity tests, preference-avoidance tests are considered important and practical, since whole-organism behavioural responses cannot be predicted from physiological and histological or other toxicity studies (Mello, 1975; Giattina and Garton, 1983).

Preference-avoidance tests exploit the above mentioned ability of fish to detect environmental conditions that are not favourable for survival. In testing the direct response to toxic

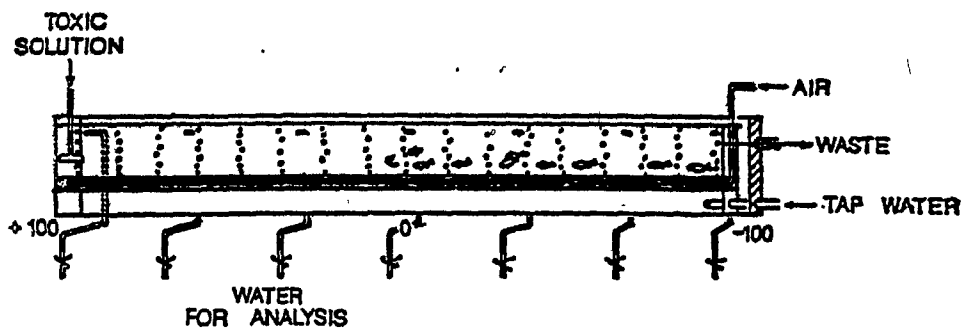
cants, it may be determined if the species can detect the toxicant, and if so, whether preference will render it more harmful, or avoidance will provide a chance for survival.

Preference-avoidance tests may be divided in two major groups, temperature preference-avoidance studies and chemical avoidance studies. Temperature, being a major factor for environmental conditions, has been studied extensively and results are found in Cherry and Cairns (1982), for different species and temperature ranges. The present study has focused on chemical avoidance.

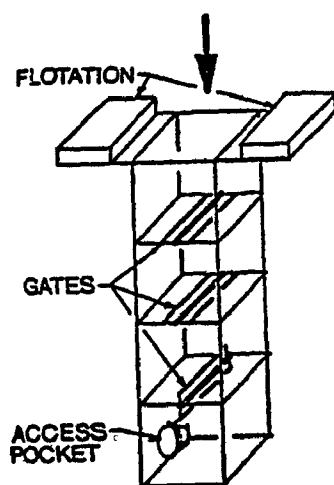
In the past, a variety of different experimental designs has been employed to expose fish to a chemical substance under simulated natural environmental conditions. The apparatus used can be classified into 3 major categories: shallow gradient, steep gradient and fluvial systems. A schematic representation of the different systems employed in such studies appear on Figure 2.1, and a review of apparatus and toxicants studied appear in Appendix A.1. All systems have distinct characteristics and advantages-disadvantages, when compared with each other. In general, equipment employing shallow gradients better represent a natural environment. In contrast, they cannot give distinct directional cues for fish orientation. Reproducibility of identical gradients during replicate experiments is generally very poor. Steep gradients on the other hand, while not simulating "normal" concentration gradients in natural systems, provide the necessary cues for directive movements. The data can be replicated with accuracy and have produced the most acceptable avoidance curves.



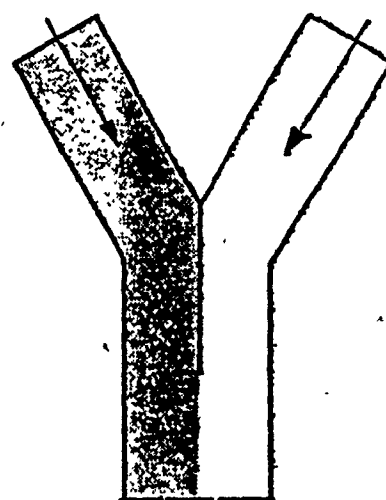
SIMPLIFIED DESIGN A Plan view



SIMPLIFIED DESIGN B - Side view

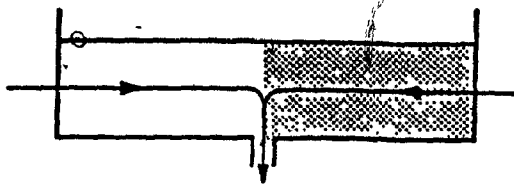


SIMPLIFIED DESIGN C - Side view

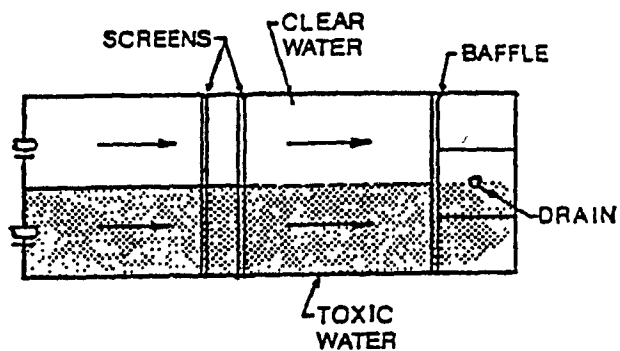


SIMPLIFIED DESIGN D - plan view

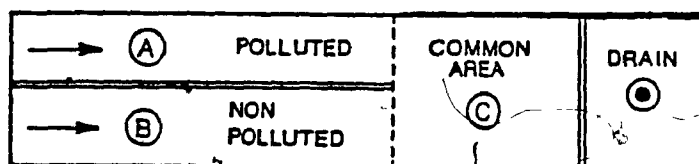
Figure 2.1 Apparati used in avoidance-preference studies (after Hadjinicolaou, 1983).



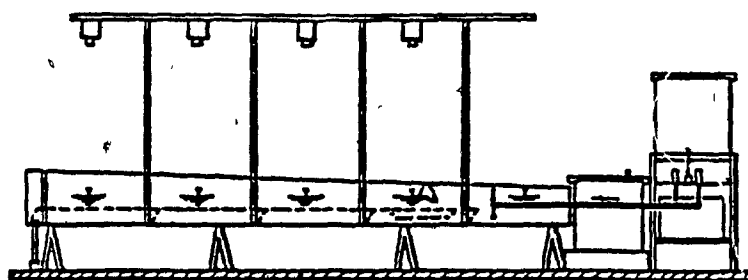
SIMPLIFIED DESIGN E - Side view



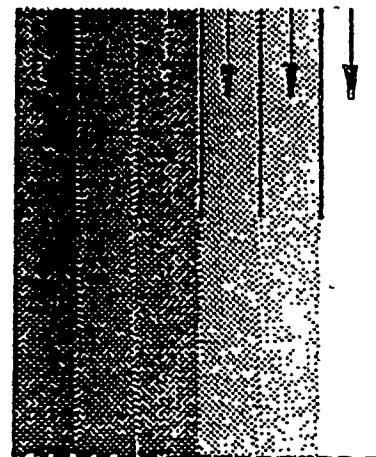
SIMPLIFIED DESIGN F - Plan view



SIMPLIFIED DESIGN G - Plan view



SIMPLIFIED DESIGN H - Side view



SIMPLIFIED DESIGN I - Plan view

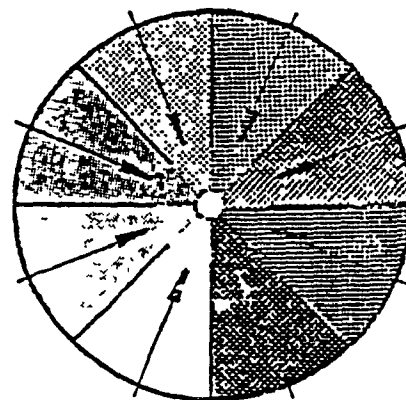
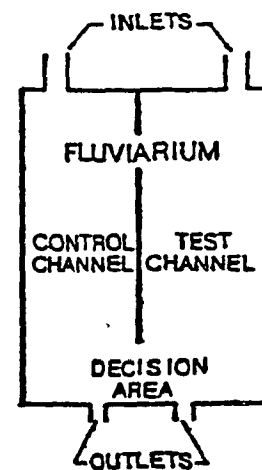
SIMPLIFIED DESIGN J
Plan viewSIMPLIFIED DESIGN K
- Plan view

Figure 2.1 (continued)

Finally, the fluvium configurations can combine both shallow and steep gradient advantages, such as the assumed distinct concentration interface between channels of different concentrations that give concrete directional cues to the organisms. As a disadvantage, concentration interfaces cannot be guaranteed, and previous fish exposure to higher concentrations may alter the organisms behaviour, yielding poor reproducibility of results during replicate experiments.

In the present study, a channel (Spraggs *et al.*, 1982 -design H, Figure 2.1), was extensively modified to exploit the advantages of both shallow and steep gradients, by analyzing and establishing hydraulic conditions to yield repetitively identical concentration profiles in the apparatus during actual experimentation for avoidance-preference evaluation.

Due to the sensitivity of fish to detect very low levels of toxicants, the most significant information provided by avoidance-preference testing is the establishment of the avoidance threshold level or avoidance threshold concentration. This level can be proposed as a Safe Concentration (S.C.) for the toxicant in question. Various authors have used the term in a different context. Some were referring to avoidance threshold as the first concentration level where significant avoidance reaction was observed (Giattina and Garton, 1983). Others used the term as the toxicant concentration effective at causing avoidance or the concentration corresponding to the intersection of the avoidance curve with the neutral line of response (usually the 50% level of number of fish in clear water or equivalently 50% of

time fish spent in clear water).

The present study uses the latter definition for the avoidance threshold level, while it is expected that authors using the first mentioned definition may propose higher concentrations as avoidance threshold levels.

2.1.4 Biological basis of behaviour relevant to fish avoidance reactions

In bioassays the "measuring probe" to evaluate the quantifiable parameter is the experimental organism. In avoidance studies, changes in behaviour is the means of quantifying the effect of the toxicant on the fish.

Behaviour of fish is affected by the previous history of the organisms, social interactions, stage of development, physical characteristics of the experimental channel, ambient temperature and territoriality of the organisms (Adler, 1975). These factors influence the repeatability of results in avoidance-preference bioassays and they were considered in the present study for decisions on fish maintenance, pre-exposure, acclimation and experimental protocol.

Mello (1975) proposed that avoidance is an integrated nervous system response, since the nervous system controls most bodily functions. A nervous system stimulation caused by the toxicant on the organism, internally or externally, induces a change in bodily functions resulting in an avoidance-preference reaction. The intensity and acuteness of avoidance reaction depends on the level of the toxicant concentration as well as on

the method of detection and transmission of the stimulus. It was suggested that olfaction plays an important role in toxicant detection. Similarly osmoregulation was proposed as a potential mechanism to produce behavioural changes.

2.1.4.1 Mode of toxic action

An element is said to be toxic if it injures the growth or metabolism of an organism, when supplied above a certain level. The most important mechanism of metal toxic action is poisoning of enzyme systems. Cu^{2+} , Hg^{2+} and Pb^{2+} have a high affinity for amino, imino and sulfhydryl groups, and can block the active sites of many enzymes in fish or replace an essential element such as Zn^{2+} (Alabaster and Lloyd, 1980; Fergusson, 1982). Metals are also readily chelated by organic molecules. Thus metal toxicity may be correlated with electronegativity, stability of sulfides, and stability of chelates. Considering the order of electronegativity of metal elements as presented below,

$\text{Hg} > \text{Cu} > \text{Sn} > \text{Pb} > \text{Ni} > \text{Co} > \text{Cr} > \text{Cd} > \text{Fe} > \text{Zn} > \text{Mn} > \text{Mg} > \text{Ca} > \text{Sr} > \text{Ba}$,

it is expected that copper should be more toxic than chromium or zinc, which is supported by findings in the literature (Alabaster and Lloyd, 1980). Based on the order of stability of their sulfides, which for the elemental form of metals can be represented by the sequence:

$\text{Hg} > \text{Cu} > \text{Pb} > \text{Cd} > \text{Co} > \text{Ni} > \text{Zn} > \text{Fe} > \text{Mn} > \text{Sn} > \text{Mg} > \text{Ca}$

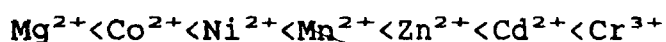
it is expected that Cu^{2+} will be more toxic than Pb^{2+} , which is suggested in the literature. The toxic action is due to the formation of insoluble sulfides by reacting with proteins, enzymes

and other ligands (Ferguson, 1982).

The order of stability of the chelates which metals form with biochemical molecules is represented by the following sequence for the elemental form of the metals:



Accordingly it is expected that Cu^{2+} will be more toxic than Ni^{2+} , which is supported from evidence in the literature (Alabaster and Lloyd, 1980). Phosphate groups of many biochemical molecules also offer potential chelating sites for metals. Phosphate being a backbone of DNA, if affected can cause incorrect genetic information to be transmitted. The outcome is that modified proteins and enzymes are produced leading to changes in the organism and producing toxic effects. Terato-genesis and birth defects are also associated with metal interference with the DNA molecule. Metal ions readily bind to phosphate in the following order:



A similar toxic action was suggested by Sprague & Drury (1969), that avoidance response of fish depends on irritants that inhibit the sulfhydryl group in enzyme systems of sensory receptors. They classified them as mercaptide forming agents such as heavy metals, oxidizing agents, and alkylating agents. Zinc which belongs to the first category, or chromium in the second category in fact produced fish avoidance. Phenol, which could not be classified in any of the above categories, does not stimulate avoidance.

Another mode of metal toxic action is impairment of O_2

uptake, causing deleterious effects on metabolic processes, or interference with swimming or respiratory ability so that actual O_2 consumption is lowered (Sprague, 1971).

Impairment of osmoregulation which regulates the salts balance of the body fluids, is the mode of action suggested for Cr (VI) toxicity on fish (Van der Putte et al., 1981).

Another proposed mode of action for a toxicant, is by combining with membrane cells, altering their permeability (e.g. Au^+ , Cd^{2+} , Cu^{2+} , Hg^{2+} , Pb^{2+} , Cr^{3+} , U^{3+}) (Alabaster and Lloyd, 1980). These elements may affect transport of Na^+ , K^+ , Cl^- or organics across membranes, and possibly rupture the cells (Strik et al., 1975).

A mode of lethal action is reduced water permeability of gills. Sprague (1968) and Van der Putte et al. (1981), suggested that toxic effects result from the actual concentration on the surface layer of the gills, which in turn depends on ambient toxicant levels and velocity of respiratory flow (Sprague, 1968).

Toxicants also act on chemoreceptive organs or motor nervous systems affecting avoidance movements. Heavy metals in particular may affect the palatal chemoreceptors and depress the response of the sugar and salt receptors (Sprague and Drury, 1969; Hara, 1979; Hara et al., 1976, 1983; Alabaster and Lloyd, 1980).

2.1.4.2 Mechanisms of action

It is proposed (Alabaster and Lloyd, 1980; Mason, 1981) that toxicants act on fish to produce their toxic effects by:

- 1) External influence on gill surface resulting in lowered oxygen availability causing hypoxia (e.g. oxidizing agents, metals, chlorine).
- 2) Internally (e.g. chloramines) producing anoxia, which is caused by converting functional blood hemoglobin to non-functional methemoglobin in O_2 transport, and
- 3) Ion regulating mechanisms of organ membranes can be responsible for toxicity of ionic species.

These mechanisms may be candidates for eliciting avoidance reactions, as well.

Most of the metals that are toxic beyond a certain concentration are essential at very low levels for normal growth. Fig. 2.2 illustrates proposed dose-response curves for essential and non-essential elements.

2.1.5 Chemical consideration of the toxicants used in the present study

It is generally accepted that only several species of the chemicals are toxic (Curtis, 1975; Fergusson, 1982; Sprague, 1969). All metals that are bound in complex chemistry produce lower toxicity reactions. Simple salts affect fish more easily (Singh and Ferns, 1978; Brown and Parsons, 1978). Also the ionic form plays an important role (hydrated ion, oxy or hydroxy species, etc.) since its reactivity depends on the form. The molecular form of the toxicant penetrates membranes more easily than the ionic form (Fergusson, 1982).

2.1.5.1 Chemistry of chromium

Chromium, with an atomic number of 24 and atomic weight 52 has oxidation states ranging from Cr(III) to Cr(VI). Most commonly, it occurs as Cr(0), Cr(III) and Cr(VI). Cr(II) is very unstable, rapidly oxidizing to Cr(III). Cr(VI) is a strong oxidant and as a result, it is easily reduced to the Cr(III) form. Only two forms, Cr(III) and Cr(VI), are found in nature.

The Cr(VI) form appears fairly stable in water, mainly due to the lack of reducing materials. The Cr(III) form is associated mainly with particulate matter, which suggests that organic particles may reduce and bind the element, leaving the Cr(VI) in solution (Cotton and Wilkinson, 1972). Other data suggest that chromium may be adsorbed on clay particles, while Cr(III) as chromate readily penetrates biological membranes.

Chromium is an essential trace element, and is found in almost all living matter as well as in soil, water and air. The water content of rivers in North America ranges from 1 to 220 $\mu\text{g/l}$ (Goulet et al., 1982), while in the vicinity of industrial discharges, levels as high as 20 mg/l have been reported (Env. Can., 1983).

Chromium occurs in most biological material in the Cr(III) form (proteins, nucleic acids, blood plasma and low molecular weight ligands). Levels up to 1 mg/g appear in liver tissue. Low concentrations of chromium have stimulatory effects, but are toxic at high concentrations (see also Fig. 2.2).

Chromium exists in wastewaters in the HCrO_4^- and CrO_4^{2-} anionic forms. Acid reduction of Cr(VI) to Cr(III) and subsequent

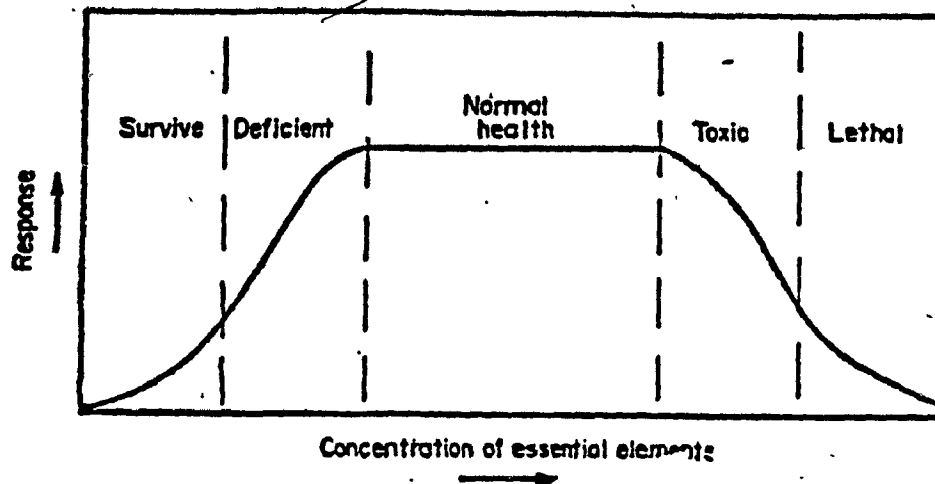


Figure 2.2.1 Dose-response curve for essential elements.
(after Fergusson, 1982)

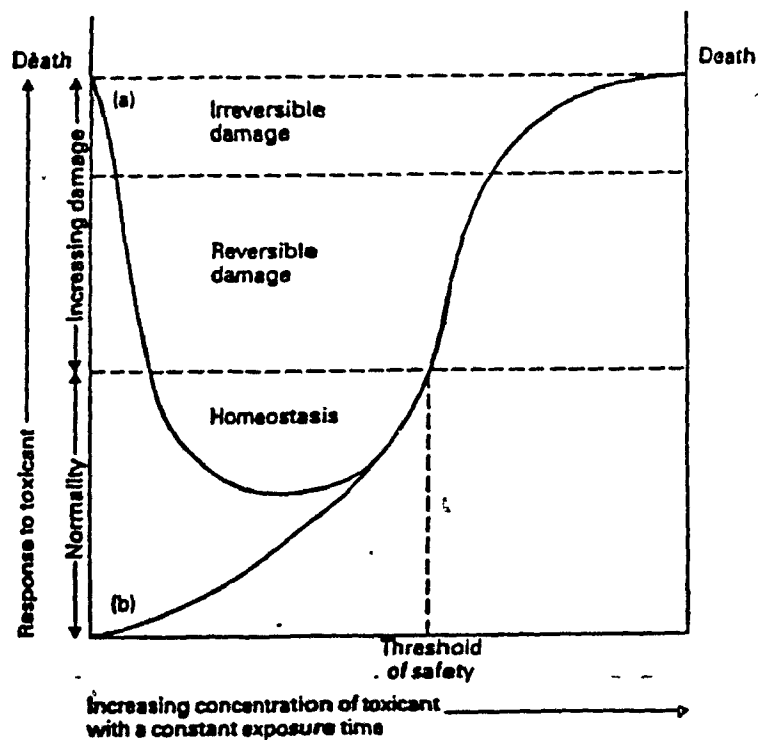


Figure 2.2.2 Dose-response curves for essential (a) and non-essential (b) toxic substances on organism's health (after Mason, 1981).

hydroxide precipitation is the most common form of Cr(VI) removal (Patterson and Minear, 1975).

Cr(VI) was found to cause impaired kidney and gill functions in trout, intestinal hemorrhages as well as swollen salt cells, slime cells and hypertrophy, hyperplasia and hemorrhages on gill lamellae (Strik et al., 1975; Van der Putte et al., 1981).

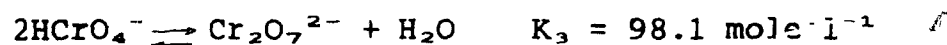
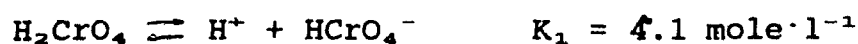
Rainbow trout can be exposed to acclimation periods in metal solutions, and although they accumulate abnormally high concentration of metal in some organs, survive as long as non-exposed fish (Alabaster and Lloyd, 1980).

Rainbow trout cannot compensate for loss of plasma ions (mainly Na^+) when exposed to Cr(VI), due to damaged kidney or gills (Van der Putte et al., 1981). On the other hand, toxicants may increase the water uptake of trout. This can result in higher resistance due to increased urine production reducing deposition of toxicant on tissues.

Chromium bioaccumulates and as is the case with other cumulative toxicants, starts to have an effect after reaching a critical level in the tissues (Mello, 1975).

Two chromium compounds were used in the present study for assessing toxicity avoidance. Chromium is a known toxic agent, but it is not known under which form and at what concentration it has an effect as may be seen by the broad ranges of effects in App. A.2. Two different salts were chosen based on their chemical characteristics, to cover the range of chemical forms in which chromium can be found in nature. Potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) and chromic nitrate ($\text{Cr}(\text{NO}_3)_3 \cdot 7\text{H}_2\text{O}$) were the forms

tested. When Cr(VI) was used, all chromium in solution was assumed to be in the hexavalent form, due to the slow reduction of Cr(VI) to Cr(III) with a half life up to 1200 hrs (Benson, 1968). The equilibria relevant to the study for Cr(VI) in aqueous solution are (Kemp, 1972)



indicating that $\text{Cr}_2\text{O}_7^{2-}$ is the predominant species under the experimental conditions, while the H_2CrO_4 is the reactive form of Cr(VI) in oxidation mechanisms.

When Cr(III) is injected in the channel CrOH^{2+} , $\text{Cr}(\text{OH})_2^+$ and Cr^{3+} are expected to exist in solution, with $\text{Cr}(\text{OH})_2^+$ being the most abundant in solution.

App. A.3. shows pH dependence and maximum concentration of the three most simple Cr(III) ions. Note that the concentration of ions with 1, 2, and 3 positive charges decrease by 1, 2, and 3 orders of magnitude for each increase of one pH unit. The kinetic inertness of Cr(III) causes the occurrence of many complexes. A dimer and soluble polymeric species also exist in the solution, but their biological activity is unknown. In nature, chromium ions are complexed with other ligands that may be soluble. Only with known composition of the medium, one may find the most abundant complexes by aid of the compilation of stability constants (Fergusson, 1982).

The Cr(III) complexes are numerous (thousands). Their principal characteristic is their relative inertness in aqueous solu-

tions. Ligand-displacement reactions of Cr(III) complexes are very slow (half-life in the order of 40 hrs). They persist for long periods of time in solution, even under thermodynamically unstable conditions (Fergusson, 1982).

2.1.5.2 Uses of chromium

Chromium is used to harden steel, manufacture stainless steel and form useful alloys. It is also used to give glass an emerald color and is widely used as a catalyst. Dichromates are used as oxidizing agents, and in tanning leather. Lead chromate is used as a pigment, and in the textile industry as mordants and by aircraft and other industries for anodizing aluminum. The refractory industry uses chromite for forming bricks and shapes. It is also used in cooling towers of large air conditioning installations.

Table 2.1 summarizes the uses of chromium in the industry along with information on other metals.

2.1.5.3 Chemistry of copper

Copper is one of the most abundant trace metals. It has an atomic number of 29 and atomic weight of 63. Copper is not encountered as free ion in the aquatic environment and its oxidation state is usually Cu^{2+} .

In aqueous media, copper forms aquo ions $[\text{Cu}(\text{H}_2\text{O})_6]^{2+}$ in the absence of other interfering agents. Alkalinity, pH and hardness are the major modifying factors in copper speciation (Spear and Pierce, 1980). Under the given experimental conditions the most

Table 2.1 Major Trace Metals in Water Effluents from a Variety of Industries (after Fergusson, 1982)

[illegible]

abundant species is expected to be CuCO_3 with Cu^{2+} and CuOH^+ found at similar concentrations but approximately 100 times less than CuCO_3 . Next is $\text{Cu}(\text{CO}_3)_2^{2-}$, with a 1000 fold lower concentration compared to CuCO_3 . Finally $\text{Cu}(\text{OH})_3^-$ exists in concentrations 1000 lower than CuCO_3 (Spear and Pierce, 1980).

With organic ligands, copper forms the most stable organic complexes. The bonding strength in copper-organic complexes was highest compared to all other divalent ions regardless of type or concentration of ligand (Spear and Pierce, 1980). Complexation capacity of organic ligands towards copper depends on pH and water hardness.

In terms of copper solubility, water hardness and pH play the major role, with decreasing solubility as pH and bicarbonate levels increase. At constant pH, solubility increases with increasing bicarbonate levels.

In general, dissolved copper levels in Canadian surface waters do not exceed $5\mu\text{g/l}$. Copper exists in nature as non-labile co-ordination complexes and adsorbed forms. Rivers have a higher assimilative capacity due to the presence of particulate matter held in suspension providing a substantial adsorptive surface. Recommended standards for public water supplies is $1000\mu\text{g/l}$, while for protection of aquatic life, the level is at $5\mu\text{g/l}$ (US EPA 1976).

In terms of bioaccumulation potential of copper, Duffus (1980) suggested no evidence of food chain magnification. He proposed that copper's toxic action is due to immediate exposure to the element. This is in agreement with Hodson et al. (1979)

who suggested that labile and not ingested or adsorbed copper is causing toxic effects. Whole body copper concentrations decrease with increasing trophic level. Consuming organisms may be capable of organ specific accumulation and metabolic regulation. On the other end of the trophic levels, plankton bioaccumulated copper at 90,000 times the ambient concentration (Spear and Pierce, 1979).

Copper inhibits oxygen consumption for aquatic biota which is proposed as a mode of toxic action (Hodson et al., 1979). Impairment of osmotic and ionic regulation is proposed as possible causes of death for invertebrates. In fish, mainly osmoregulatory impairment is associated with death. For sublethal concentrations, the lowest levels of toxicant concentration causing physiological and reproductive impairment were comparable with upper levels of toxicant concentrations responsible for behavioural (avoidance) reactions (Spear and Pierce, 1979). (Also in Fig. 2.3.)

A tabulation of the available information on copper and the toxicity of various pollutants is found in Beitinger and Freeman, 1983, and Hara et al., 1983. According to the data, fish are affected by copper well below lethal levels and some species could disappear without direct evidence of mortality (Hodson et al., 1979). Fish also demonstrated a potential for acclimation in copper resulting in increased tolerance at lethal levels (Hodson et al., 1979; Dixon and Sprague, 1981).

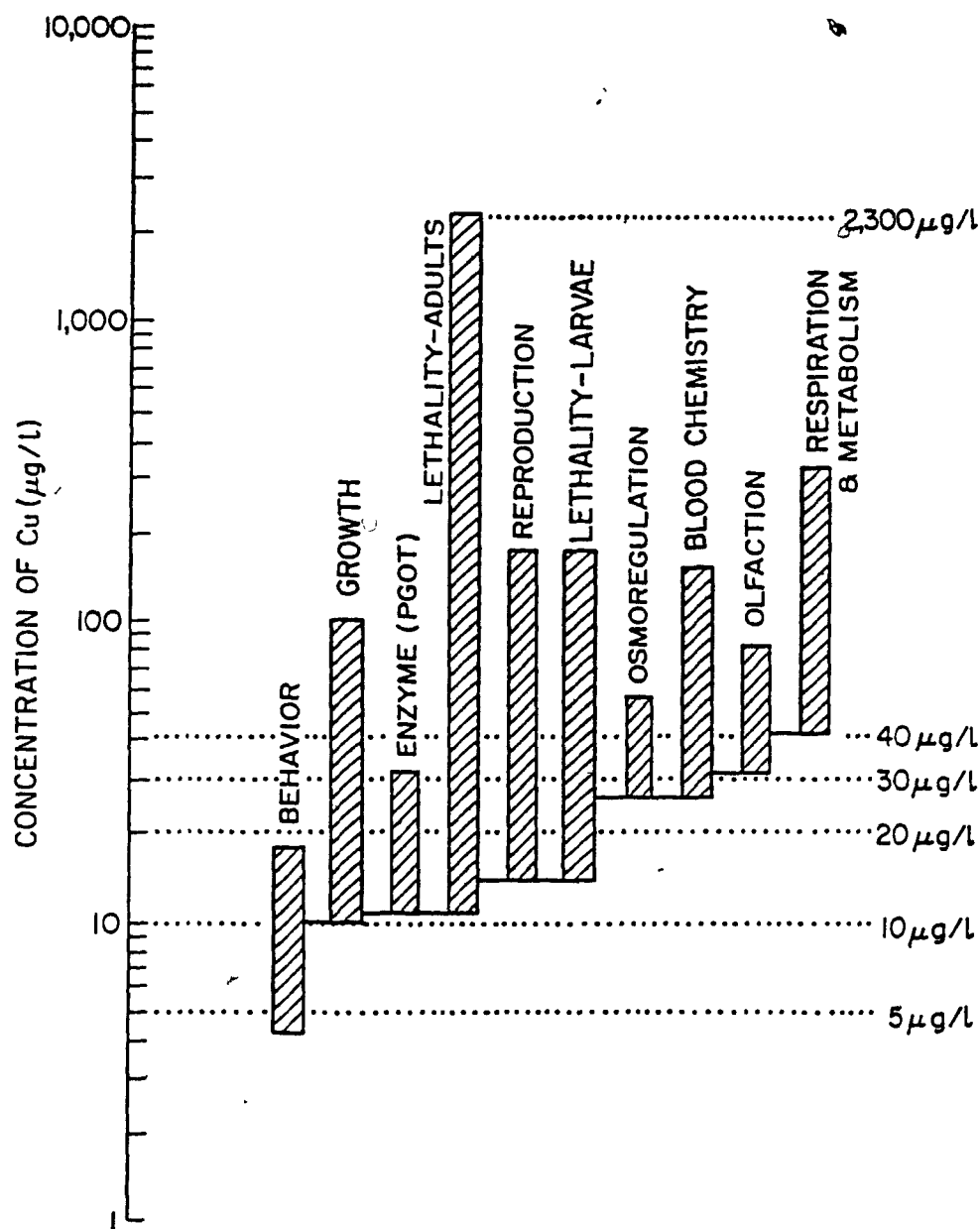


Figure 2.3 The ranges of Cu threshold concentrations known to elicit various effects in freshwater fish (after Hodson *et al.*, 1979).

2.1.5.4 Sources of copper

Copper found in water is a waste product of human activities such as in the textile industries, as an anti-fouling agent in paints, in the manufacture of electrical products, in electroplating and smelting. Copper compounds are also used as fungicides, pesticides, algaecides and piscicides.

2.2 Approach in the present study

2.2.1 Need for the study

Various investigators (see 5.2.2) have expressed a need for research on the impact of previous short or long term exposure and adaptation to the effect of toxicants at sublethal levels. A development of a protocol for sublethal testing, especially when studying fish avoidance reactions was necessary. More information was also needed on the mechanisms by which fish detect and avoid chemicals. Information on the sublethal effects of chromium and particularly avoidance, was scarce and inconclusive and information on lethal levels varied widely (App. A.2).

2.2.2 Avoidance-preference as a tool for assessing toxicant impact on organisms

Behaviour has been recognized as a method of assessing the condition and well being of an organism (Hodson et al., 1979). Certain behavioural characteristics may be associated with normality, while others may be associated with illness, injury, and fatigue (Cherry and Cairns, 1980).

Avoidance-preference is considered one of the most sensitive predictors of toxicant impact on organisms. Comparison of avoidance data with lethal threshold values provide more sensitive estimates for safe levels of toxicity (Buikema et al., 1982). In addition, concentrations at which fish start exhibiting avoidance reactions may be correlated with threshold reproductive impairment concentrations (Buikema et al., 1982; Hodson et al., 1979) (Fig.2.3).

2.2.3 The element of pre-exposure in the present study

In natural situations, background concentrations of toxicants are present in the water, while bioassays were normally conducted using organisms reared in purified water. Extrapolation of lab results were questionable in terms of their applicability in a natural ecosystem.

The use of pre-exposed fish in the present study provides information on the modification of fish behaviour due to pre-exposure, and the range of pre-exposure concentrations that can be handled by fish homeostatic mechanisms, without causing irreversible effects (Fig.2.4).

2.2.4 Factors affecting the outcome of avoidance tests

The measurable parameter in the present study is fish avoidance reaction. In a laboratory simulation of natural conditions, the following expression can be established:

$$\begin{array}{l} \text{Fish} \\ \text{Avoidance} \\ \text{Reaction} \end{array} = \text{fn} \left(\begin{array}{l} \text{System Hydraulics, System Chemistry,} \\ \text{Organism Biology and Social Behaviour,} \\ \text{Experimental Procedures} \end{array} \right) \quad (2.1)$$

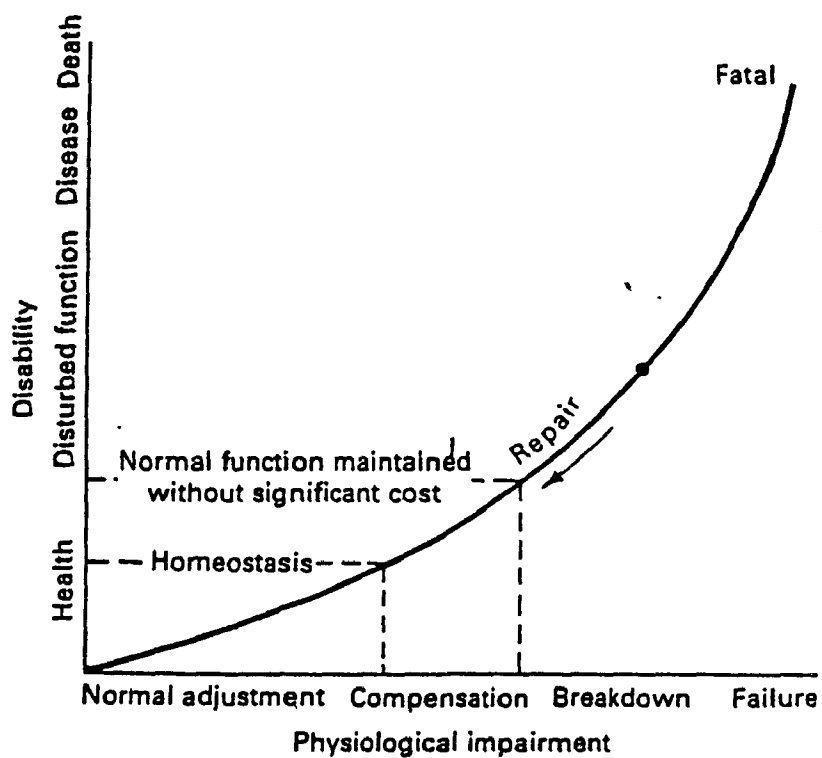


Figure 2.4 The relationship between physiological impairment following increasing exposure to pollutants and the consequent disability of an organism (after Lloyd, 1972).

All parameters noted in equation 2.1 have a significant impact on the outcome of the avoidance-preference tests. They directly affect the transference and transformation of the toxicant in the water (Hydraulics, Chemistry, Biology) while repeatability of avoidance data lies with two factors that were not given the appropriate attention in the past (Social behaviour, Experimental procedures).

2.2.5 Critical parameters for avoidance-preference testing

The goal of any experimental design is to yield reliable and reproducible results. It is believed that poor reproducibility of avoidance data in the past is the result of insufficient consideration of the parameters involved, especially those involving the experimental protocol.

A list of avoidance-preference classification parameters is presented on Table 2.2. Critical parameters are those considered essential for the reproducibility of results. At the hydraulic level the reproducibility of the concentration field depends on the time to reach steady-state in the channel. This is the situation when the concentration field is stable throughout the channel. Steady-state time is different from retention time, which is the time it takes the injected toxicant to travel from the cross-section of injection to the channel end gate. In the present study retention time was of the order of 6 minutes, while steady state was established after 15 minutes.

Standard water quality and known speciation of the toxicant are imperative to yield reproducible avoidance results.

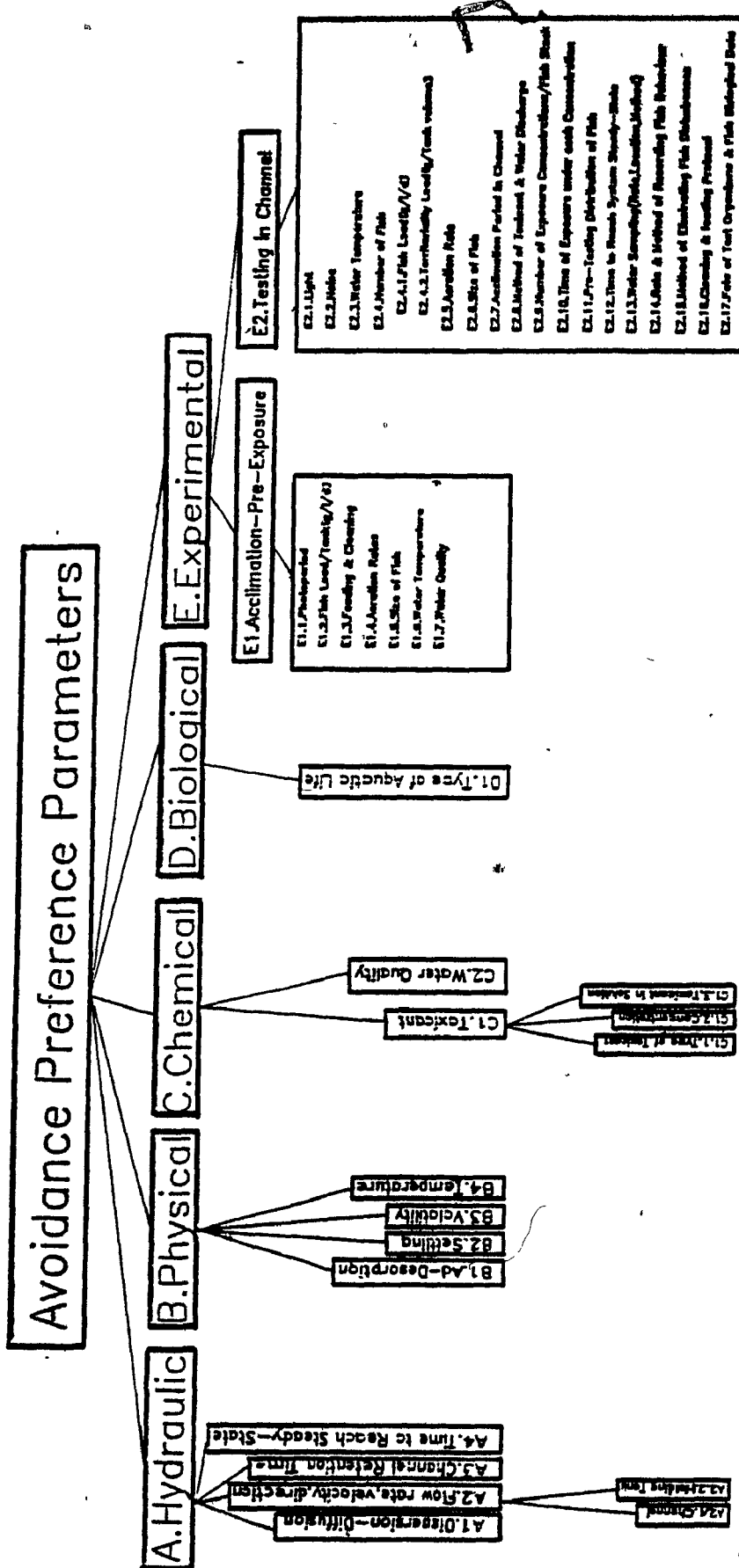


Table 2.2 Avoidance preference classification parameters

For a fixed experimental protocol the following parameters are critical to the reproducibility of results:

Territoriality load expressed as [total fish weight / tank volume]. Availability of adequate space for normal social behaviour is highly significant to the reproducibility of results. If this parameter is violated, avoidance reactions are masked due to aggressiveness of individuals in the process to establish their territorial space. This parameter is affected by the size and developmental stage of the organisms.

Channel acclimation period [T]. Sufficient time should be provided for the organisms to familiarize with the geometry of the avoidance apparatus as well as to recover from the stressful experience during their introduction in the channel.

Fish distribution prior to actual testing. (A.P.D.) Each group of individuals used during experimentation does not necessarily distribute similarly throughout the channel. Any fixed consideration of initial fish distribution, such as 50/50 in the past, introduces a random bias factor if the distribution is not actually the assumed one, resulting in poor data reproducibility.

Time for fish to reach steady-state. This is the time it takes fish to establish their positions in the channel, manifested through infrequent position changes and relaxed swimming. It is longer than the time to reach channel steady-state. The sequence for the present study was 6 minutes retention, 15 minutes channel steady-state, 20 minutes fish steady-state. Readings obtained prior to fish steady-state are prone to errors and poor reproducibility of results.

3.0 Materials and Methods

3.1 Materials

The present study employed a continuous flow-through system. This system consisted of 5 basic components (Figure 3.1):

- . Water Treatment (1)
- . Holding and Pre-exposure Facilities (2)
- . Temperature Control (3)
- . The Channel (4)
- . Data Acquisition (5)

A brief description of each component follows.

3.1.1 Water treatment

Since the available water source is the city of Montreal water supply, and standard water quality is a prerequisite for this type of experiment, an additional purification system was incorporated to guarantee standard water quality in the system. It consisted of:

- (a) an auto-activated carbon filter (A.C.) (DURO AAC-24) with automatic multi-valve control for backwashing, and
- (b) ultra-violet light water sterilizer (Aquafine MP-2-SL).

The City of Montreal water passed through the A.C., where organics, amines, taste, color and chlorine were removed (efficiency up to 95%) and was then sterilized by the ultra-violet lights (>99% efficiency in microorganism reduction), to ensure that no bacteria may affect the fish, in the holding and pre-exposure tanks as well as during the experiments in the channel.

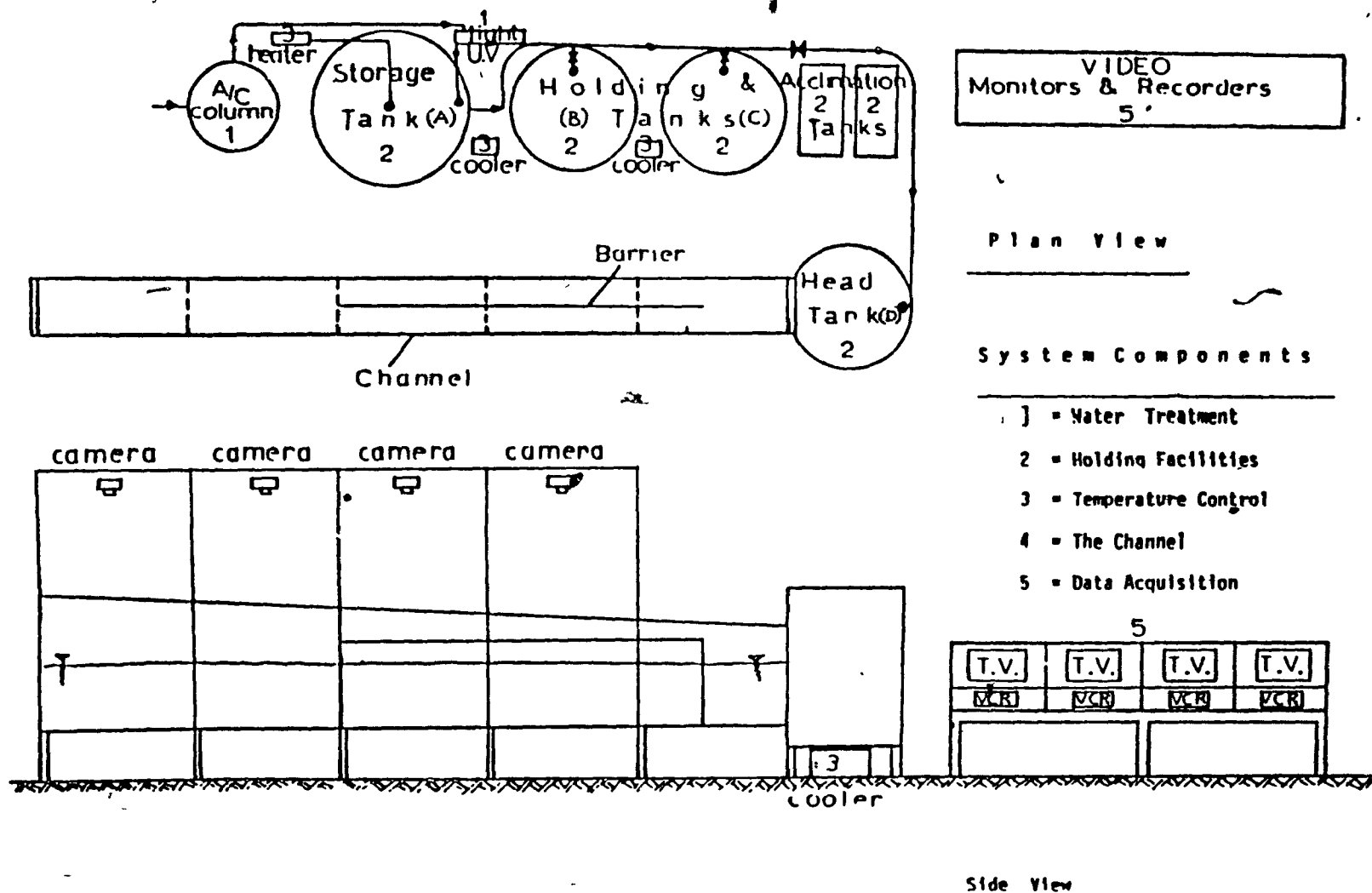


Figure 3.1 Experimental set-up.

3.1.2 Holding and pre-exposure facilities

The facility was comprised of:

- . 4 polyethylene drums (tanks A,B,C,D)
- . 2 rectangular tanks
- . 7 flowmeters
- . 4 air pumps
- . 3 water pumps.

Tank A, with a capacity of 1900 litres, was the storage reservoir for purified water. The temperature was regulated accordingly, and a floater configuration controlled the water level while preventing gas supersaturation during the winter months, which may cause gas bubble disease to the fish. It also served as an emergency reservoir of water in the event of temporary system failure or when the A.C. filter was backwashed. The required head for water circulation in the system was provided by a magnetic drive pump (March DP-GT-MD) located downstream from Tank A at a capacity of 130 l/min.

Tank C was used as holding tank for the non-exposed fish population, and had a capacity of 1200 litres. A flowmeter was attached with a 2.54 cm pipe to provide adjustable continuous flow. Air flow was regulated by an independent air pump (WISA model 200) and distributed through air stones. Standard 5.08 cm stand pipes control the overflow in the center of the drums and allow for drainage during cleaning.

Tank B (identical with Tank C) together with the two rectangular tanks (capacity 400 litres each) were used for pre-exposing fish to toxicants. Air was provided through independent air

pumps (Hagen Optima) and airstones. The drain configuration in Tank B was similar to that of Tank C, while for the rectangular tanks a 10 cm valve was allowing for drainage, which was accomplished through a perforated circular center drain, mounted flush with the bottom of the tanks. Water was regulated by flowmeters.

Tank D, capacity 1200 litres, was the head tank for the test channel. It receives 11-72 l/min water regulated by a flowmeter located downstream from Tank C. A 30 x 40 hole on the side of the tank, 25 cm from the rim, allows the water to enter in the channel. Two more centrifugal pumps were used to introduce the toxicant in the toxicant discharge zone of the channel, and create identical flow conditions in the clear water zone compared to those in the toxicant discharge, to eliminate bias factors due to differential discharge conditions. The rate of discharge was regulated by flowmeters, downstream from each pump.

3.1.3 Temperature control

A submerged heating unit (KTL-318-1 WMMR) was installed in Tank A, with an automatic temperature control device (Chromalox HCP-60). It provided a constant temperature of $14.5 \pm 1^{\circ}\text{C}$ to the system from October to May.

In the summer, portable cooling units were provided for each of the fish pre-exposure tanks (batch exposure), while water for the system and Tank B (continuous flow) was cooled down to 14.5°C by a large capacity cooling unit (60,000 BTU) immersed in Tank A.

Waterproof temperature probes were used in conjunction with a YSI Model 44 Tele-Thermometer to obtain temperature readings on

the different system components.

3.1.4 Test channel

The test channel was 9.15 m long, 30.5 cm wide, and the depth of flow was 30.00 cm. It was divided into 5 sections of 1.83 m each (Fig. 3.2). The sidewalls of the first section were made of plexiglass, while the other sections were constructed of glass.

The flow was split into two streams in the first upstream section of the channel by a 6.35 mm thick plexiglass barrier placed on the center line of the channel, and extending from the middle of the first section to the end of the third (4.60 m). It was partly glued at the bottom, partly removable, to allow for easier cleaning when the water was drained. This separation device is introduced to give the fish a choice between the toxicant discharge zone and the clear water side of the channel (Fig. 3.2).

18 fish were used per experimental run and were confined via two perforated honeycomb gates within the 3 mid sections of the channel. The first upstream section was used to accommodate toxicant and clear water injections through multiport diffusers, and provide space for complete mixing of the toxicant with the main water stream through a multistage impeller (Fig. 3.3). The last downstream section was used as a buffering zone to provide an undisturbed flow field within the testing region (3 mid sections). The drain was of an overflow configuration over the end gate of the channel. The end gate was removable for rapid drain-

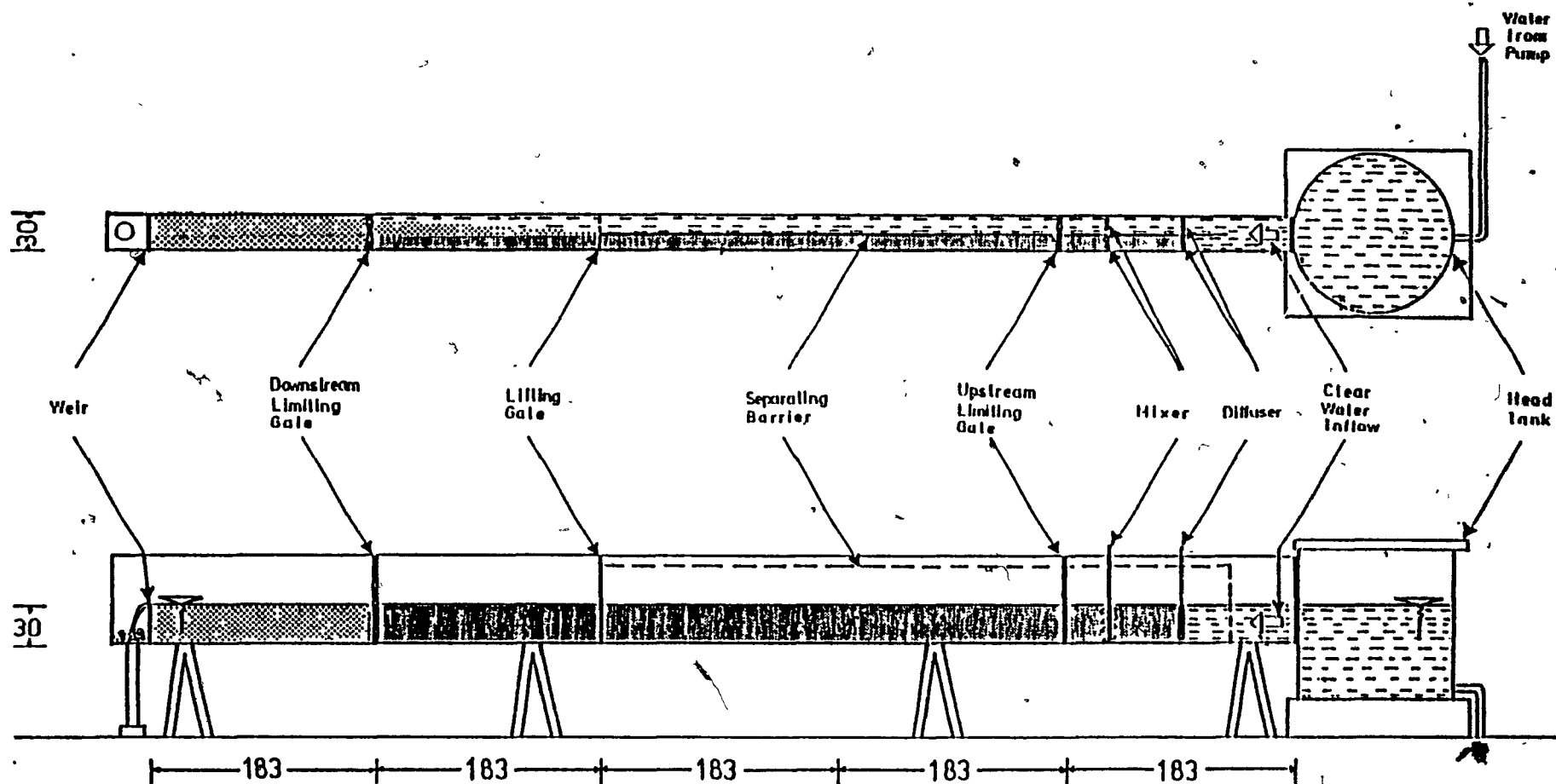


Figure 3.2 The avoidance-preference channel (all dimensions in cm).

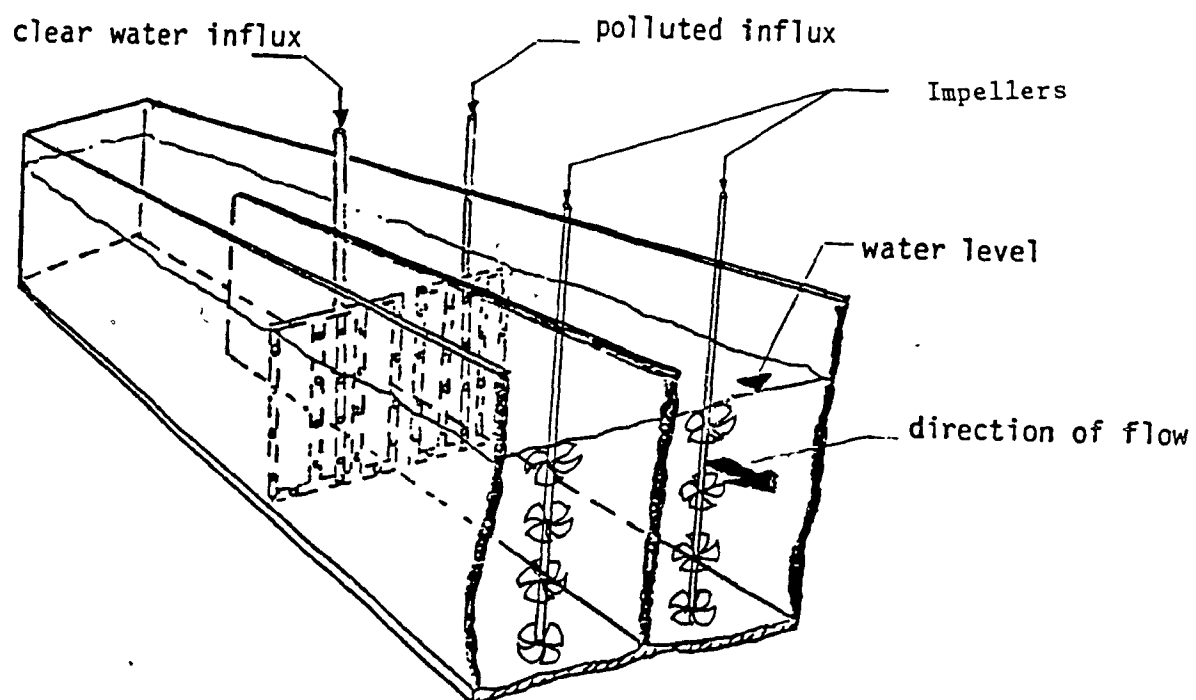


Figure 3.3 Details of diffusers and mixing impellers.

ing and ease in channel cleaning.

The third section of the channel, which was undivided, provided fish with adequate space and a constant shallow gradient concentration field. This allowed fish to select the concentration they preferred, ranging from the level within the toxicant discharge zone to that in the clear water zone.

A lifting gate was installed downstream from the edge of the barrier to confine fish in the non-separated section during the injection of the first concentration of the toxicant prior to establishing steady-state conditions in the channel. After 15 minutes (time to reach steady state), the gate was lifted, allowing swimming throughout the testing area of the channel.

The bottom of the channel was covered with teflon inserts kept in place with silicone glue. Squares were drawn on the inserts defining thus a reference system for monitoring the fish position. Each square was 10 cm x 10 cm, forming a 18 x 3 square grid on the bottom within the area of interest for the present study.

To allow for a three-dimensional view of the fish position in the channel, mirrors were placed along one side of the 3 sections, at a 45° angle. They also served as visual barriers so as not to disturb the fish from external sources. Additional portable separators were used to protect fish from disturbances during actual testing in the channel with consequent influence on their behaviour. Three video cameras, positioned over the channel, were used to record the sequence of events during each experimental run.

3.1.5 Data acquisition

During an experimental run, data were collected for the events in the three mid-sections of the channel. Three cameras were positioned over these sections at 2.9 m above the channel, on moveable trolleys. Their visual field was set at wide angle setting, for a coverage of 5.49 m (1.83 m/camera), providing total coverage of the area of interest.

Events are monitored on T.V. screens, and simultaneously recorded on tapes through three VCR's. This allowed for further evaluation/analysis of the experimental data. All video gear were Panasonic.

3.1.6 Organisms

Yearling rainbow trout were used in the bioassay. They were obtained from a Quebec commercial hatchery. Fish when received were between 5-7 cm long and weighed 2-3 g. They were maintained under flowthrough conditions and O₂ concentrations at 90% of saturation level, for a minimum of two weeks in clear water prior to introducing them in the pre-exposure tanks. Density of fish in all holding and pre-exposure tanks never exceeded 0.75 g per litre of water per day, compared to the 1-2 g/l/d suggested by bioassay standards (EPS, 1980; APHA, 1980).

The water temperature in all pre-exposure tanks was maintained at 14.5 ± 1.0°C (McCauley et al., 1977). In the winter a simple flexible duct system supplied cold outside air which was regulated with air vents to maintain the temperature in the tanks at the desired level. During the summer portable coolers were

used to maintain the temperature at the pre-determined level.

3.2 Methods

3.2.1 Channel characteristics

The flow characteristics and concentration distribution in the channel were established through tracer studies using methylene blue injections. Flow rates for toxicant injections were determined from preliminary experiments to obtain uniform mixing and avoid settling of the injected solution at low toxicant flows. The channel discharge was set at the actual experimental level of 28.5 l/min in each separated section, yielding a velocity of 1.1 cm/s in the entire channel.

Methylene blue concentrate was pumped into the toxicant discharge zone through a multiport diffuser (Fig. 3.3). Complete mixing was achieved via a multistage impeller located 30 cm downstream from the diffuser cross-section (Fig. 3.3).

Sampling was performed in order to establish lateral, longitudinal and depth profiles throughout the channel under actual experimental conditions. Samples were drawn simultaneously from 8 different cross-sections spaced along the length of the channel. Five samples/depth at 4 different depths were drawn per cross-section using a suction pump, pipettes and flexible tubing over a 60 minute period. All samples were analyzed spectrophotometrically at 652 nm. The dilution capacity of the channel was established by comparing the sample concentration to the concentration of the methylene blue concentrate.

The dye concentration was constant with depth in the non-

separated zone. In the separated toxicant discharge zone, the concentration was uniform throughout at steady state. The dilution capacity of the channel in the separated section was established to be $S = 0.0116$ (S = channel measured concentration / stock solution concentration) at steady-state. The discharge rates were 57 l/min for the channel and 400 ml/min for the toxicant flow.

3.2.2 Fish maintenance

Fish were received at a length between 5-7 cm and were maintained in holding tank C for 2 weeks prior to pre-exposure and testing. Fish were fed twice daily for fish up to 8 cm of length and once a day for fish greater than 8 cm.

Fish were always maintained at a fish load per tank less than 1 g/l/d.

3.2.3 Pre-exposure of the fish to toxicants

Fish were transferred to the pre-exposure tanks where they were exposed to sublethal levels of toxicant, for a minimum period of 7 weeks. Solutions were changed daily in the pre-exposure tanks. This pre-exposure period was extended in the case of Cr(VI) up to 20 weeks due to mortality at the 3.0 mg/l pre-exposure level. The rationale was to perform avoidance tests on stable populations. A population was considered stabilized (steady-state of pre-exposure), when no apparent differences in mortalities were observed between pre-exposed and non-exposed fish for two weeks.

Sampling of pre-exposure solutions was performed randomly prior and after changing the solution in order to establish the rate of toxicant loss in the tank over the period of a day.

3.2.4 Avoidance-Preference protocol

Experiments were performed in the channel using rainbow trout with an average test weight of 50 (range 37-64) g and overall length 14 (range 12-17) cm. The water temperature was maintained at $14.5 \pm 1^{\circ}\text{C}$ and the water characteristics averaged 100 mg/l total hardness as CaCO_3 , 50 mg/l alkalinity as CaCO_3 and $\text{pH} = 7.2$ (see also App. B.1).

3.2.4.1 Channel acclimation

Prior to testing, fish were acclimated for 2 or 7 days in the experimental channel under clear water flow through conditions.

3.2.4.2 Avoidance-Preference testing with single and step function increasing concentrations

Individual concentrated solutions of the toxicant were prepared for each of the concentrations scheduled for testing. They were delivered to the channel by means of a pump and flow regulator.

Prior to starting the experiment, fifteen readings of the fish distribution were taken one minute apart. They were used as the pre-testing reference fish distribution. The fish were then restricted to the third non-separated section of the channel for

15 minutes prior to toxicant injection. After 15 minutes from initiation of the toxicant injection (Channel steady-state condition), fish were permitted free movement in the channel. Avoidance reaction readings were recorded at fish steady-state distribution, which for the present apparatus and conditions was established at 20 minutes. 15 readings at 1 minute intervals (21-35 minutes from initiation of toxicant injection) were considered adequate to establish the avoidance-preference reaction of a population exposed at the set toxicant concentration.

During single concentration testing, the recording of data was extended to 60 minutes to account for probable differences in reactions due to longer recording periods. Data suggested that once fish had reached steady state distribution (after 20 minutes from injection) extension in time of exposure at each level beyond 35 minutes was not justified.

During multiple concentration or step function increasing concentration testing, a new higher concentration level of toxicant was introduced in the channel following a previous 35 min channel exposure period. This step was repeated depending on the number of concentration levels being tested. Fish tended to maintain positions on either side of the channel. Occasionally, fish were positioned in the mixing zone (Fig 4.1), and considered as avoiding or not depending on their location relative to the channel centre line.

Throughout the testing in the channel, clear water was injected within the clear water zone at identical discharge conditions as in the toxicant discharge zone.

Fish that were used for 2 or 7 days channel acclimation periods were discarded following testing.

3.3 Data evaluation and analysis

Each experimental point on the avoidance curves presented in this study is based on 3 replicate experiments and 15 readings per experiment recorded at system steady state. The total of 45 readings were combined to produce a mean and a standard deviation value that was plotted on the avoidance curve. Statistical significance of all results was assessed at the 0.05 probability level. The sequence for handling and analyzing raw data is described below.

The 15 readings taken before injection of the toxicant were combined to yield the average pre-testing distribution (A.P.D.). The significance of this parameter was outlined in section 2.2.5.

The fish distribution as a percentage was calculated by Eq. (3.1)

$$\% \text{ fish in clear water zone} = \left[0.5 \times \frac{\text{N.F.P.} - \text{A.P.D.}}{\text{A.P.D.}} \right] \times 100 \quad (3.1)$$

where N.F.P. = actual number of fish in the toxicant zone
A.P.D. = average pre-testing distribution

The mean of 15 readings was calculated for each test concentration during a single experiment (5 concentrations per experiment). This process was repeated for each of the three replicate experiments.

A one way analysis of variance (ANOVA) was performed on each

set of the 3 replicates for data obtained under identical conditions. Thus the similarity of mean avoidance reactions from the three replicate experiments was established. % of fish in clear was used as the dependent variable and the number of replicate experiment as the independent variable in the ANOVA. In all cases, no significant differences were detected between means obtained from the 3 replicates. All data from the 3 replicates were combined, and the mean of all 45 readings (3 x 15), obtained under identical conditions, were used to produce the avoidance curves (% fish in clear vs. toxicant concentration in the channel). A regression analysis was performed on each set of data that produced the final avoidance curves using a SAS non-linear procedure in order to establish data trends (linear or otherwise) and inflection points.

In experiments involving testing pre-exposed populations under identical conditions (2 series per toxicant), a one-way ANOVA was performed with pre-exposure level as the independent variable and avoidance reaction as the dependent one. The procedure suggested whether pre-exposure led to differences in fish avoidance behaviour, while all other parameters were identical. On the ANOVA tables, if $(PR > F) < 0.05$, then the means of the compared populations are considered significantly different.

The validity of results using an ANOVA requires that several assumptions be satisfied. Variances associated with each treatment in the experiment are to be equal. Each treatment population should be normally distributed. The effects in the model must behave in an additive fashion and the errors are to be stat-

istically independent.

All the above assumptions were satisfied by the data sets. In addition, the experimental procedure introduced a time scale factor, only within each experimental run, since more than one effect was applied on the same population. ANOVA may be applied with a time scale factor since:

a) Experiments performed with single concentration per experiment yielded similar means in avoidance reactions, and

b) all populations considered in the ANOVA were subjected to identical treatment in the channel for each series of experiments involving identical sequence in concentrations and time exposure at each level.

A Duncan's multiple comparison test was performed on the set of data used for the avoidance curves. It served to classify the mean avoidance values into different categories, once it was established by the ANOVA that population means were statistically different. The values needed for the Duncan's test were selected based on a 0.05 probability level and in most cases 176 degrees of freedom. For DUNCAN results, only populations whose means are characterized with different letters (e.g. A,B,C) are considered significantly different.

Both restrictions for the validity of Duncan's test results, i.e. equal sample sizes and population means not significantly different between them, were satisfied by all sets of data. A multiple-linear regression procedure was used where regression analysis was needed, such as in the case of establishing the trend of changing avoidance threshold values with pre-exposure

level. Values of R^2 from regression analyses ranged between 0.88 - 0.99. For avoidance threshold variations $R^2 = 0.73$ for Cr(VI) and $R^2 = 0.82$ for Cr(II). All analyses of data were performed on the SAS system installed on the main frame of McGill University.

4.0 Results

4.1 Characterization of hydraulic channel and establishment of experimental test conditions

4.1.1 Channel hydraulics

Measurements are obtained in any laboratory experiment using adequate and reliable instrumentation. In the present study, the primary instrument for running the experiment, apart from the fish, is the experimental channel. The equivalent of a laboratory instrument calibration is the determination of the dispersion-dilution characteristics of the channel under actual experimental conditions. This was determined using methylene blue tracer injection.

Figure 4.1 demonstrates the lateral and longitudinal dilution characteristics of the hydraulic channel once the channel reached steady-state conditions. The dilution field may be translated into a concentration field by simply multiplying the dilution factor in the channel by the concentration of the stock that is injected into the toxicant discharge zone. Under actual experimental conditions, the concentration of any injected toxicant was established to be constant throughout the toxicant discharge zone after the channel reached steady-state. The time to reach steady-state was established at 15 minutes after the commencement of tracer injection.

For all experimental runs the dilution factor in the toxicant discharge zone of the channel was $S = 0.0116$, where

$$S = \frac{\text{toxicant discharge (1/min)}}{\text{channel discharge in the toxicant zone (1/min)}}$$

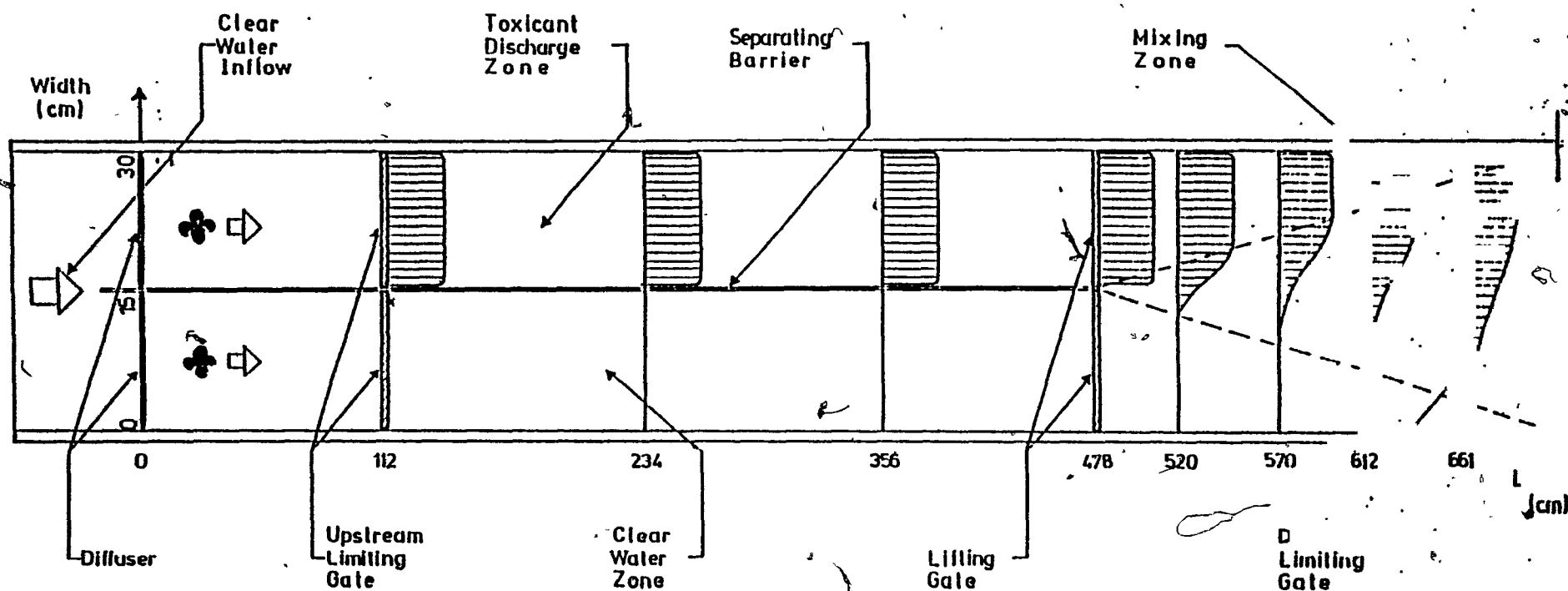


Figure 4.1 Lateral concentration distributions along the experimental channel for the selected test conditions (Dilution ratio in the toxicant discharge zone is constant at $S = 0.0116$, where $S = \text{toxicant discharge/channel discharge in the toxicant zone.}$)

The mixing zone at the downstream end of the channel can be characterized by three regions (Fig.4.1). All mixing took place within the dashed line prismatic region downstream from the edge of the dividing barrier. Outside the broken lines of Fig.4.1, both clear and toxicant stream concentrations were maintained at the separated stream levels.

The advantage of establishing the detailed concentration field under experimental conditions is that fish positions can be directly translated to actual toxicant concentration levels which fish prefer or avoid during experimentation. Thus, only occasional sampling is required for toxicant levels in the channel, simply to verify consistency between theoretically expected and actual toxicant concentrations. Overall deviations of actual vs. expected levels of toxicant in the channel ranged between -7 and +13% of the theoretically expected concentrations (See App.B.2).

4.1.2 Experimental test procedures

A battery of preliminary experiments was performed to establish

- a) Optimum number of test organisms based on fish load (g/l/day) and territoriality load (g/experimental channel volume)
- b) Experimental protocol (single concentration experiments vs. step function increasing concentration testing)
- c) Effect of length of acclimation of fish in the channel
- d) Effect of shallow versus steep concentration gradient in the experimental channel

4.1.2.1 Optimum number of fish

Standards for bioassays suggest, that fish should be exposed to a minimum of 1-2 litres of water per gram of fish per day (EPS, Env. Can.1980). Water flow in the hydraulic channel yielded 82 l/g/d throughout experimentation, far beyond that required by standards.

Since the present study concerns behavioural patterns in fish, the optimum number of test organisms in the channel should be defined in terms of the channel capacity to handle the population of fish with minimum aggressive behaviour. A minimum number of fish is required for statistical validity and meaningful results, since behaviour of one fish in a tank, as was the case for most avoidance studies in the past, is obviously a poor representation of an actual environmental situation. However, as the fish load increases, aggression may alter fish distribution due to territoriality load.

It was observed by several researchers (Gibson, 1978; Chiszar et al., 1975) that fish exhibit an aggressive behaviour when introduced into a confined space. For data reproducibility it was necessary to determine the maximum number of organisms that could co-exist in the channel without apparent fighting for territorial space, based on fish size and experimental apparatus and conditions. Experiments were performed using 15 to 30 fish per experiment and the results are presented in Fig.4.2.

When 30 fish were introduced to the channel, the level of their reaction to a ten fold increase of toxicant concentration was not significantly different over the range of toxicant con-

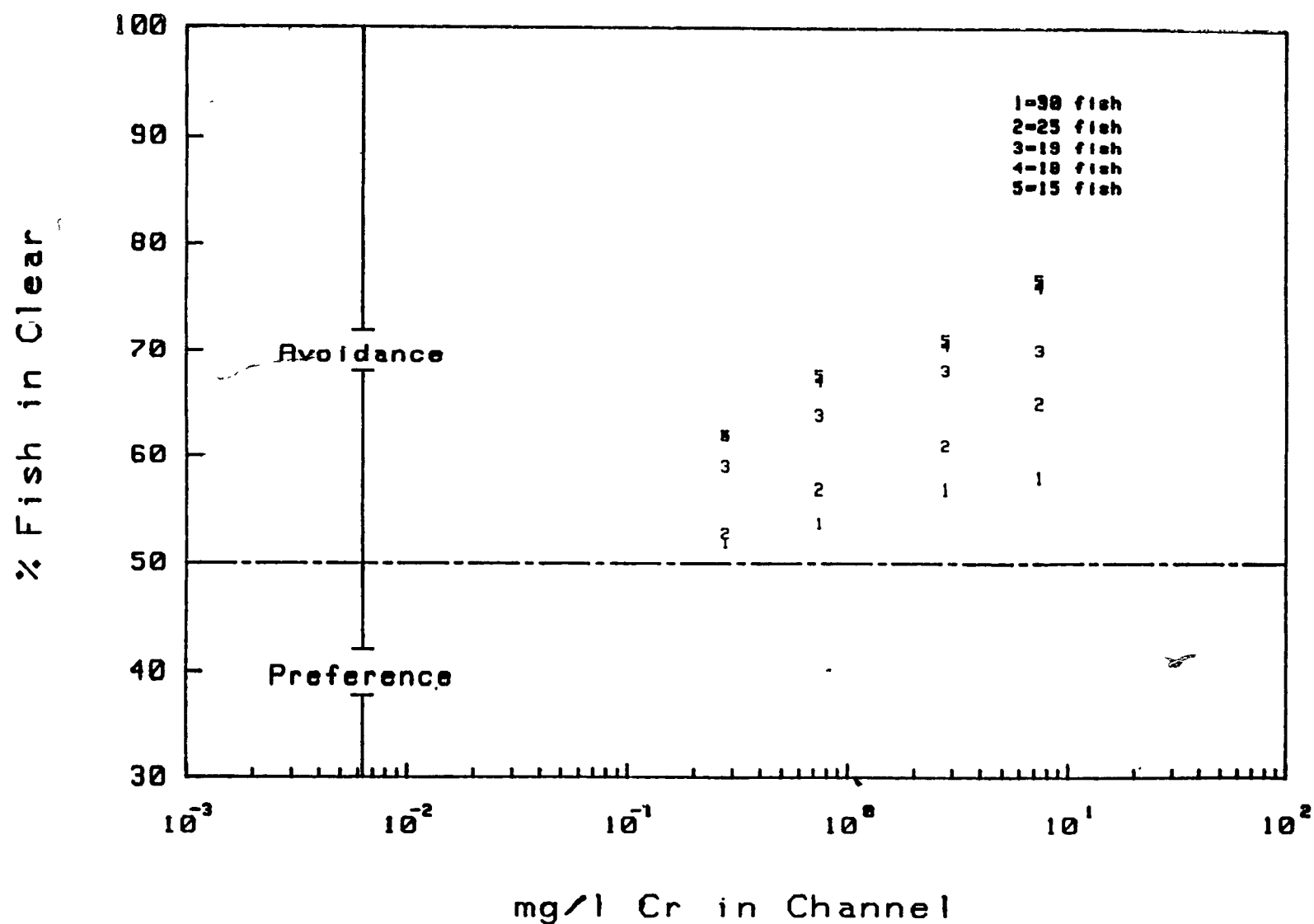


Figure 4.2 Avoidance reactions of different numbers of fish in the channel exposed under identical conditions to Cr(VI).

centrations used. In addition, fighting for territorial space was evident at all times, when fish were reacting to the presence of the toxicant attempting to evacuate the toxicant discharge zone. Eventually some fish were forced by other individuals to retreat to the position they occupied in the toxicant discharge zone prior to the toxicant injections, thus masking actual fish avoidance reaction levels. Therefore, it was decided that 30 fish of the size and age used cannot co-exist in the available volume of the tank and still provide useful avoidance data, since territoriality instincts masked their real avoidance reactions.

Similar results were obtained with 25 fish in the channel. Although avoidance reaction increased with higher toxicant concentrations in the channel, results were not significantly different for the different channel concentrations. Comparison to results with 30 fish in the channel also yielded no significant differences (Table 4.1).

Fish did react differently when exposed to higher concentrations of the toxicant in the channel, when 20 fish were used in the experiment. Again, aggressive behaviour was evident for the highest concentration to which fish were being exposed, resulting in some organisms retreating to the positions they occupied prior to toxicant injection.

When 15 fish were used in the channel, the results obtained were significantly different between all higher toxicant concentration levels (Table 4.1). There was no evidence of consistent aggressive behaviour in the channel, indicating that the volume of the tank was providing adequate territorial space for all



Cr(VI) Number of Fish in Channel 1=30 2=25 3=19 4=18 5=15	Channel Concentrations [mg/l]			
	0.3	0.8	3.0	8.0
	ANOVA	ANOVA	ANOVA	ANOVA
	F=48.36 PR >	F=73.27 F = 0 .	F=85.65 0 0 0 1	F=105.7
	DUNCAN	DUNCAN	DUNCAN	DUNCAN
	A 5 C 2 A 4 C 1 AB 3	A 5 C 2 A 4 CD1 B 3	A 5 C 2 A 4 D 1 AB 3	A 5 C 2 A 4 D 1 B 3

Table 4.1 Results of the ANOVA procedure and DUNCAN tests, from populations subjected to identical channel exposure to Cr(VI), with only variable the number of fish in the channel. (N=225, $\alpha = 0.05$, $DF_{ANOVA}=224$, $DF_{DUNCAN}=221$)

organisms.

The optimum number of fish was established at 18 under actual experimental conditions. The larger the number of organisms involved, the less weight each organism bears for the evaluation of the mean population reaction. Statistically, results based on 15 or 18 fish were not significantly different (Table 4.1). Mean avoidance values obtained using 19 fish were consistently lower although not statistically different (Table 4.1) compared to results based on 18 fish over the range of concentrations used. Therefore 18 was established as the maximum number of fish that the channel could handle to yield reproducible and representative results for avoidance preference reactions, under the set of experimental conditions used throughout this study. This yields a territoriality load of 2.09 g/l. For easier reference, the territoriality load can be established at 2 g/l.

4.1.2.2 Single versus step function increasing concentration testing

Two methods have been reported in the literature for running actual avoidance preference tests. One single concentration per experiment and step function exposure of the fish population to two or more concentrations of the toxicant. (Sprague, 1969, 1970; Sprague and Drury, 1969; Giattina et al., 1982; Scherer, 1975; Cherry et al., 1977).

The advantage of obtaining a larger set of information from a single population using step function testing is indisputable. The only major drawback was the questionable method for statisti-

cal analysis of such data, since widely used statistical tests presume independence of population to yield valid results.

For the present study, experiments exposing 18 fish to:

a) 1 concentration per experiment; b) 3 concentrations; c) 5 concentrations; and d) 6 concentrations per experiment, are presented on Fig.4.3.

Results were obtained running experiments employing one concentration per experiment for 0.3, 0.6, 0.8, 1.0 and 3.0 mg/l Cr(VI). The levels of avoidance reactions from single concentration experiments were almost identical for all concentration levels compared to those obtained from the multiple exposure experiments. Statistical tests suggested no significant differences between results obtained using either method (Table 4.2). In addition, one single concentration experiment with 0.6 mg/l Cr(VI) in the channel, yielded results that matched the level suggested by the established fish avoidance reaction curve (see also Fig. 4.5). This, in turn, suggests that results obtained by either method are compatible and the avoidance reaction curve can closely predict fish avoidance reactions for the entire concentration range employed in the present study. Experiments, using three concentrations per experiment, were performed employing 0.3, 0.8 and 3.0 mg/l Cr(VI) levels in the channel. The subsequent data for avoidance reactions yielded no statistically different results compared to either cases of single concentration or 5 concentrations per experiment (Table 4.2).

Results obtained using 5 concentrations (0.08, 0.3, 0.8, 3.0 and 8.0 mg/l Cr(VI) per experiment and those obtained using 1 or

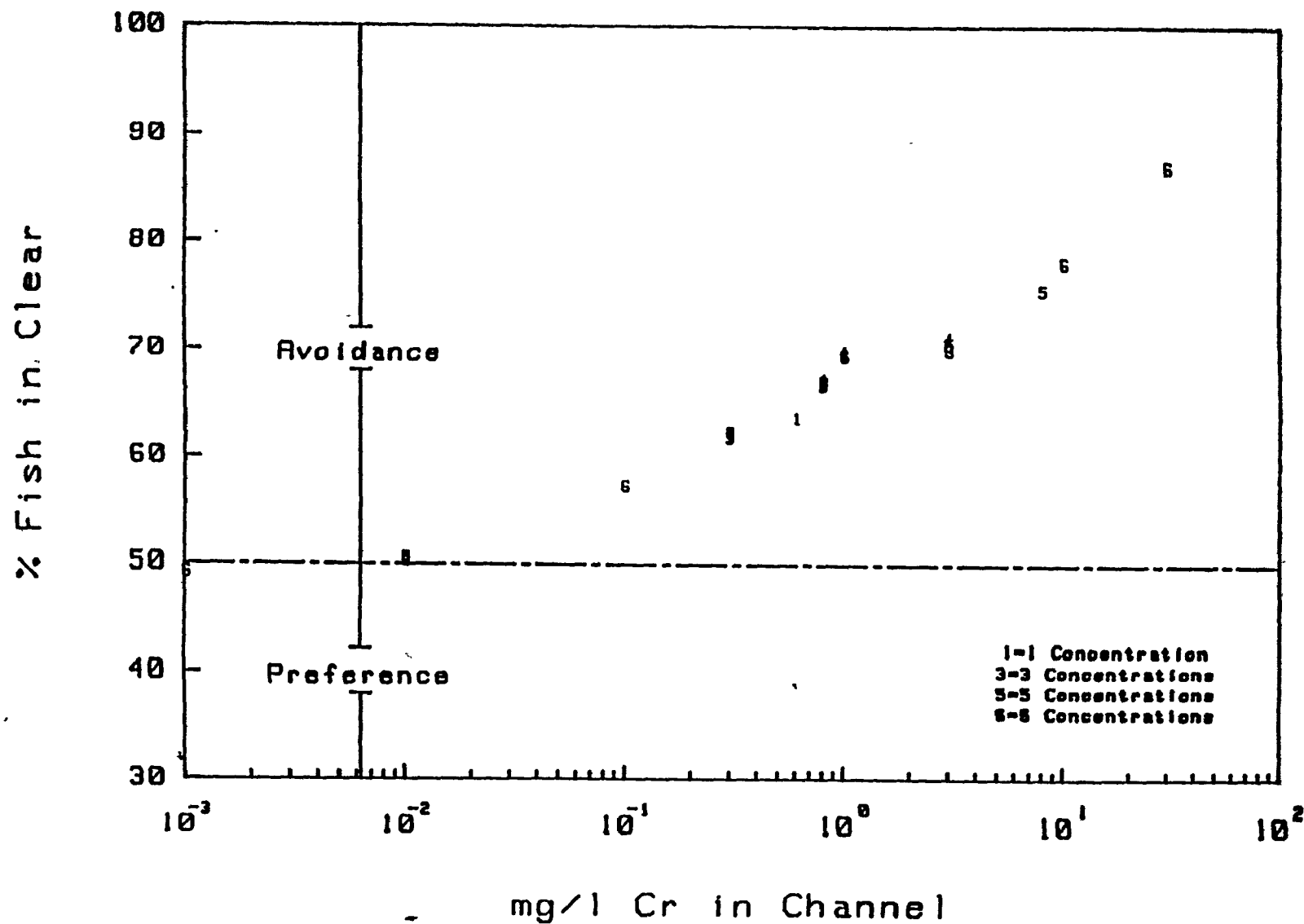


Figure 4.3 Avoidance-preference reactions of rainbow trout exposed to Cr(VI), using single and step function concentration methods of introducing the toxicant in the channel.

Cr(VI)	Channel Concentration [mg/l]		
	0.3	0.8	3.0
	ANOVA	ANOVA	ANOVA
	F=0.39	F=0.23	F=0.47
	PR>F=.83	PR>F=.93	PR>F=.71
	DUNCAN	DUNCAN	DUNCAN
1=1			
3=3	A 3 A 1	A 1 A 3	A 1 A 5
5=5	A 5	A 5	A 3

Table 4.2 Results of ANOVA procedure and DUNCAN tests, from populations subjected to identical chemical concentrations, with only variable the method of introduction of the toxicant in the channel (single vs. step function). (N=135, $\alpha = 0.05$, $DF_{ANOVA}=134$, $DF_{DUNCAN}=132$)

3 concentrations per experiment were not statistically different (Table 4.2).

The sequence of concentrations used for 6 levels per experimental run was 0.001, 0.01, 0.1, 1.0, 10.0 and 30.0 mg/l Cr(VI). Reaction at the only common level, 1.0 mg/l Cr(VI) for 1 conc./exp. yielded similar results (Table 4.2). No statistical comparison can be drawn for any other condition, due to different concentration levels used for the previous cases (1-5 concentrations/experiment). Again, the overall trend of avoidance reaction, over the range of concentrations employed is compatible with the one suggested by fish reactions established from experiments using 1-5 concentrations per experiment.

Overall, results obtained at steady state under the same experimental conditions, yielded no statistically significant differences due to the method of introducing the toxicant (single versus step function increasing concentration). More efficient multiple exposure of 5 or 6 levels per experimental run was adopted for the bulk of this project.

4.1.2.3 Channel acclimation period

Different periods of time have been used for acclimating fish in experimental tanks prior to avoidance testing (Sprague, 1968; Sprague and Drury, 1969; Scherer, 1975; Ishio, 1964; Biesinger et al., 1976; Giattina et al., 1981; Bogardus et al., 1976; Beitinger and Freeman, 1985.). Previous investigators employed channel acclimation periods ranging from 10 min to two days. To establish the effect of channel acclimation on avoidance, exper-

iments were performed with $\text{Cu}(\text{NO}_3)_2$ as toxicant, following acclimation times of a) 20 minutes b) 1 day and c) 2 days. Results for all acclimation times are presented in Fig.4.4. Three replicate experiments were performed following each acclimation period to establish reproducibility of results.

Results following 20 minutes of channel acclimation (0 days) gave avoidance reactions over a ten fold increase of toxicant concentrations, that were not significantly different. This suggests an indifference to the toxicant. In addition, the apparent trend of the avoidance reaction was not the same for all replicate experiments (Table 4.3).

Although there is a tendency for the avoidance to increase with increasing concentration, the increase was insignificant. Other stressors mask the actual avoidance reaction of the organisms, such as the netting experience during fish introduction in the channel. Fish have not yet settled after 20 minutes in the channel. This was manifested through fish being highly mobile and alternating places quite frequently compared to the rate of position change after a two day acclimation period.

Avoidance reaction of fish was evaluated after one day of acclimation in the channel and was significantly higher (Table 4.3) than avoidance reaction after 20 minutes acclimation in the channel, except at the $11.25 \mu\text{g/l}$ level. Results obtained from the replicate experiments did not behave consistently, being in some instances significantly different (Table 4.3). The overall trends suggested similarities for higher concentrations, but poor reproducibility and statistical differences indicated that

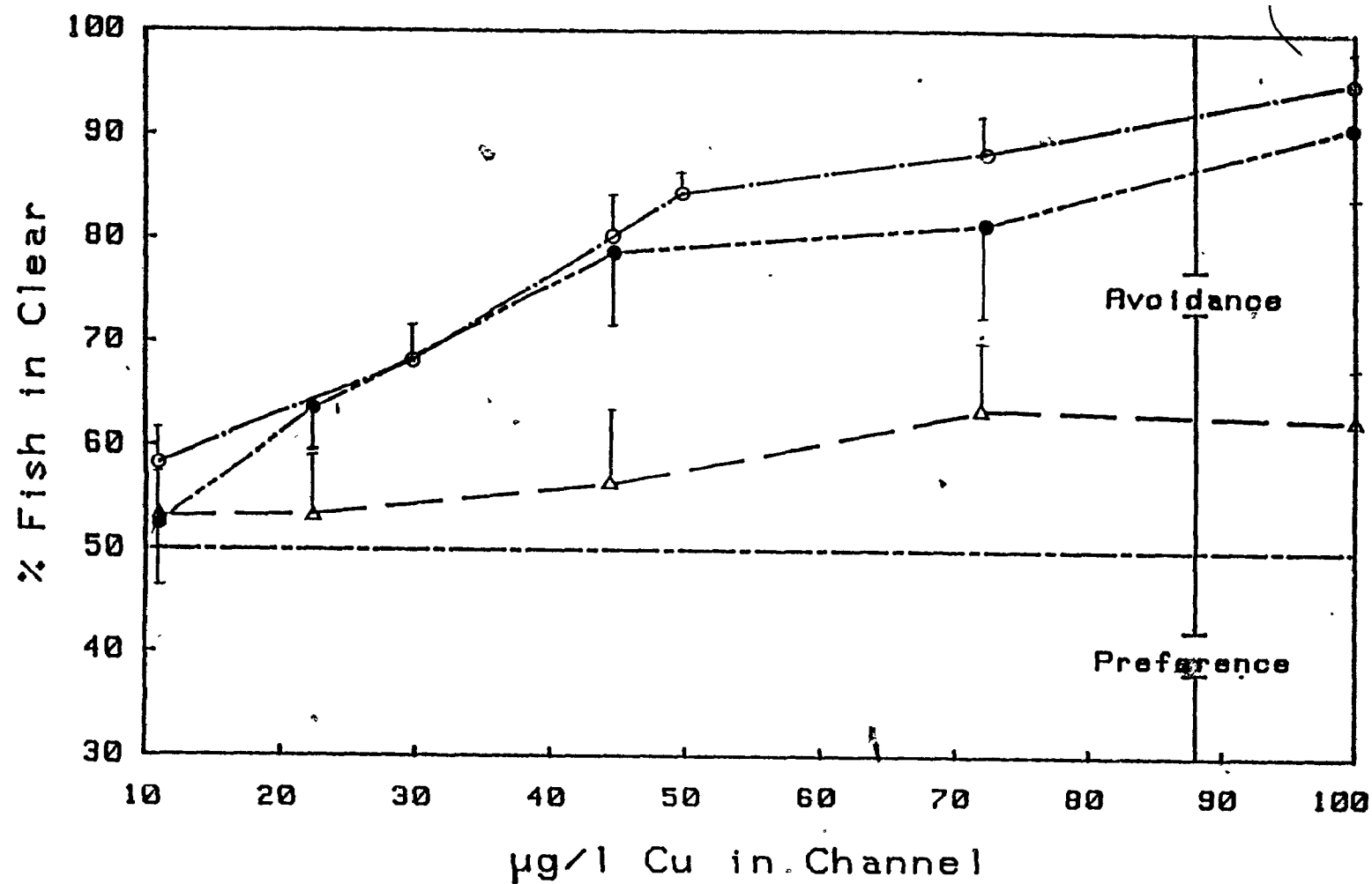


Figure 4.4 Avoidance-preference reactions of rainbow trout exposed to Cu(II), after 0 (Δ), 1 ($\bullet$) and 2 ($\circ$) days acclimation in the experimental channel.

Acclimation in Channel	Cu(II) Channel Concentration [ug/l]				
	11.25	22.5	45.0	72.5	100.0
	A	N	O	V	A
0 Days 1=Replicate #1 2=Replicate #2 3=Replicate #3	F=1.33 PR>F=.23	F=19.9 PR>F=.0001	F=2.99 PR>F=.0011	F=29.53 PR>F=.0001	F=43.15 PR>F=.0001
	D	U	N	A	N
	A 1 A 3 A 2	A 2 B 1 A 3	A 3 B 2 AB 1	A 2 C 3 B 1	A 3 B 1 B 2
1 Day 1=Replicate #1 2=Replicate #2 3=Replicate #3	A	N	O	V	A
	F=5.41 PR>F=.002	F=2.24 PR>F=.042	F=13.64 PR>F=.0001	F=50.84 PR>F=.0001	F=5.63 PR>F=.033
	D	U	N	A	N
	A 3 B 2 B 1	A 1 B 3 A 2	A 1 B 3 A 2	A 3 B 2 B 1	A 1 B 3 AB 2

Table 4.3 Results of the ANOVA procedure and DUNCAN tests from populations subjected to identical channel exposure, with only variable the length of their acclimation period. ($\alpha = 0.05$. For individual cases, i.e. 0,1,2 days. $N=45$, $DF_{ANOVA}=44$, $DF_{DUNCAN}=42$. For comparisons, $N=135$, $DF_{ANOVA}=134$, $DF_{DUNCAN}=132$)

Acclimation in Channel	Cu(II) Channel Concentration [µg/l]				
	11.25	22.5	45.0	72.5	100.0
	A	N	O	V	A
2 Days 1=Replicate #1 2=Replicate #2 3=Replicate #3	F=1.07 PR>F=.42	F=1.19 PR>F=.18	F=2.21 PR>F=.057	F=6.99 PR>F=.0005	F=0.89 PR>F=.87
	D	U	N	C	A
	A 1 A 3 A 2	A 1 A 3 A 2	A 1 A 2 A 3	A 2 B 1 A 3	A 1 A 2 A 3
Comparison 1=0 Days 2=1 Day 3=2 Days	A	N	O	V	A
	F=15.19	F=20.64 PR >	F=54.28 F = 0.0001	F=109.83	F=220.25
	D	U	N	C	A
	A 3 B 2 B 1	A 3 B 1 A 2	A 3 B 1 A 2	A 3 C 1 B 2	A 3 C 1 AB 2

Table 4.3 (continued)

fish after one day acclimation in the channel were not yet ready to behave consistently and reliably in terms of avoidance reactions.

This in turn suggested that within one day of acclimation in the channel fish had not completely forgotten the netting experience. The inconsistency of results, between the replicate experiments, indicated that fish were not fully acclimated in the channel environment. Avoidance behaviour based on one day acclimation periods appear variable with large standard deviations and therefore is considered unacceptable to provide reliable avoidance data.

Results after two days acclimation are presented on Fig.4.4. The reproducibility as well as the consistency in trends and overall behaviour of fish stocks before and during the experiment suggest that the two day period is adequate for fish to a) overcome the netting experience and b) to become familiar to the new environment. Results yielded no significant differences at any level (Table 4.3), and consistent curve trends. In addition, fish behaviour prior to starting the experiment indicated no stressful reactions. The fish appeared relaxed and content with their established territorial space. Aggressive behaviour was not observed.

4.1.2.4 Effect of concentration gradient on fish avoidance reactions (shallow vs. steep)

In the past, two methods were employed for creating the toxicant concentration zones in avoidance tanks, each with its own

apparatus design. One was a completely separated toxicant zone from clear water zone, resulting in a steep gradient. The alternative was a concentration gradient formed as parallel streams, assumed immiscible, with different concentrations in increasing manner (see Section 2.1.3).

Differences in avoidance reactions (see also Section 5.1.1) were proposed by Westlake et al., 1974, between results obtained on apparatus with steep compared to shallow concentration gradients. The present avoidance channel combined both steep and shallow gradient characteristics (Section 4.1.1). To determine if behavioral differences could be accounted for by steep or shallow gradients in the apparatus, results were evaluated for two different cases; when fish occupied the separated zones and when fish mainly occupied the non-separated section of the channel.

Since the pre-testing distribution was always considered in the calculation of the net fish avoidance (% fish in clear), data based on populations occupying the separated or non-separated zones respectively yielded statistically similar results (Table 4.4). This suggests that fish avoidance reactions evaluated at steady-state conditions are mainly triggered by the absolute level of the toxicant rather than by the concentration gradient in the channel.

4.2 Avoidance Behaviour of fish exposed to $K_2Cr_2O_7$

Figures 4.5 to 4.11 present the results of fish avoidance reactions to the presence of $K_2Cr_2O_7$ (Cr(VI)).

Cr(VI) Data from Fish in Steep or Shal- low Gradients in Channel 1=1st Occasion 2=2nd Occasion 3=3rd Occasion	Channel Concentration [mg/l]		
	0.1	0.3	0.8
	ANOVA	ANOVA	ANOVA
	F=0.49 PR>F=.53	F=1.02 PR>F=.36	F=0.88 PR>F=.42
	DUNCAN	DUNCAN	DUNCAN
	A 3 A 1 A 2	A 1 A 3 A 2	A 2 A 1 A 3

Table 4.4 Results of ANOVA procedure and DUNCAN tests from comparison of DATA obtained in shallow or steep gradient concentrations. (N=45, $\alpha = 0.05$, $DF_{ANOVA}=44$, $DF_{DUNCAN}=42$)

Concentrations in the channel ranged from 0.001 to 30 mg/l as Cr(VI), while pre-exposure levels ranged from 0 (non-exposed) to 3.0 mg/l as Cr(VI). All results for avoidance-preference are presented in terms of per cent fish present in clear water versus the level of toxicant in the channel expressed in mg/l of Cr(VI) on a logarithmic scale. Values higher than 50% indicate avoidance, whereas values lower than the 50% level suggest preference for the toxicant. At 30 mg/l as Cr(VI), the pH in the channel dropped to 6.6. This level is not considered to produce any effect on fish avoidance behaviour (Barton et al., 1985).

4.2.1 Avoidance behaviour of Rainbow Trout which have not been exposed to Cr(VI)

The avoidance behaviour of trout which had not been previously exposed to Cr(VI) is illustrated in Figure 4.5. No significant avoidance was observed at the lowest test concentrations 0.001 and 0.01 mg/l Cr, while at 0.08 mg/l, 54% of the fish population preferred the clear water zone. Avoidance was more pronounced with increasing concentration in the channel. Two linear relationships on a log-normal scale as plotted in Figure 4.5 can be represented by equations 4.1 (Table 4.5). These expressions yield an avoidance threshold level of 0.026 mg/l, and an avoidance response of 91% at the 96 hour LC50 value of 100 mg/l proposed by USEPA (1976). Avoidance threshold is defined as the effective concentration beyond which avoidance reaction begins and increases steadily with increasing level of toxicant concentrations. In practice, it is defined by the point of intersec-

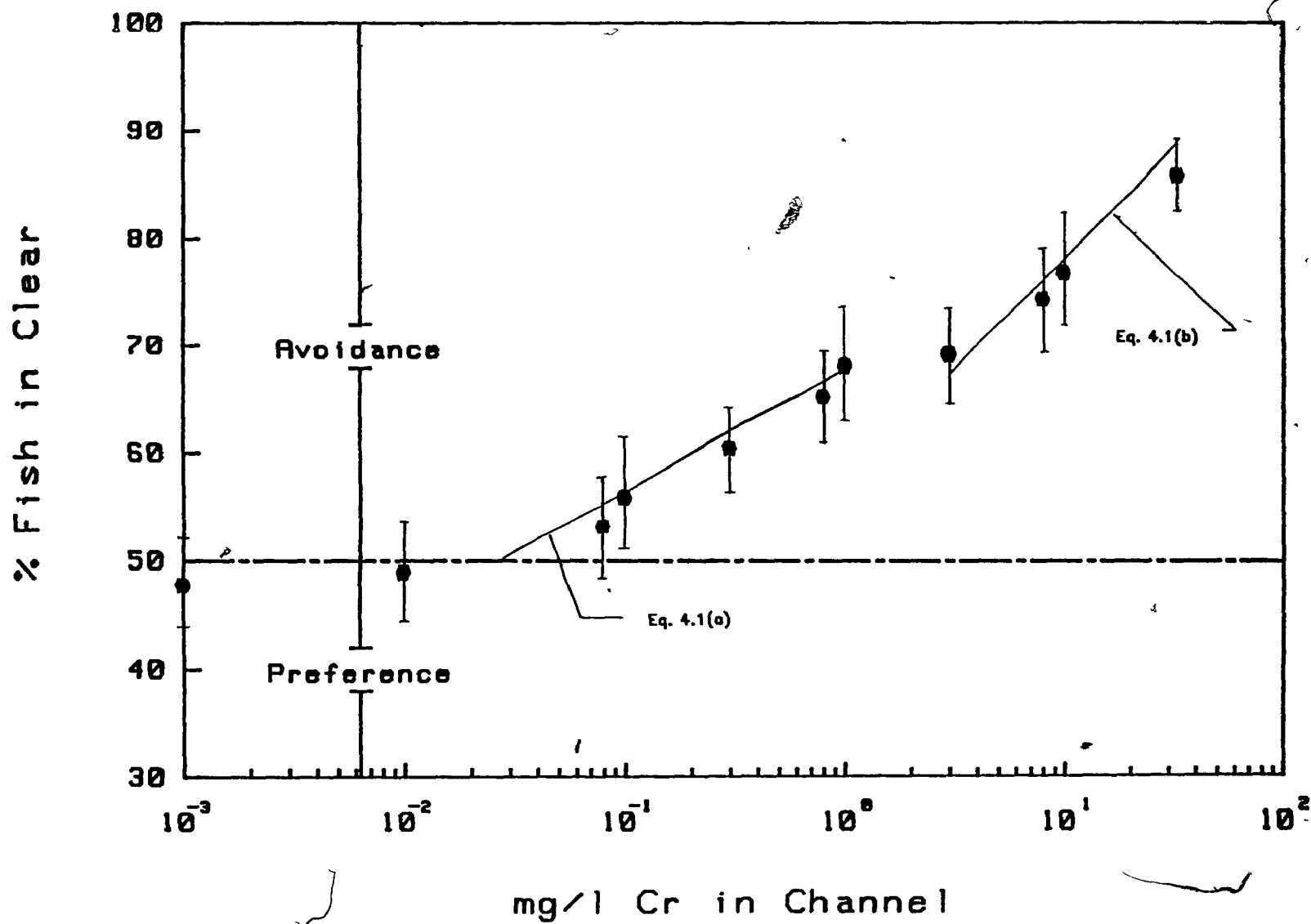


Figure 4.5 Avoidance reaction of non pre-exposed rainbow trout to $K_2Cr_2O_7$.

		% Avoidance to Cr(VI) =		Equation #	Threshold (mg/l)
Cr(VI) P-E [mg/l]		Below ABP*	Above ABP*		
Control		61.4 + 4.4log[Cr(VI)] (a)		4.1	0.026
0.01	N/A		66.98 + 11.97log[Cr(VI)] (b) 59.87 + 8.59log[Cr(VI)]	4.2	0.071
0.1	N/A		57.96 + 12.32log[Cr(VI)]	4.3	0.22
0.3	N/A		57.64 + 9.96log[Cr(VI)]	4.4	0.17
0.8	N/A		36.78 + 16.7log[Cr(VI)]	4.5	5.8
1.0	N/A		46.35 + 13.8log[Cr(VI)]	4.6	1.8
Threshold Avoidance Variation	log[Threshold Avoidance] = 0.201 + 0.746log[P-E]			4.7	

* ABP=Avoidance Breakpoint Level
 [P-E]=Pre-Exposure Concentration
 [Cr(VI)]=Chromium Concentration in the Channel

Table 4.5 Equations and threshold avoidance levels based on data
 obtained from avoidance tests on rainbow trout exposed
 to Cr(VI).

tion of the 50% avoidance line and the avoidance curve.

4.2.2 Avoidance behaviour of trout which have been pre-exposed to Cr(VI)

Figures 4.6 to 4.11 present results from fish populations pre-exposed to different levels of $K_2Cr_2O_7$. All pre-exposed populations were tested after two and seven days channel acclimation periods except for the case of 3.0 mg/l Cr(VI) pre-exposure level. In that case, mortality of the population was significantly higher than the controls (36% vs. 1.5% for the controls). There were not sufficient numbers of fish to run both sets of experiments after two and seven days acclimation in the channel.

Two single linear relationships were used to best fit the data points on a log-normal scale and are presented along with the threshold avoidance value on Table 4.5.

The avoidance behaviour of rainbow trout pre-exposed at 0.01 mg/l Cr is presented in Figure 4.6. Fish pre-exposed at 0.01 mg/l Cr(VI) are sensitized by the toxicant compared to the response of fish which had not been pre-exposed to Cr(VI). This was evident as a slightly higher avoidance when exposed to the two lowest channel concentrations (0.001 and 0.01 mg/l Cr(VI)) (Fig. 4.5 and 4.6). Statistically this difference is not considered significant. Similar sensitization, observed in lethal bioassays, is mentioned by other researchers for populations pre-exposed to low levels of toxicants (Weis and Weis, 1983; Dixon and Sprague, 1981). At channel concentrations within the range of 0.001-0.1 mg/l, there was no significant reaction of the fish.

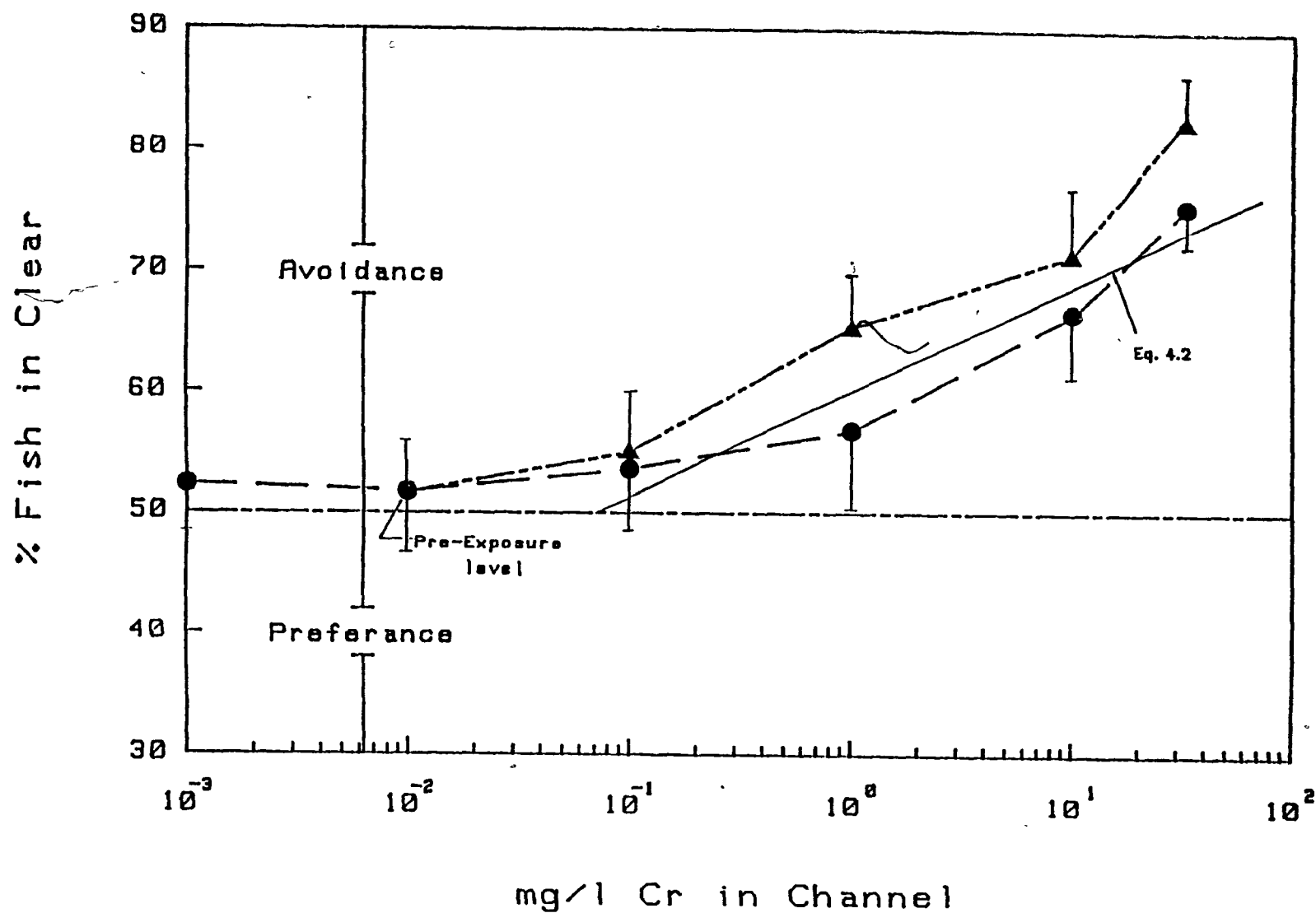


Figure 4.6 Avoidance reaction of rainbow trout pre-exposed to 0.01 mg/l Cr(VI) (●) and after 7 days of acclimation in clear water (▲).

The actual mean of % fish in clear at 0.01 mg/l Cr in the channel was 50.9% (ST.D. = 6.2) compared to 51.3% (ST.D. = 4.0) and 52.7% (ST.D. = 6.2) for 0.001 and 0.1 mg/l Cr(VI) respectively, indicating a minimum avoidance reaction at concentrations equal to the pre-exposure level. Beyond that particular level, all avoidance reactions were lower compared to avoidance reactions recorded from non-exposed populations.

For channel test concentrations higher than the pre-exposure level, the fish avoidance behaviour increased with increasing levels of chromium. This behaviour can be represented by equation 4.2 (Table 4.5) Based on Eq. 4.2 the calculated avoidance threshold value of 0.071 mg/l Cr is higher than that derived for the non-exposed population. Results presented in Figure 4.6 also indicate that the avoidance response of fish following a 7 day channel acclimation period yielded an avoidance reaction curve that was similar to that of a non-exposed population, with no differences suggested from statistical tests at any level of Cr(VI) in the channel (Table 4.6).

The avoidance behaviour of rainbow trout pre-exposed at 0.1 mg/l Cr(VI) is presented in Figure 4.7. At concentrations well below the pre-exposure level (0.001 mg/l) a slight preference 45.2% (ST.D. = 6.3) was observed. At the next higher test concentration, fish exhibited a slight avoidance 54.0% (ST.D. = 4.5). When the toxicant level in the channel reached the pre-exposure level, an inflection point was observed on the avoidance curve, with 46.3% (ST.D. = 4.1) of fish in clear water. As concentrations of Cr in the channel increased above the pre-exposure

		Channel Concentrations [mg/l]				
Cr(VI) P-E		0.08	0.3	0.8	3.0	8.0
Series (I)			A N	O	V A	
	1=Control	F=1.39 PR>F=.08	F=1.07 PR>F=.13	F=0.98 PR>F=.22	F=1.57 PR>F=.06	F=2.29 PR>F=.048
	2=0.3 mg/l		D U	N C	A N	
	3=0.8 mg/l	A 1 A 3 A 2	A 1 A 3 A 2	A 1 A 3 A 2	A 3 A 2 A 1	A 1 A 2 A 3
Series (II)		0.01	0.1	1.0	10.0	30.0
	1=Control		A N	O	V A	
	2=.01 mg/l	F=3.63 PR>F=.001	F=3.02 PR>F=.002	F=57.63 P R > F = . 0 0 0 1	F=68.22	F=74.73
	3=0.1 mg/l		D U	N C	A N	
	4=1.0 mg/l	A 4 B 1 B 2 B 3	A 3 A 4 A 1 AB2	A 1 AB2 A 3 C 4	A 1 B 2 B 3 C 4	A 1 B 3 B 2 C 4

Table 4.6 Results of ANOVA procedure and DUNCAN tests, on pre-exposed populations acclimated for 7 days in clear water as compared with reactions of non pre-exposed rainbow trout. (N=135, $\alpha = 0.05$, $DF_{ANOVA}=134$, $DF_{DUNCAN}=132$)

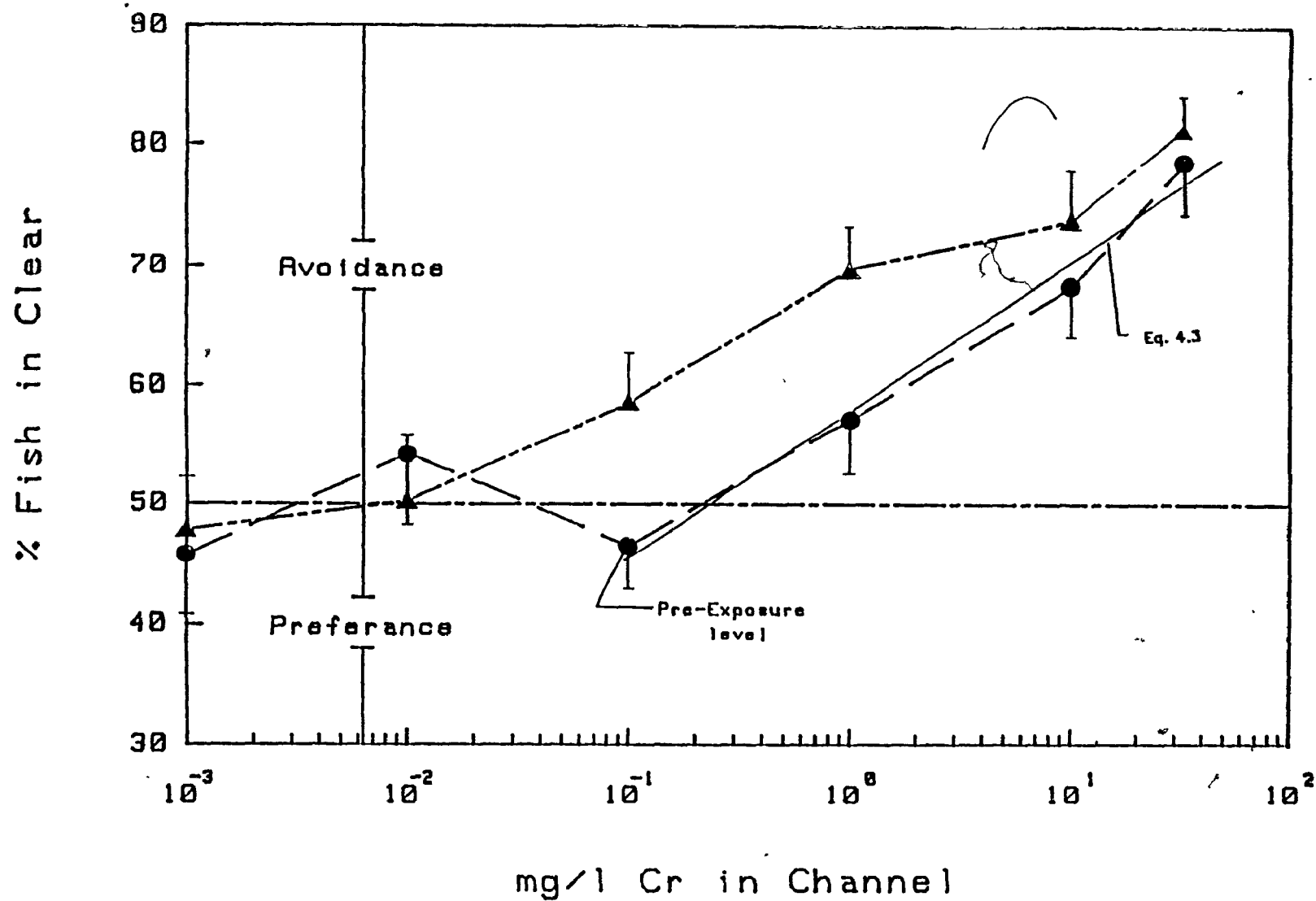


Figure 4.7 Avoidance reaction of rainbow trout pre-exposed to 0.1 mg/l Cr(VI) (●) and after 7 days of acclimation in clear water (▲).

level so did the avoidance reaction of the fish. Equation 4.3 (Table 4.5) represents the avoidance behaviour of rainbow trout for concentrations equal to or greater than the 0.1 mg/l pre-exposure level.

The actual threshold value of 0.22 mg/l was higher than in the case of the non-exposed population. Results presented in Figure 4.7 and Table 4.6 following a 7 day channel acclimation period, indicate that the avoidance response of fish pre-exposed at 0.1 mg/l was similar to that of the non-exposed population.

The avoidance behaviour of trout pre-exposed to 0.3 mg/l Cr(VI) is presented in Figure 4.8. At the lowest concentration tested (0.08 mg/l), the avoidance response was not statistically different (Table 4.7) than that of the non-exposed population. However, at channel test concentrations equal to or greater than the pre-exposure level, the response was significantly different from that of the non-exposed population. (Table 4.7) Again, a behavioural change was observed at the point where the level of Cr(VI) in the channel was similar to the pre-exposure concentration. An inflection point in the avoidance curve was observed at this concentration which suggests that the degree of avoidance is a minimum at concentrations equal to the pre-exposure level, with increasing avoidance at concentrations less than or greater than pre-exposure concentration levels. At channel test concentrations higher than the pre-exposure level, the fish avoidance behaviour can be represented by equation 4.4 (Table 4.5). This expression yields an avoidance threshold level of 0.17 mg/l Cr(VI), which is higher than for non-exposed fish. Results pre-

	Channel Concentrations [mg/l]				
Cr(VI) P-E	0.08	0.3	0.8	3.0	8.0
Series (I)	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
1=Control	F=43.39	F=58.96	F=330.7	F=250.5	F=195.6
2=0.3 mg/l		PR >	F = 0 . 0 0 0 1		
3=0.8 mg/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
4=3.0 mg/l	A 2 B 3 A 1 B 4	A 1 C 3 B 2 D 4	A 1 B 4 B 2 C 3	A 1 C 4 B 2 D 3	A 1 C 4 B 2 C 3
Series (II)	0.01	0.1	1.0	10.0	30.0
1=Control	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
2=.01 mg/l	F=34.98	F=16.21	F=232.3	F=223.5	F=207.1
3=0.1 mg/l		PR >	F = 0 . 0 0 0 1		
4=1.0 mg/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
	A 2 B 3 A 1 B 4	A 1 B 4 A 2 C 3	A 1 C 4 B 2 C 3	A 1 C 4 B 3 D 2	A 1 C 4 B 3 D 2

Table 4.7 Results of ANOVA procedure and DUNCAN tests, on populations subjected to identical channel exposure to Cr(VI), with only variable the level of pre-exposure (N=180, $\alpha = 0.05$, $DF_{ANOVA} = 179$, $DF_{DUNCAN} = 176$)

sented in Figure 4.8 and Table 4.6 indicate that the avoidance response behaviour of fish acclimated for 7 days in the channel clear water following the 0.3 mg/l pre-exposure period was similar to that of the non-exposed population.

The avoidance behaviour pattern of trout pre-exposed to 0.8 mg/l Cr(VI) is presented in Figure 4.9. At the lowest channel test concentration (0.08 mg/l), no significant difference in response was observed from a pre-test fish distribution. At higher channel test concentrations, increasing preference for the toxicant was observed, reaching an inflection point on the curve representing maximum preference at the pre-exposure concentration. This preference behaviour was in sharp contrast to the avoidance response of a non-exposed population or populations pre-exposed to lower concentrations.

Equation 4.5 describes fish avoidance behaviour for concentrations higher than 0.8 mg/l Cr(VI), the pre-exposure level (Table 4.5).

The experimentally determined avoidance threshold value is 5.8 mg/l Cr(VI), higher than the threshold avoidance of non-exposed populations. After 7 days acclimation in channel clear water following the pre-exposure period at 0.8 mg/l, the avoidance response of the fish (Figure 4.9 and Table 4.6) was similar to that of a non-exposed population.

The avoidance behaviour of rainbow trout pre-exposed at 1.0 mg/l Cr(VI) is presented in Figure 4.10. At a concentration well below the pre-exposure level, a slight preference was exhibited 43.7% (ST.D. = 3.7) at 0.01 mg/l Cr(VI). At a concentration

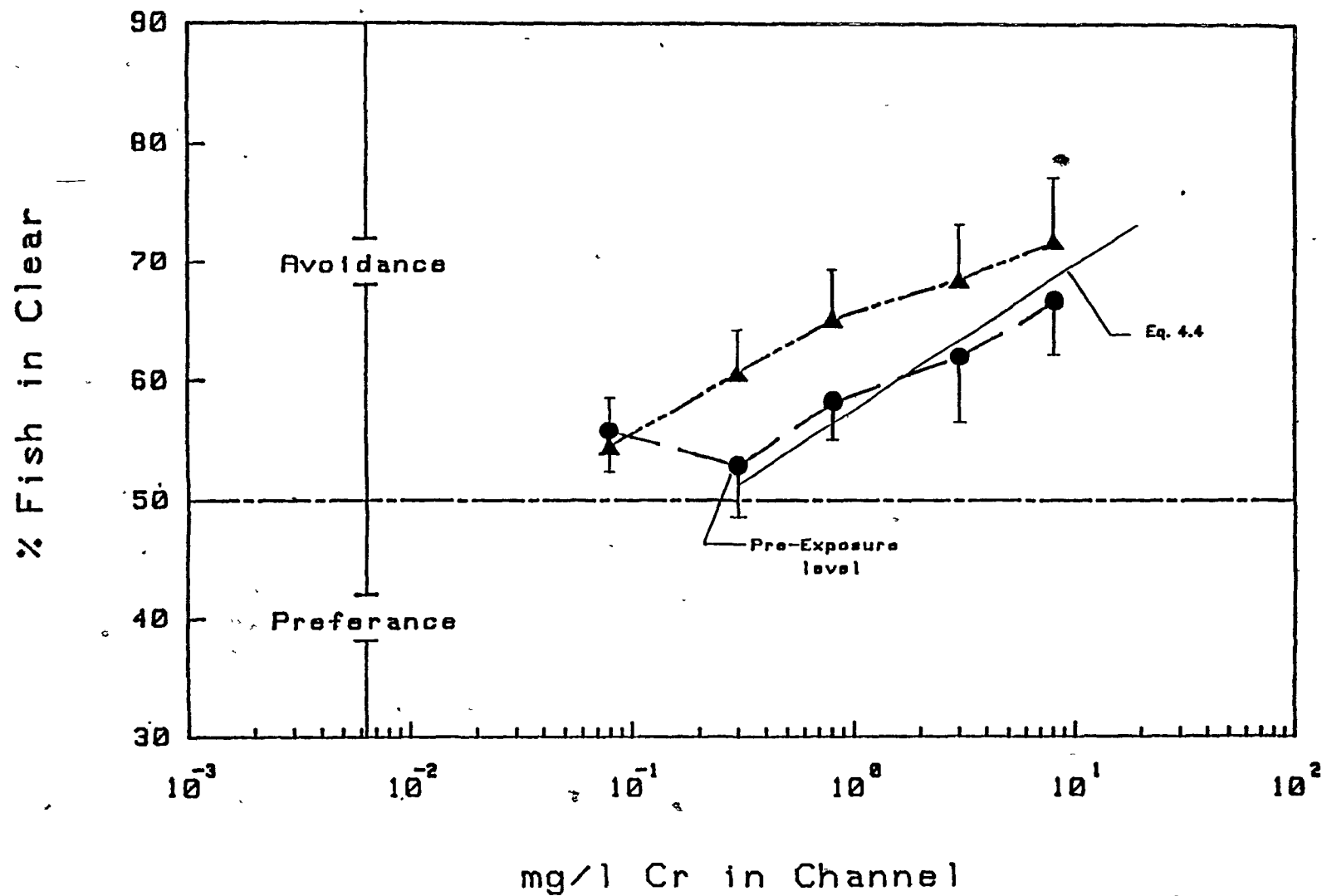


Figure 4.8 Avoidance reaction of rainbow trout pre-exposed to 0.3 mg/l Cr(VI) (●) and after 7 days of acclimation in clear water (▲).

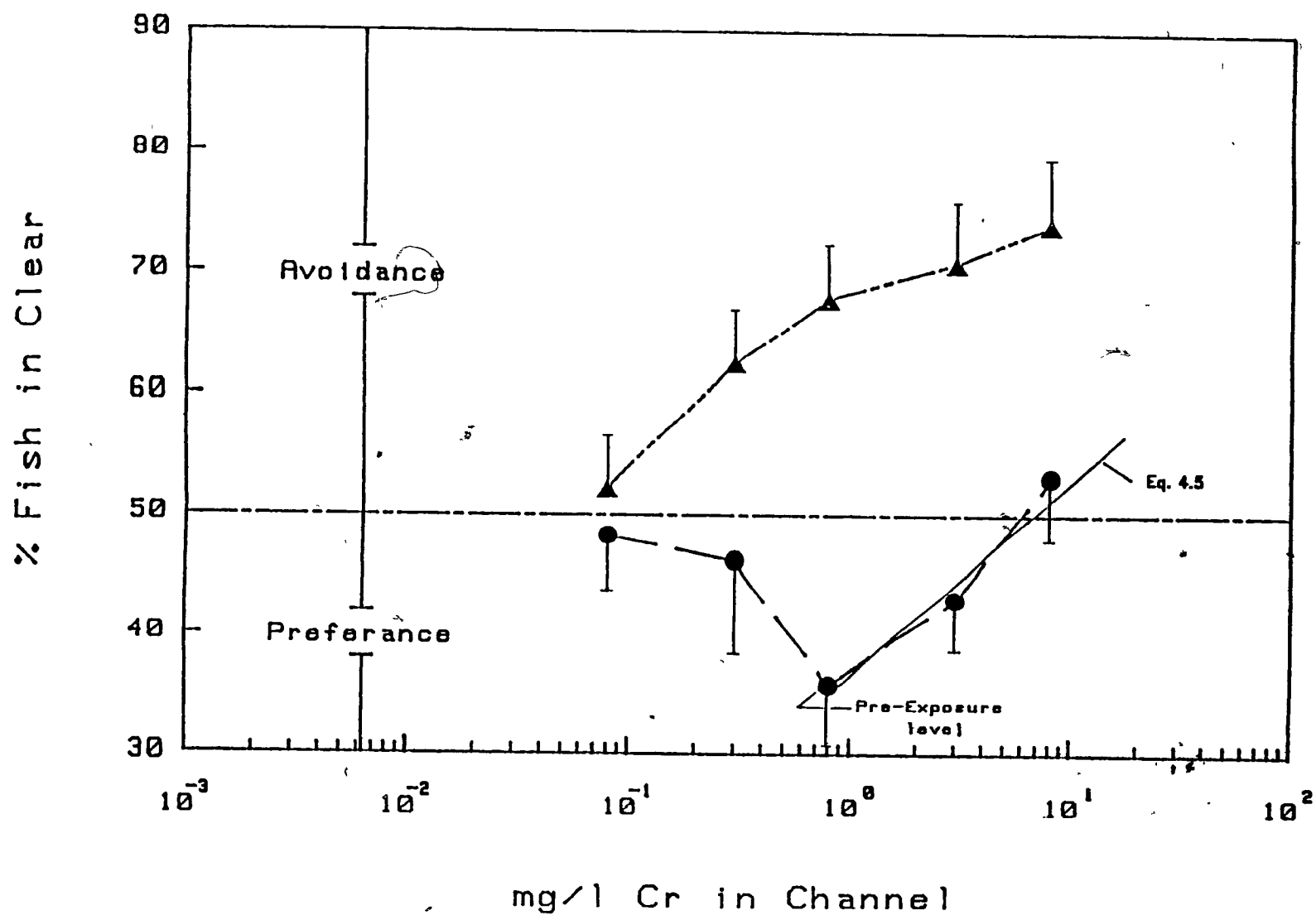


Figure 4.9 Avoidance reactions of rainbow trout pre-exposed to 0.8 mg/l Cr(VI) (●) and after 7 days of acclimation in clear water (▲).

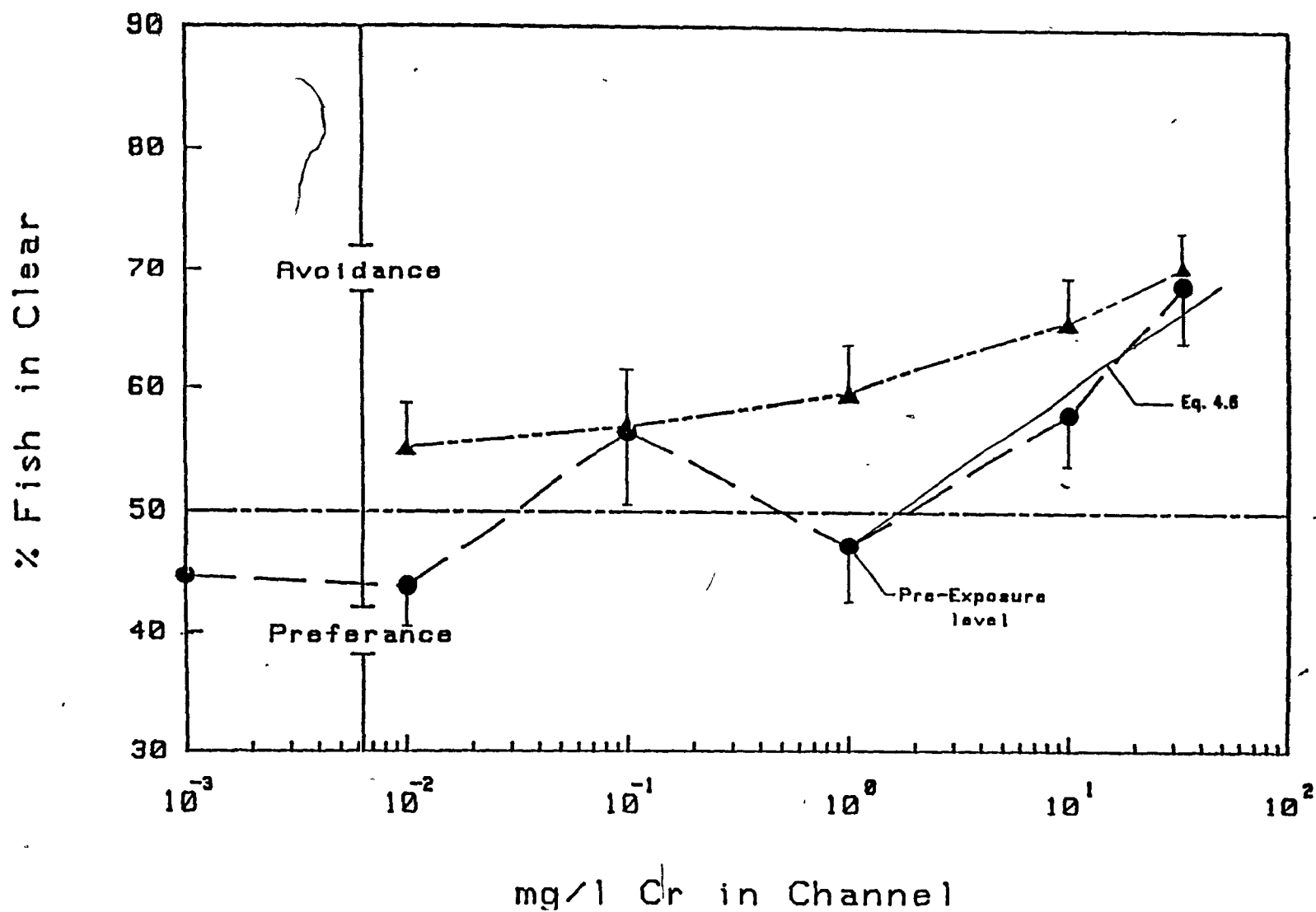


Figure 4.10 Avoidance reaction of rainbow trout pre-exposed to 1.0 mg/l Cr(VI) (●) and after 7 days of acclimation in clear water (▲).

10-fold lower than the pre-exposure level, a net population shift was noticed towards the clear water which was again reversed as the concentration approached the level of pre-exposure. From then on a clearly increasing avoidance with channel concentration levels was exhibited. Equation 4.6 describes the avoidance behaviour of rainbow trout pre-exposed at 1.0 mg/l for concentrations higher than the pre-exposure level (Table 4.5).

The actual threshold avoidance value was 1.8 mg/l Cr(VI), again higher compared to that of non-exposed populations. Results obtained after 7 days acclimation of fish in channel clear water indicate that fish behaviour was not similar to that of the non-exposed population (Fig.4.10 and Table 4.6). Such behaviour suggested that fish had not fully recovered their chemoreceptive capacity within a 7 day clearance period.

The effect of pre-exposure at 3.0 mg/l Cr(VI) on the avoidance response behaviour of the fish is presented in Figure 4.11. An increase in the degree of avoidance was observed with increasing channel test concentrations reaching a maximum avoidance at the highest test concentration below the pre-exposure level (0.8 mg/l). An inflection point on the avoidance curve was again observed at a channel test concentration equal to the pre-exposure level. Lack of a larger number of experimental points beyond the pre-exposure level makes the proposal of a predictive equation meaningless.

Avoidance threshold values from all pre-exposure levels are presented in Figure 4.12. There was a linear increase in threshold values with increasing levels of pre-exposure with slight devia-

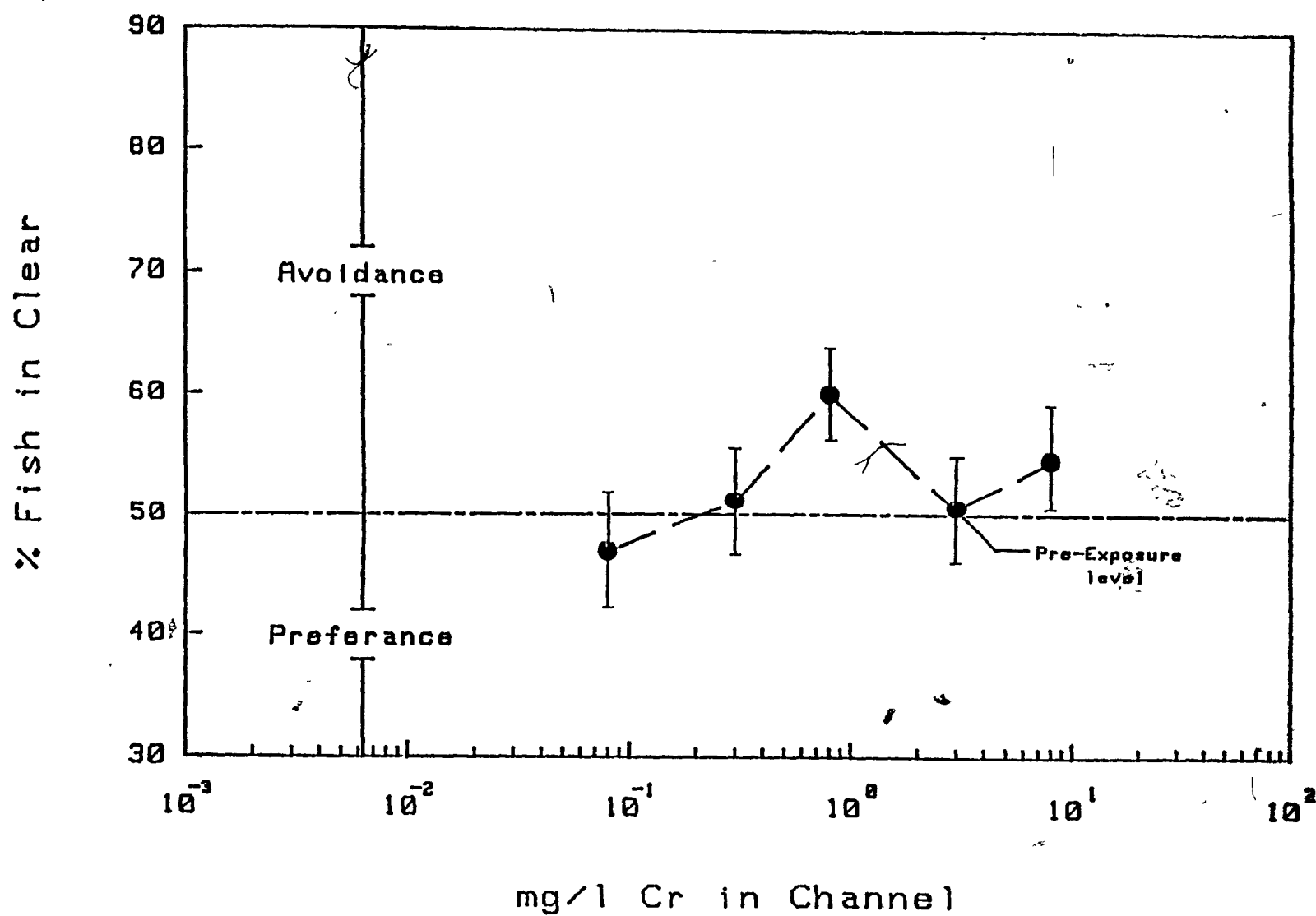


Figure 4.11 Avoidance reaction of rainbow trout pre-exposed to 3.0 mg/l Cr(VI).

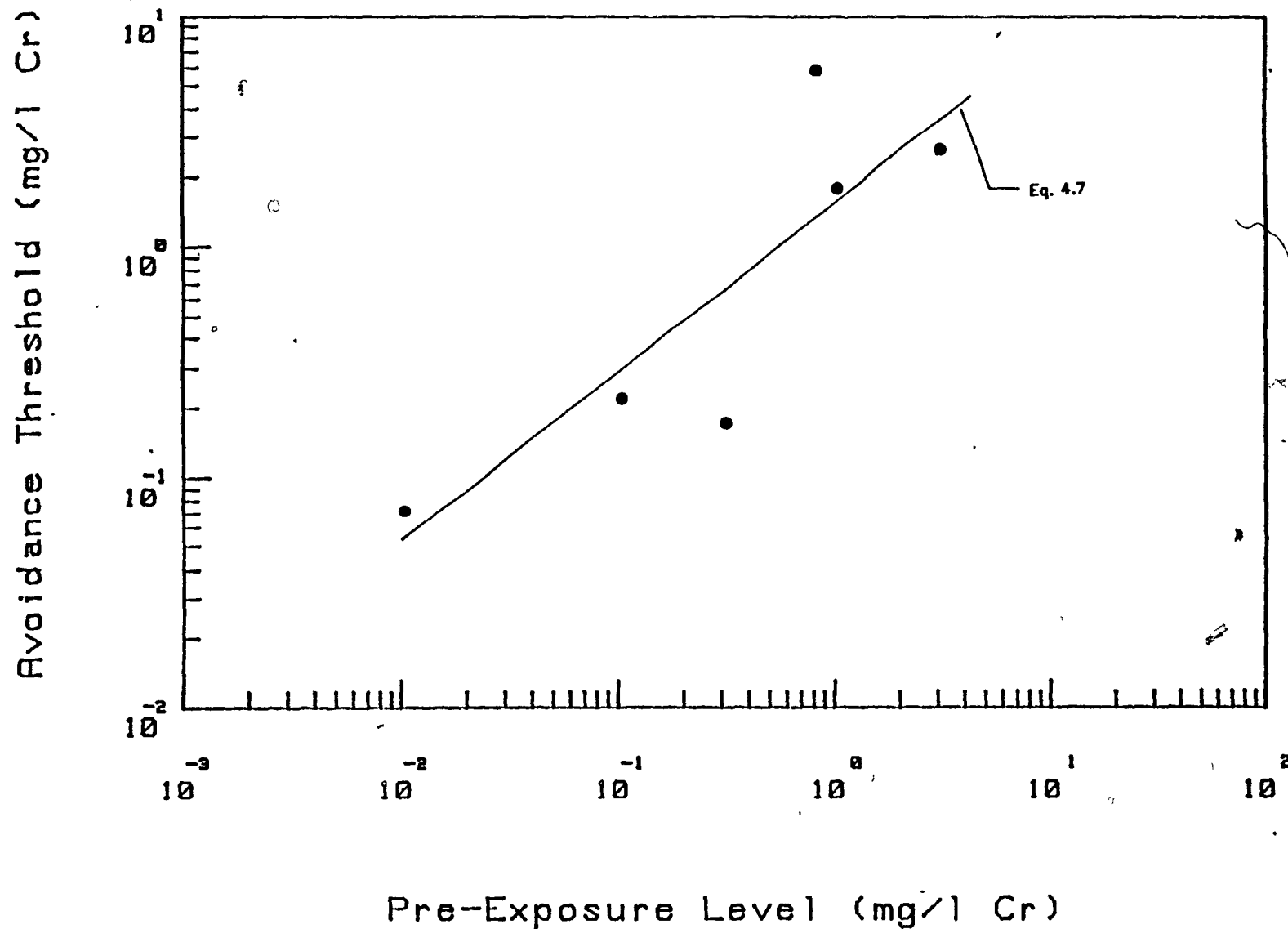


Figure 4.12 Rainbow trout avoidance threshold variation with increasing level of pre-exposure to Cr(VI).

tions at 0.3 and 0.8 mg/l. Equation (4.7) (Table 4.5) describes the change in avoidance threshold values vs. the level of pre-exposure.

Overall it was observed that populations pre-exposed between 0.1 and 3.0 mg/l as Cr(VI) yielded avoidance curves characterized by similar trends (see figures 4.6 to 4.11). In all cases the avoidance curves exhibited an inflection point, that coincided with the pre-exposure level of the population. Avoidance behaviour of pre-exposed populations for test concentrations equal or greater than the respective pre-exposure level was always lower and statistically different than that of a non-exposed population (Table 4.7). Avoidance reactions decreased with increasing levels of pre-exposure, for concentrations beyond the respective pre-exposure level. Such reactions reached a minimum for a population pre-exposed at 0.8 mg/l Cr(VI), where preference reactions were mainly recorded for concentrations up to 5.8 mg/l, the avoidance threshold for that pre-exposure concentration. Fish populations pre-exposed beyond the 0.8 mg/l Cr(VI) level exhibited higher avoidance reaction levels compared to the 0.8 mg/l Cr(VI) pre-exposed population. Beyond the 1.0 mg/l Cr(VI) pre-exposure level, the trend, again, was decreasing avoidance reactions with increasing levels of pre-exposure for concentrations higher than the respective pre-exposure level.

Avoidance threshold values increased with increasing level of pre-exposure as illustrated on Figure 4.12 where a linear relationship may be observed. Fish avoidance behaviour after 7 days acclimation in clear water in the channel was in general,

significantly different than the same population's reaction after 2 days in the channel. For all populations pre-exposed up to and including the 0.8 mg/l Cr(VI) level, avoidance reactions were similar to the non-exposed population. (Table 4.6).

Fish pre-exposed beyond the 0.8 mg/l Cr(VI) level, after 7 days channel acclimation, reacted statistically different than the non-exposed population (Table 4.6). Mathematical expressions for threshold value variations and avoidance reactions, for values beyond their respective pre-exposure level are summarized in Table 4.5.

4.3 Avoidance-Preference testing with trivalent chromium (Cr(III))

Figures 4.13 to 4.19 illustrate fish avoidance reaction using a trivalent form of chromium as $\text{Cr}(\text{NO}_3)_3$.

Experimental concentrations of $\text{Cr}(\text{NO}_3)_3$ ranged from 0.001 mg/l to 30 mg/l as $\text{Cr}(\text{III})$, while pre-exposure levels ranged from 0 (non-exposed) to 3.0 mg/l as $\text{Cr}(\text{III})$. At 30 mg/l as $\text{Cr}(\text{III})$, the pH change was insignificant (from pH 7.3 to 7.14).

There was no mortality during the entire pre-exposure period at any concentration level, when fish were exposed to $\text{Cr}(\text{NO}_3)_3$. This observation suggested toxicity differences between the two chromium compounds. Mathematical expressions and threshold avoidance values were obtained to best represent the data, and are tabulated in Table 4.8.

Cr(III) P-E [mg/l]	% Avoidance to Cr(III) =		Equation #	Threshold (mg/l)
	Below ABP*	Above ABP*		
Control	$58.76 + 2.61 \log [\text{Cr(III)}] \text{ (a)}$	$41.93 + 35.2 \log [\text{Cr(III)}] \text{ (b)}$	4.8	0.0026
0.01	$66.1 + 6.13 \log [\text{Cr(III)}] \text{ (a)}$	$34.65 + 39.0 \log [\text{Cr(III)}] \text{ (b)}$	4.9	0.012
0.1	N/A	$65.24 + 16.0 \log [\text{Cr(III)}]$	4.10	0.029
0.3	N/A	$2.26 + 54.58 \log [\text{Cr(III)}]$	4.11	0.37
0.8	N/A	$29.30 + 33.2 \log [\text{Cr(III)}]$	4.12	4.05
1.0	N/A	$45.72 + 35.7 \log [\text{Cr(III)}]$	4.13	1.32
3.0	N/A	$45.27 + 7.18 \log [\text{Cr(III)}]$	4.14	4.05
Threshold Avoidance Variation	$\log [\text{Threshold Avoidance}] = 0.184 + 1.16 \log [\text{P-E}]$		4.15	

* ABP=Avoidance Breakpoint Level

[P-E]=Pre-Exposure Concentration

[Cr(III)]=Chromium Concentration in the Channel

Table 4.8 Equations and threshold avoidance levels based on data obtained from avoidance tests on rainbow trout exposed to Cr(III).

4.3.1 Avoidance behaviour of trout which have not been pre-exposed to Cr(III)

Figure 4.13 illustrates the avoidance curve of a non-previously exposed population after injection of Cr(III) in the channel. No significant avoidance response was observed within the range 0.001 and 1.0 mg/l Cr(III) in the channel.

Further increase in toxicant concentrations beyond the 1.0 mg/l Cr(III) resulted in an abrupt change in avoidance reaction, especially beyond the 3.0 mg/l Cr(III) level. Throughout the range of concentrations used, avoidance reaction increased with increasing levels of $\text{Cr}(\text{NO}_3)_3$ in the channel. Two linear mathematical expressions are proposed for the two distinct ranges of avoidance reaction and are included in Table 4.8.

The avoidance threshold value for Cr(III) can be established at 0.0026 mg/l, while 100% avoidance can be expected for the 96h-LC50 value of 100 mg/l Cr proposed by USEPA (1976).

4.3.2 Avoidance preference behaviour of trout which have been pre-exposed at different levels of Cr(III).

Figure 4.14 presents results obtained from populations pre-exposed to 0.01 mg/l Cr(III). The general avoidance reaction trends were similar to the non-exposed population. Fish appeared more sensitive at 1.0 mg/l Cr(III), compared to the avoidance reaction of the non-exposed population. A slightly higher mean avoidance reaction was observed at 1.0 mg/l Cr(III), compared to non-exposed fish, although not statistically different (Table 4.9).

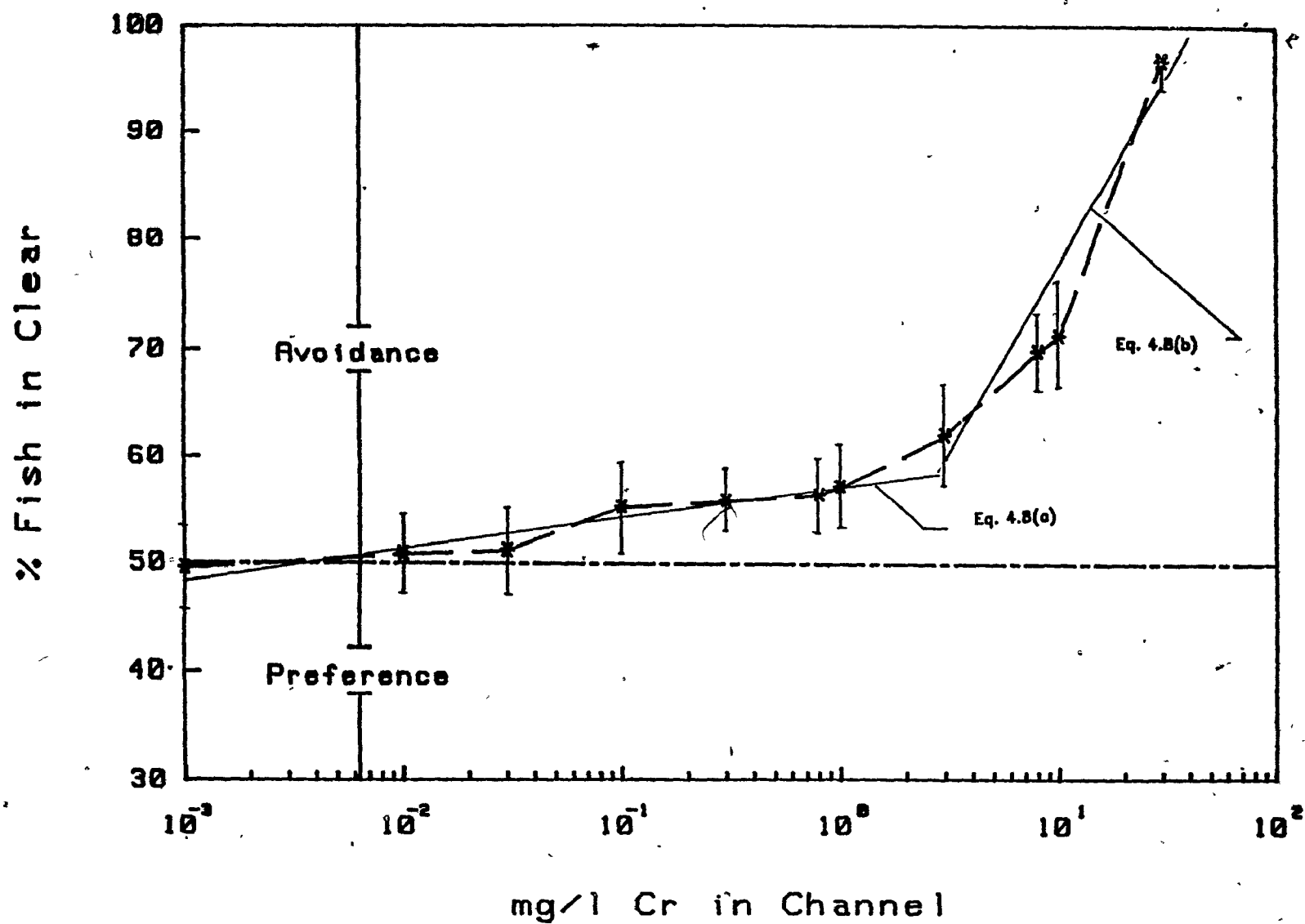


Figure 4.13 Avoidance reaction of non pre-exposed rainbow trout to $\text{Cr}(\text{NO}_3)_3$

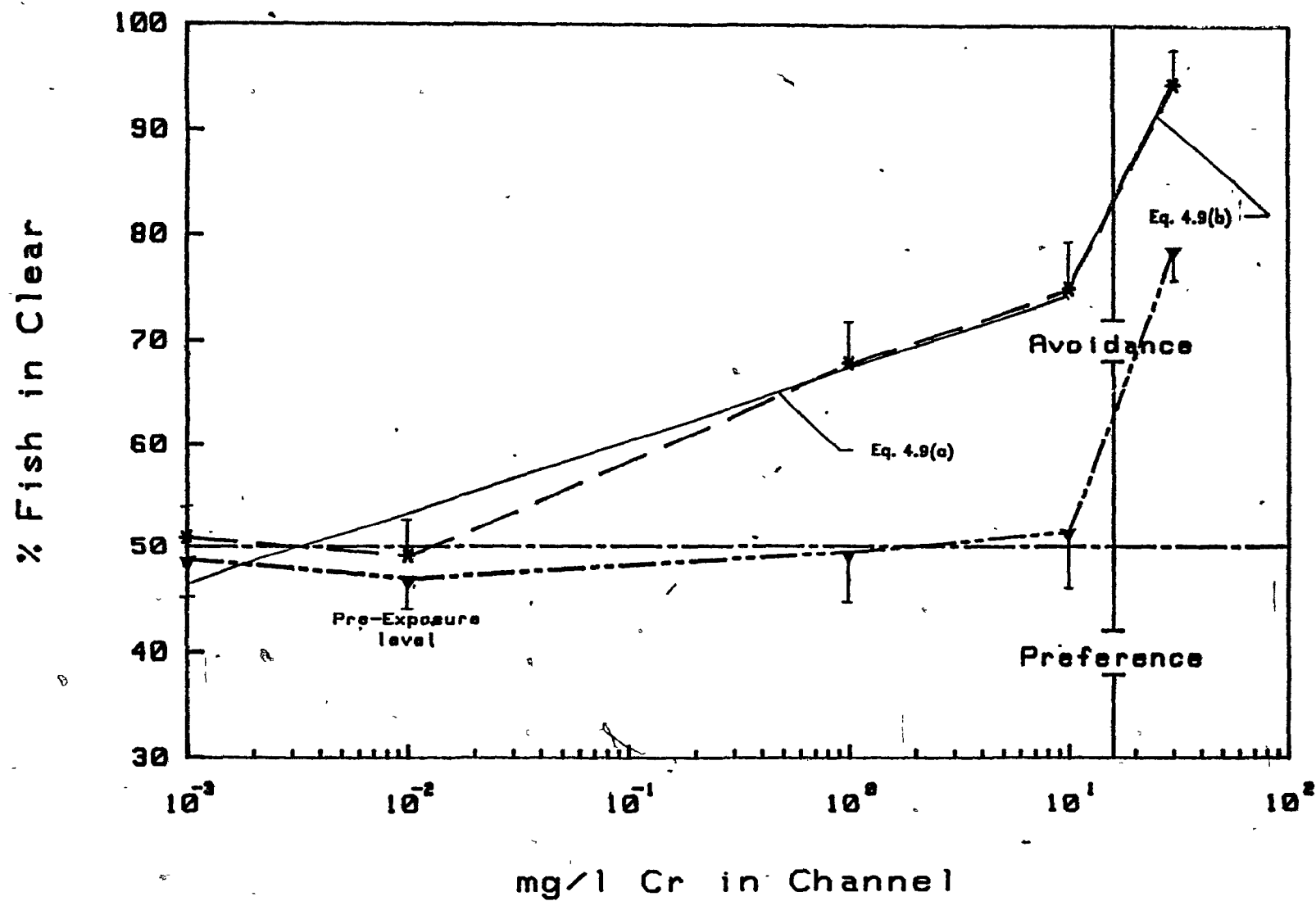


Figure 4.14 Avoidance reaction of rainbow trout pre-exposed to 0.01 mg/l Cr(III) (*) and after 7 days acclimation in clear water (▼).

	Channel Concentrations [mg/l]				
Cr(III) P-E	0.01	0.3	0.8	3.0	30.0
Series (I)	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
1=Control	F=0.87	F=61.26	F=98.95	F=95.22	F=246.1
2=0.3 mg/l	PR>F=.459	PR >	F = 0 . 0 0 0 1		
3=0.8 mg/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
4=3.0 mg/l	A 4 B 3 A 2 B 1	A 1 C 2 B 4 D 3	A 1 B 4 A 2 C 3	A 1 C 2 B 3 D 4	A 1 C 3 B 2 C 4
Series (II)	0.01	0.1	1.0	10.0	30.0
1=Control	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
2=.01 mg/l	F=17.22	F=24.26	F=84.43	F=38.63	F=5.50
3=0.1 mg/l		PR >	F = 0 . 0 0 0 1		PR>F=.001
4=1.0 mg/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
	A 2 C 4 B 3 C 1	A 1 B 4 A 2 B 3	A 4 B 1 B 2 C 3	A 3 B 4 B 2 C 1	A 3 A 2 A 1 B 4

Table 4.9. Results of ANOVA procedure and DUNCAN tests, on populations subjected to identical channel exposure to Cr(III), with only variable the level of pre-exposure (N=180, $\alpha = 0.05$, $DF_{ANOVA} = 179$, $DF_{DUNCAN} = 176$).

For concentrations beyond the 10.0 mg/l level, avoidance reactions were lower than the non-exposed population. The overall trend beyond the pre-exposure level was increasing avoidance with increasing toxicant level. Again fish avoidance reaction was at a minimum for toxicant level in the channel coinciding with the pre-exposure concentration, as in all cases with Cr(VI). Results from populations after a 7 day channel acclimation period yielded an avoidance reaction curve with similar trend characteristics to that of the non-exposed. On the other hand, avoidance levels were in general significantly lower (Table 4.10) compared to reactions of similar populations acclimated for 2 days in the channel.

Results after two days suggest that a 0.01 mg/l Cr(III) pre-exposure level does not significantly affect fish avoidance behaviour compared to that of non-exposed.

Mathematical expressions and threshold values are summarized in Table 4.8.

Figure 4.15 presents results obtained from a population pre-exposed at 0.1 mg/l Cr(III) after 2 and 7 days channel acclimation periods. In the range 0.01 - 1.0 mg/l Cr(III) after a 2 day channel acclimation period, fish did not exhibit dramatic changes in avoidance reaction, but were sensitized compared to the non-exposed population, exhibiting higher avoidance reactions. Minimum reaction was recorded at the level of pre-exposure, as observed in all previous cases. Avoidance reactions within the above mentioned range were significantly different compared to non-exposed fish (Table 4.9). Beyond the 1.0 mg/l Cr(III) level,

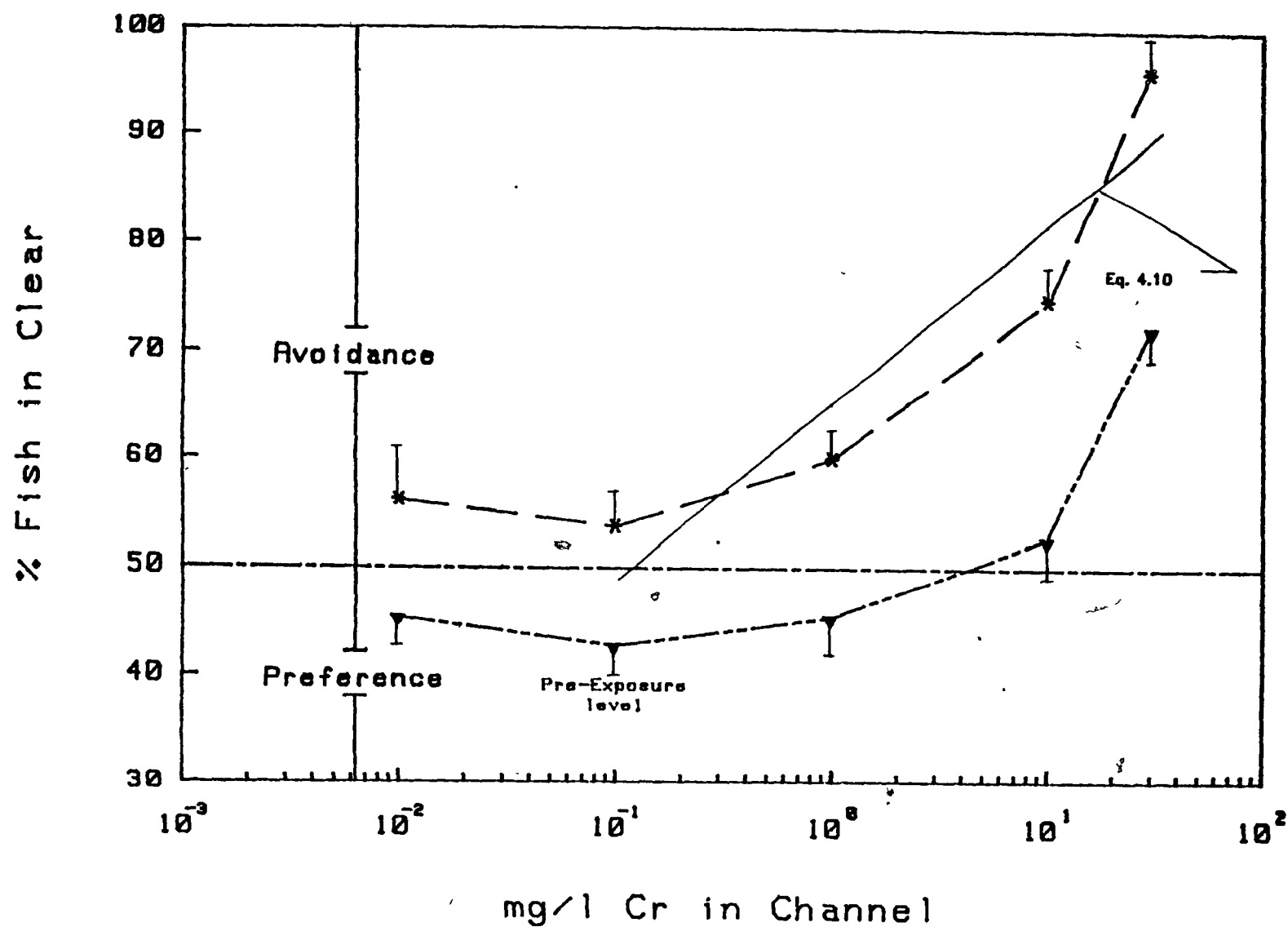


Figure 4.15 Avoidance reaction of rainbow trout pre-exposed to 0.1 mg/l Cr(III) (*) and after 7 days acclimation in clear water (▼).

fish avoidance reaction was different compared to that of lower levels in the channel and those of the non-exposed populations, except at 30.0 mg/l Cr(III), where DUNCAN's test classified results in the same class (A) (Fig. 4.13 and 4.15 and Table 4.9).

Avoidance reaction trends for the 0.1 mg/l Cr(III) pre-exposure level were similar for 2 and 7 days channel acclimation periods. Values after 7 days were consistently lower than after 2 days (Table 4.10). Beyond the 1.0 mg/l Cr(III) level, reactions were significantly lower after 7 days compared to similar acclimation in the case of the 0.01 mg/l Cr(III) pre-exposure level. Mathematical expressions and threshold values are provided in Table 4.8. Threshold avoidance values increase with level of pre-exposure, suggesting a higher tolerance for higher pre-exposure levels, as is suggested by various researchers (see 5.2.2).

Figure 4.16 presents results obtained from a population pre-exposed at 0.3 mg/l Cr(III) after 2 and 7 days channel acclimation periods. Minimum avoidance reaction at the pre-exposure level for both curves suggests recognition of familiar environment as observed with all pre-exposed populations, even after 7 days clearance of the toxicant. Beyond their pre-exposure level, avoidance reactions increase with increasing levels of toxicant in the channel. In the present case, avoidance reactions followed similar trends compared to the non-exposed but avoidance values were significantly lower (Table 4.9) except at the 0.8 mg/l Cr(III) level. This in turn suggests an increasing level of tolerance with increasing pre-exposure levels. Similarly, avoid-

Pre-Exposure level [mg/l]	Cr(III) Channel Concentration [mg/l]				
	0.01	0.1	1.0	10.0	30.0
	A	N	O	V	A
0.01	F=4.95 PR>F=.029	F=2.22 PR>F=.014	F=477.5 PR>F=.0001	F=368.6 PR>F=.0001	F=305.4 PR>F=.0001
	D	U	N	C	A
	A 1 B 2	A 1 A 2	A 1 B 2	A 1 B 2	A 1 B 2
0.1	A	N	O	V	A
	F=162.4 PR>F=.0001	F=169.8 PR>F=.0001	F=78.83 PR>F=.0001	F=376.2 PR>F=.0001	F=278.7 PR>F=.0001
	D	U	N	C	A
1.0	A	N	O	V	A
	F=14.2 PR>F=.0003	F=0.73 PR>F=.3941	F=2.15 PR>F=.1461	F=10.19 PR>F=.002	F=0.36 PR>F=.5485
	D	U	N	C	A
	A 1 B 2	A 2 A 1	A 1 A 2	A 2 B 1	A 2 A 1

Table 4.10 Results of ANOVA procedure and DUNCAN tests, on pre-exposed populations acclimated for 2 days as compared with reactions after 7 day acclimation of the same populations in clear water. (N=90, $\alpha = 0.05$, $DF_{ANOVA}=89$, $DF_{DUNCAN}=88$)

Pre-Exposure level [mg/l]	Cr(III) Channel Concentration [mg/l]				
	0.01	0.1	1.0	10.0	30.0
	A	N	O	V A	
0.3 (Where 0.1mg/l read 0.3mg/l)	F=3.87 PR>F=.0523	F=0.66 PR>F=.4187	F =48.86 PR>F=.0001	F=41.41 PR>F=.0001	F=12.18 PR>F=.0007
	D	U	N C	A	N
	A 2 A 1	A 2 A 1	A 1 B 2	A 1 B 2	A 1 B 2
0.8 (Where 1.0mg/l read 0.8mg/l)	A	N	O	V A	
	F=4.69 PR>F=.0331	F=0.66 PR>F=.4196	F=3.80 PR>F=.0545	F =117.66 PR>F=.0001	F=78.61 PR>F=.0001
	D	U	N C	A	N
	A 2 A 1	A 2 A 1	A 1 A 2	A 2 B 1	A 2 B 1
3.0 (Where 1.0mg/l read 3.0mg/l)	A	N	O	V A	
	F=57.01 PR>F=.0001	F=1.80 PR>F=.1837	F=2.00 PR>F=.1604	F=63.60 PR>F=.0001	F=0.27 PR>F=.8068
	D	U	N C	A	N
	A 1 B 2	A 1 A 2	A 2 A 1	A 2 B 1	A 2 A 1

Table 4.10 (continued)

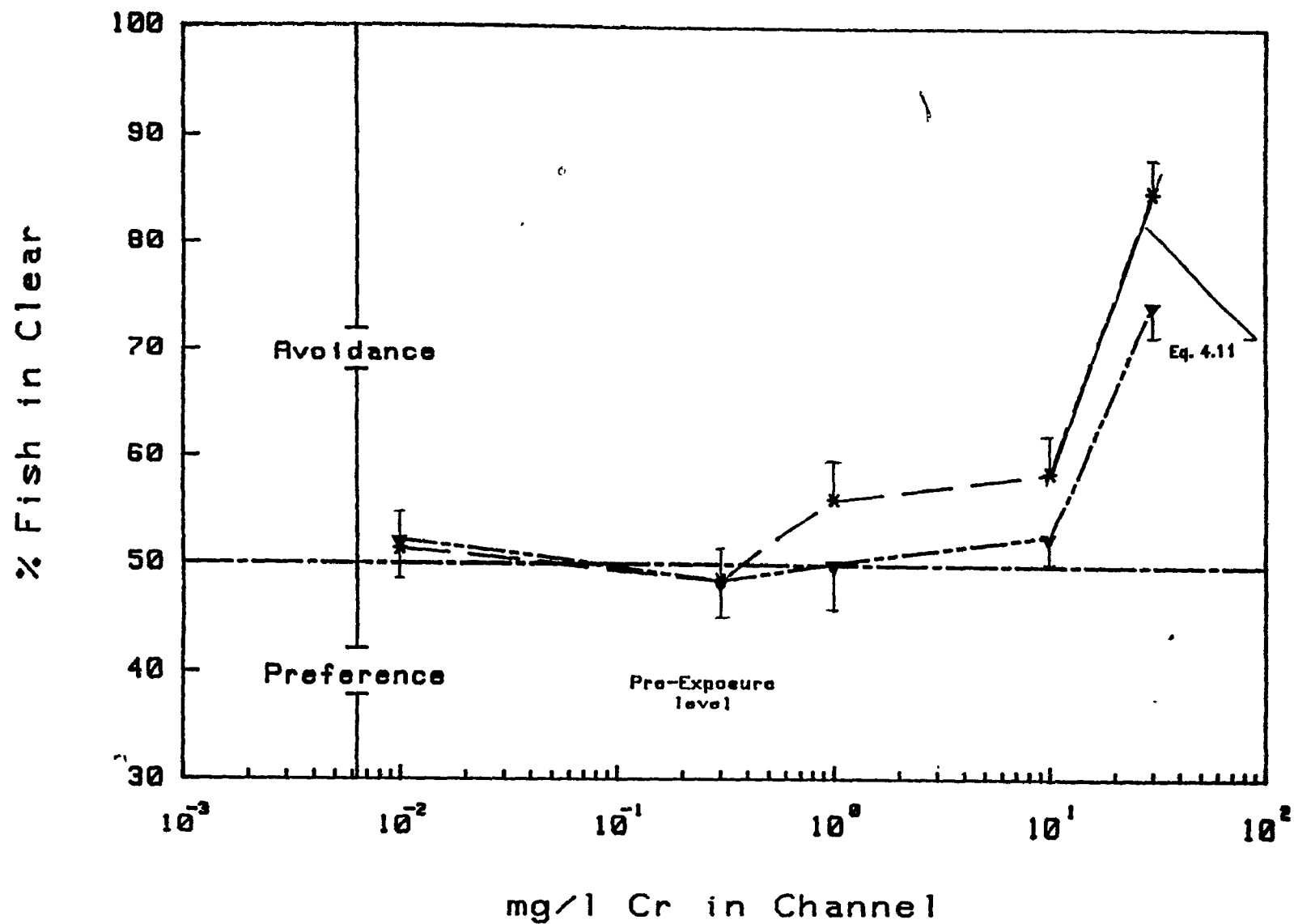


Figure 4.16 Avoidance reaction of rainbow trout pre-exposed to 0.3 mg/l Cr(III) (*) and after 7 days acclimation in clear water (∇).

ance threshold followed the same trend, increasing with the level of pre-exposure.

Avoidance reactions beyond the pre-exposure level after 7 days channel acclimation period follow the same general trends as for the 2 day channel acclimation experiments with mean avoidance values significantly lower after 7 days channel acclimation.

Populations pre-exposed between 0.01 and 0.3 mg/l Cr(III) share several common characteristics. Fish avoidance behaviour at or below 1.0 mg/l Cr(III) in the channel and after 2 day channel acclimation periods was similar to that of a non-exposed population with the same avoidance reaction slope. Abrupt change in avoidance reaction was observed at the same level as for the non-exposed population. Beyond 1.0 mg/l Cr(III), the avoidance curve was parallel to that of the non-exposed population. After 7 days channel acclimation, avoidance reactions of pre-exposed fish were significantly reduced compared to the reactions after 2 days channel acclimation. In addition, after 7 days of clearance fish still reacted favourably to the level of their pre-exposure.

Avoidance threshold values and mathematical expressions to fit the data are included in Table 4.8.

Figures 4.17 to 4.19, present results obtained using populations pre-exposed at 0.8, 1.0 and 3.0 mg/l Cr(III). All three avoidance curves yielded similar trends and behaviour. In all cases the avoidance curve exhibited an inflection point that coincided with their respective pre-exposure concentration level. This behaviour suggested that fish do recognize and are attracted to a familiar environment. It also suggested that for the case

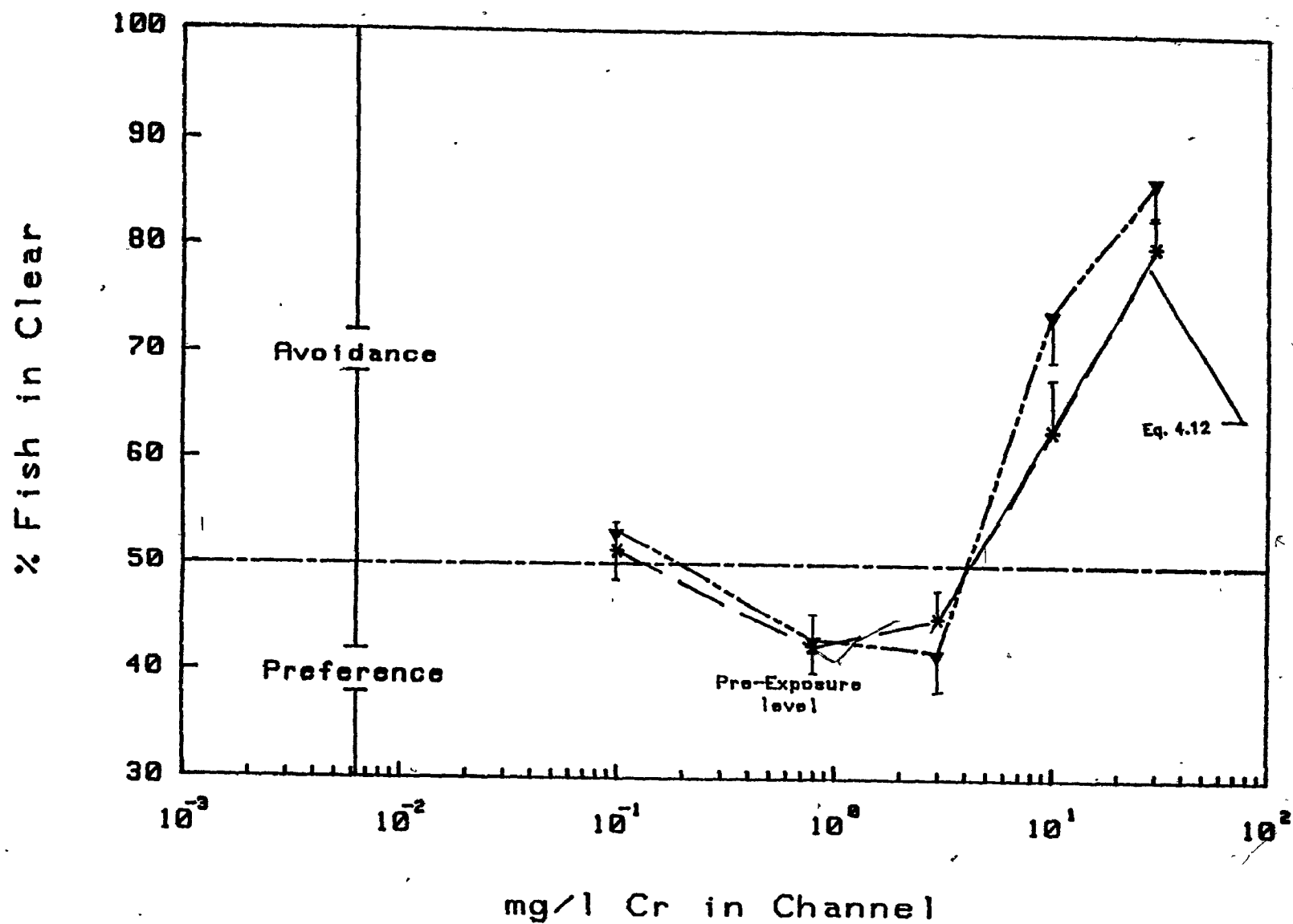


Figure 4.17 Avoidance reaction of rainbow trout pre-exposed to 0.8 mg/l Cr(III) (*) and after 7 days acclimation in clear water (▼).

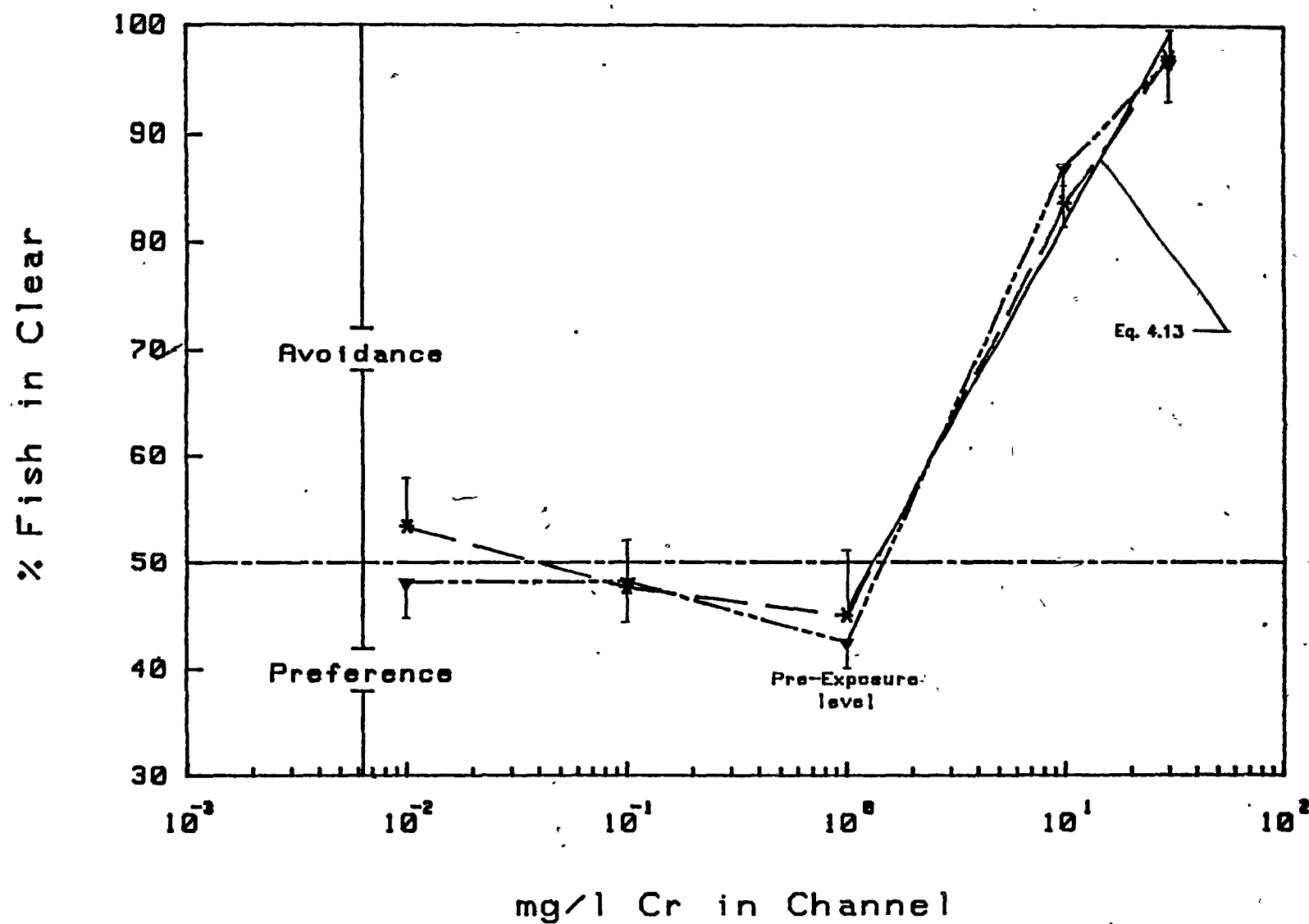


Figure 4.18 Avoidance reaction of rainbow trout pre-exposed to 1.0 mg/l Cr(III) (*) and after 7 days acclimation in clear water (▼).

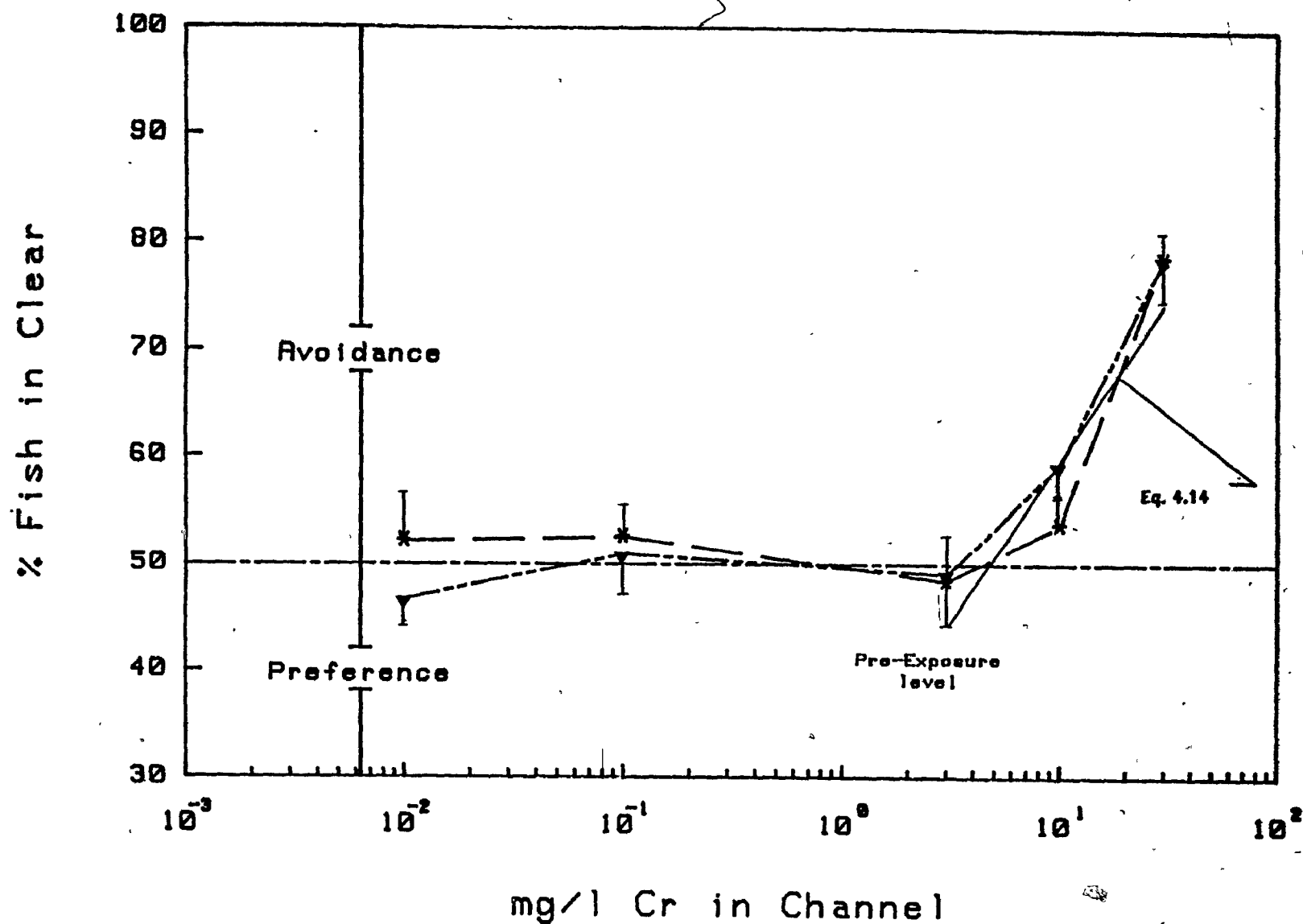


Figure 4.19 Avoidance reaction of rainbow trout pre-exposed to 3.0 mg/l Cr(III) (*), and after 7 days acclimation in clear water (▼).

of Cr(III), even 7 days in clear water were not sufficient to erase the memory of the level of pre-exposure.

In all three cases, (0.8, 1.0 and 3.0 mg/l Cr(III)), after 7 days in clear water, the avoidance reaction was similar for two and seven days channel acclimation periods (Table 4.10).

Considering all pre-exposure levels, the lowest avoidance reaction was observed at concentrations coinciding with the pre-exposure level.

Avoidance reactions decreased with increasing level of pre-exposure, considering values beyond the respective pre-exposure level. A minimum to such reactions was reached at a pre-exposure level of 0.8 mg/l Cr(III) which is the same as for the Cr(VI) case suggesting a generally similar mechanism of triggering avoidance reactions. Fish populations pre-exposed at 1.0 mg/l Cr(III), exhibited a new higher avoidance reaction compared to the 0.8 mg/l Cr(III) population. At pre-exposure levels beyond the 1.0 mg/l Cr(III), the trend again was lower avoidance reactions with increasing levels of pre-exposure, for concentrations beyond the respective pre-exposure level.

Avoidance threshold values increased linearly with increasing levels of pre-exposure (Fig. 4.20). For populations pre-exposed between 0.8-3.0 mg/l Cr(III), fish avoidance behaviour beyond the respective pre-exposure level after 7 days acclimation was slightly higher compared to that after 2 days acclimation. Mathematical expressions for avoidance curves and avoidance threshold variation are summarized in Table 4.8.

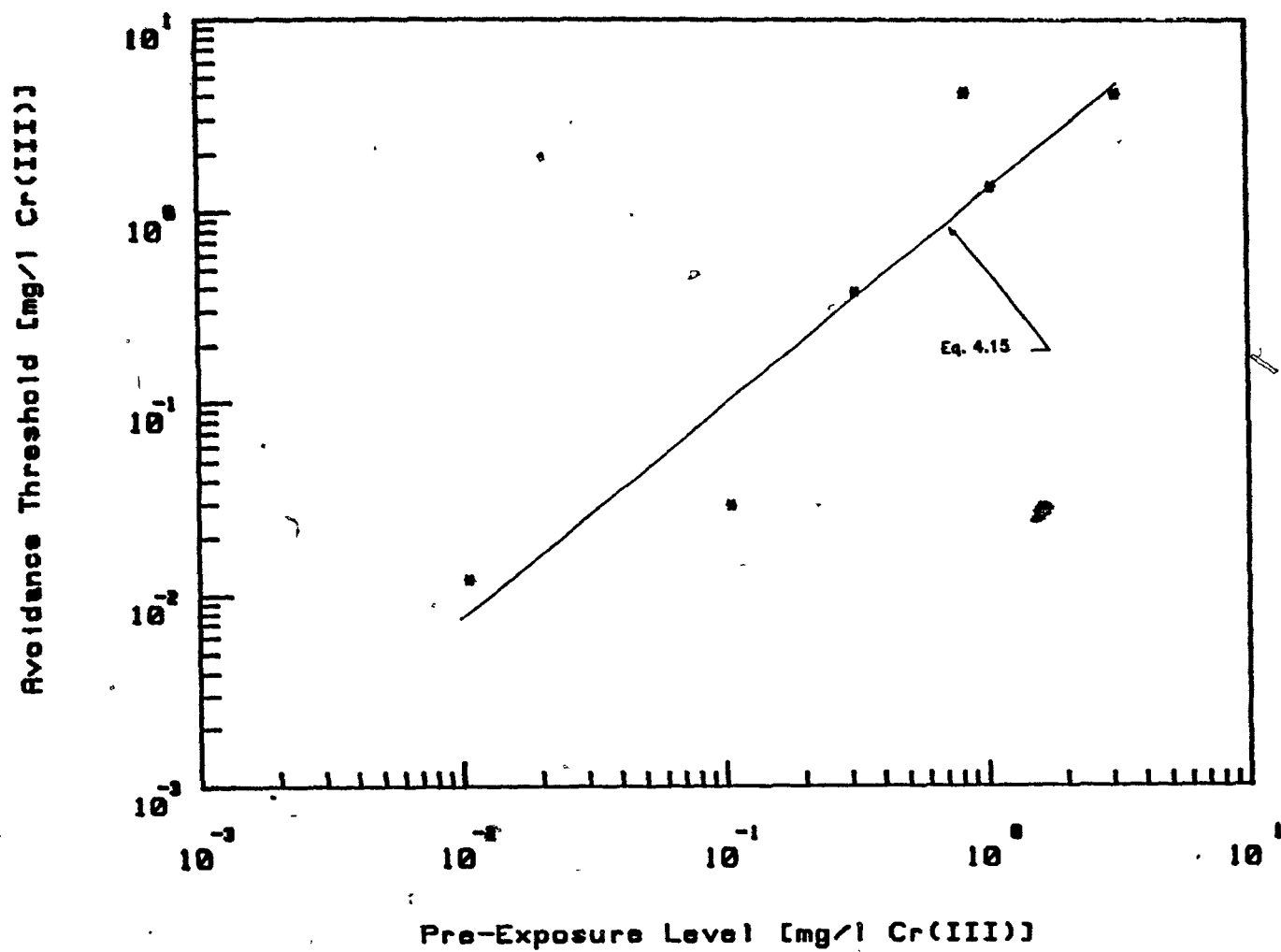


Figure 4.20 Rainbow trout avoidance threshold variation with increasing level of pre-exposure to Cr(III).

4.4 Avoidance preference testing with copper (Cu(II))

Figures 4.21 to 4.26 present results of fish avoidance reactions, using copper in the form of $\text{Cu}(\text{NO}_3)_2$.

Experimental concentrations ranged from 11.25 to 100 $\mu\text{g/l}$ as Cu(II), while pre-exposure levels ranged from 0 to 72.5 $\mu\text{g/l}$ as Cu(II). At 100 $\mu\text{g/l}$ no significant drop of pH was observed (pH from 7.4 to 7.1). The ranges of concentrations in the channel and the pre-exposure levels, were decided based on information about lethal levels for copper. Results are presented in the same format as for the chromium compounds. (fish in clear vs. $\mu\text{g/l}$ Cu(II) in the channel). The horizontal (x-axis) is in normal scale.

Figure 4.21 presents results obtained from a non-exposed population, while Figures 4.22 to 4.26 refer to results from pre-exposed fish to different levels of $\text{Cu}(\text{NO}_3)_2$. Again tests were performed after 2 and 7 day channel acclimation periods.

There was no mortality during the entire pre-exposure period at any concentration level. This suggests that for the given water quality, pre-exposure period and levels of Cu(II) used, concentrations were sublethal.

Mathematical expressions to best fit data and threshold avoidance values are presented in tabular form on Table 4.11.

4.4.1 Avoidance behaviour of Rainbow Trout which have not been pre-exposed to Cu(II)

For a non-exposed population, the avoidance curve (Fig. 4.21) consists of two distinct linear branches. Two linear

Cu(II) P-E [ug/l]		% Avoidance to Cu(II) =		Equation #	Threshold (ug/l)
		Below ABP*	Above ABP*		
Control		$50.85 + 0.7[Cu(II)]$ (a)	$76.45 + 0.14[Cu(II)]$ (b)	4.16	2.1
22.5		N/A	$80.1 + 0.22[Cu(II)]$	4.17	14.1
30.0		N/A	$38.67 + 0.4[Cu(II)]$	4.18	31.8
45.0		N/A	$54.03 + 0.15[Cu(II)]$	4.19	3.2
50.0		N/A	$81.65 + 0.21[Cu(II)]$	4.20	0.0
72.5		$48.94 + 0.83[Cu(II)]$ (a)	$40.49 + 0.49[Cu(II)]$ (b)	4.21	3.7
Threshold Avoidance Variation	Threshold Avoid. = $0.52 + 0.68[P-E]$		N/A	4.22	

* ABP=Avoidance Breakpoint Level
 [P-E]=Pre-Exposure Concentration
 [Cu(II)]=Copper Concentration in the Channel

Table 4.11 Equations and threshold avoidance levels based on data obtained from avoidance tests on rainbow trout exposed to Cu(II).

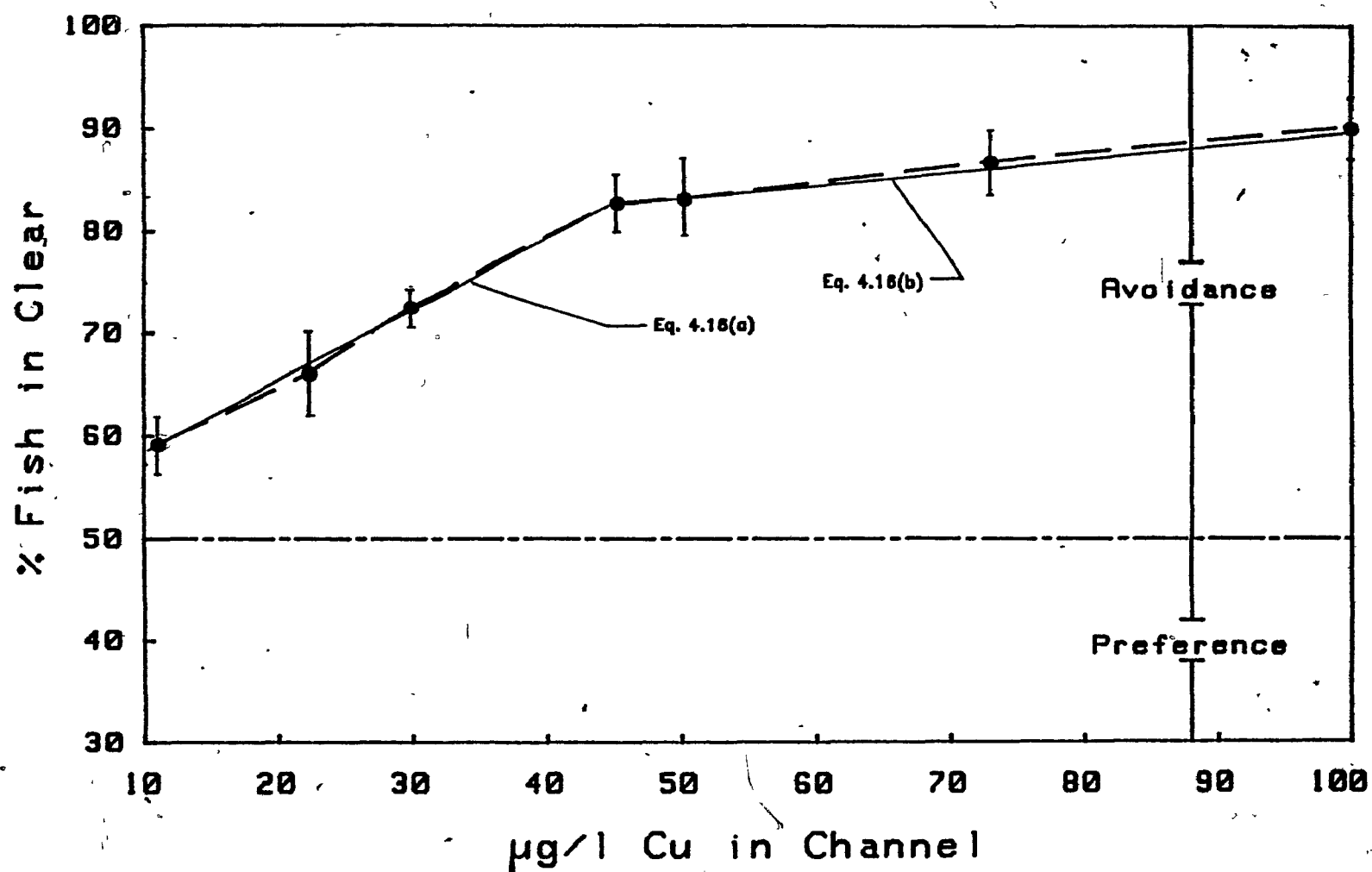


Figure 4.21 Avoidance reaction of non-pre-exposed rainbow trout to $\text{Cu}(\text{NO}_3)_2$

mathematical expressions are proposed for those two distinct ranges of avoidance reaction (Table 4.11). Fish avoidance to Cu(II) increased with increasing concentration levels. The avoidance curve inflection point occurred at 45 $\mu\text{g/l}$.

For the concentration range below 45 $\mu\text{g/l}$ Cu(II) avoidance clearly increased with increasing level of Cu(II) in the channel.

Beyond 45 $\mu\text{g/l}$ Cu(II), avoidance reactions did not increase significantly, although mean avoidance values followed an increasing trend with increasing Cu(II) concentration in the channel.

For a non-exposed population, using the established mathematical expression to fit the experimental data, a threshold avoidance value of 2.1 $\mu\text{g/l}$ Cu(II) can be proposed.

4.4.2 Avoidance behaviour of rainbow trout which have been pre-exposed to Cu(II)

Figures 4.22 to 4.26 present results from populations pre-exposed between 22.5 to 75 $\mu\text{g/l}$ Cu(II). All avoidance reaction curves after 2 days acclimation followed similar trends and characteristics compared to the non-exposed avoidance curves. In all cases, the avoidance reaction curve after a 2 day channel acclimation period exhibited an inflection point, that coincided with the pre-exposure level of the population. Avoidance behaviour of pre-exposed populations for channel concentrations beyond their respective pre-exposure level, was significantly different compared to avoidance reactions of non-exposed populations (Table 4.12).

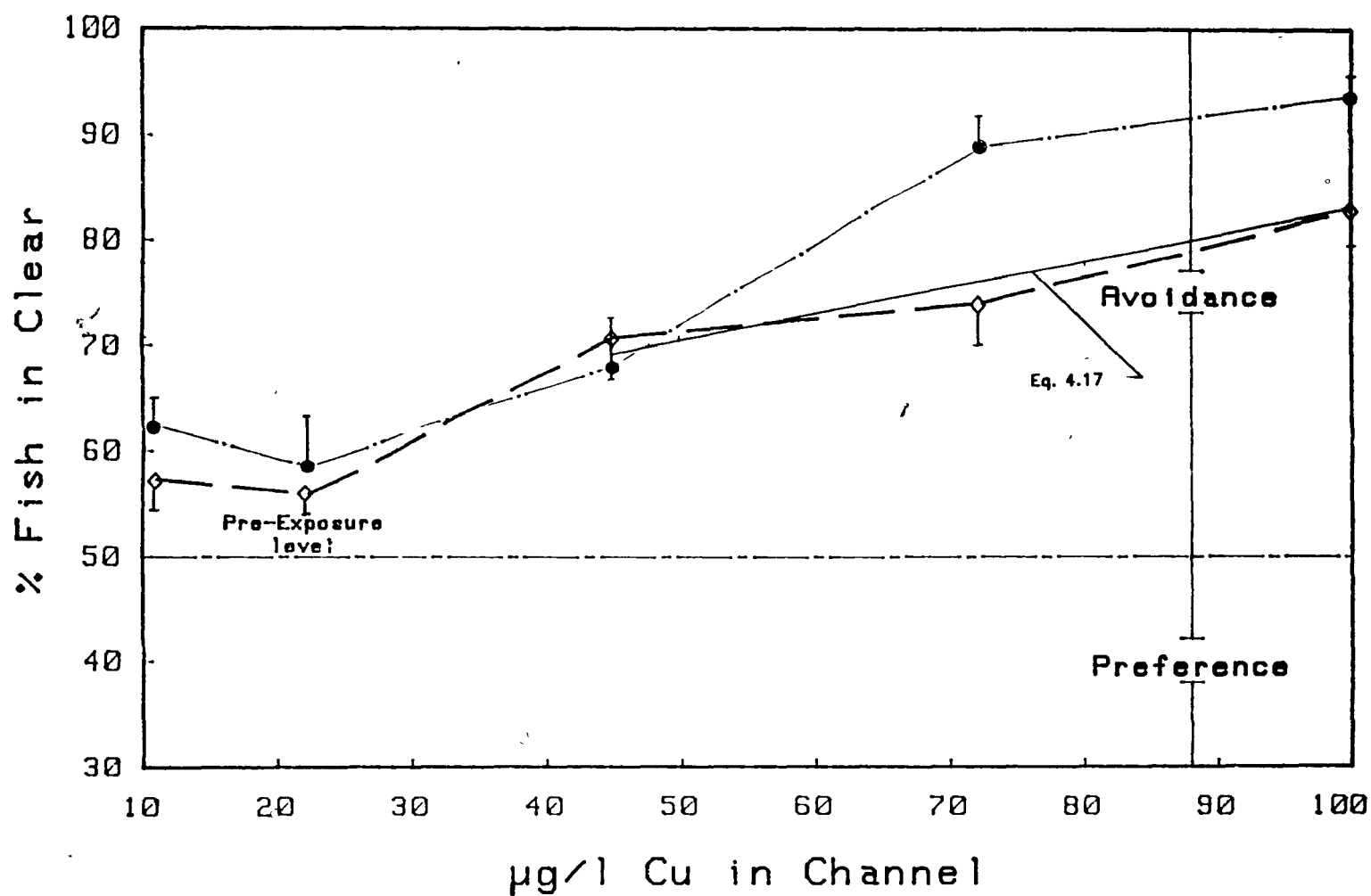


Figure 4.22 Avoidance reaction of rainbow trout pre-exposed to 22.5 µg/l Cu(II) (●) and after 7 days acclimation in clear water (◇).

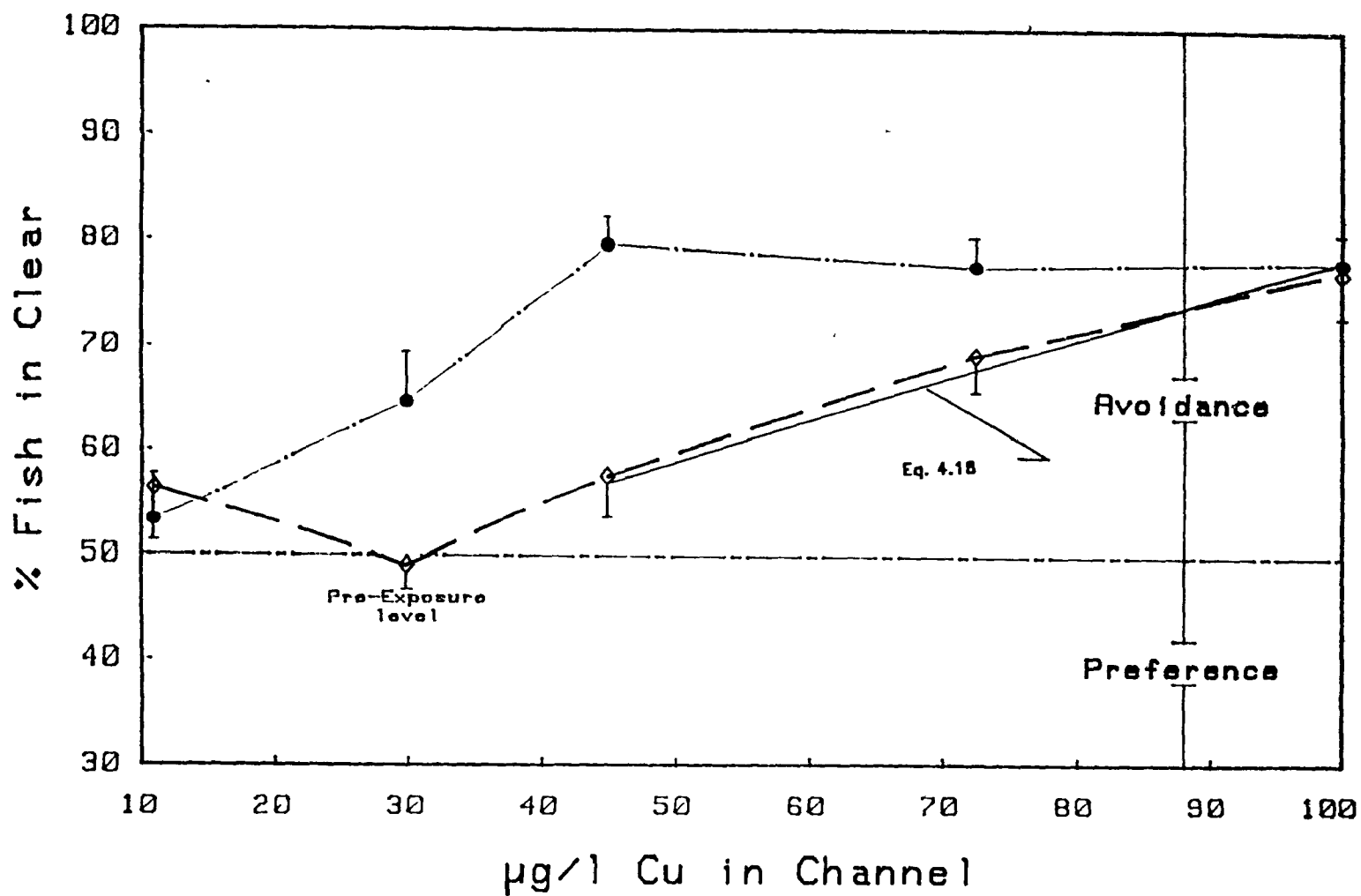


Figure 4.23 Avoidance reaction of rainbow trout pre-exposed to 30.0 µg/l Cu(II) (●) and after 7 days acclimation in clear water (◇).

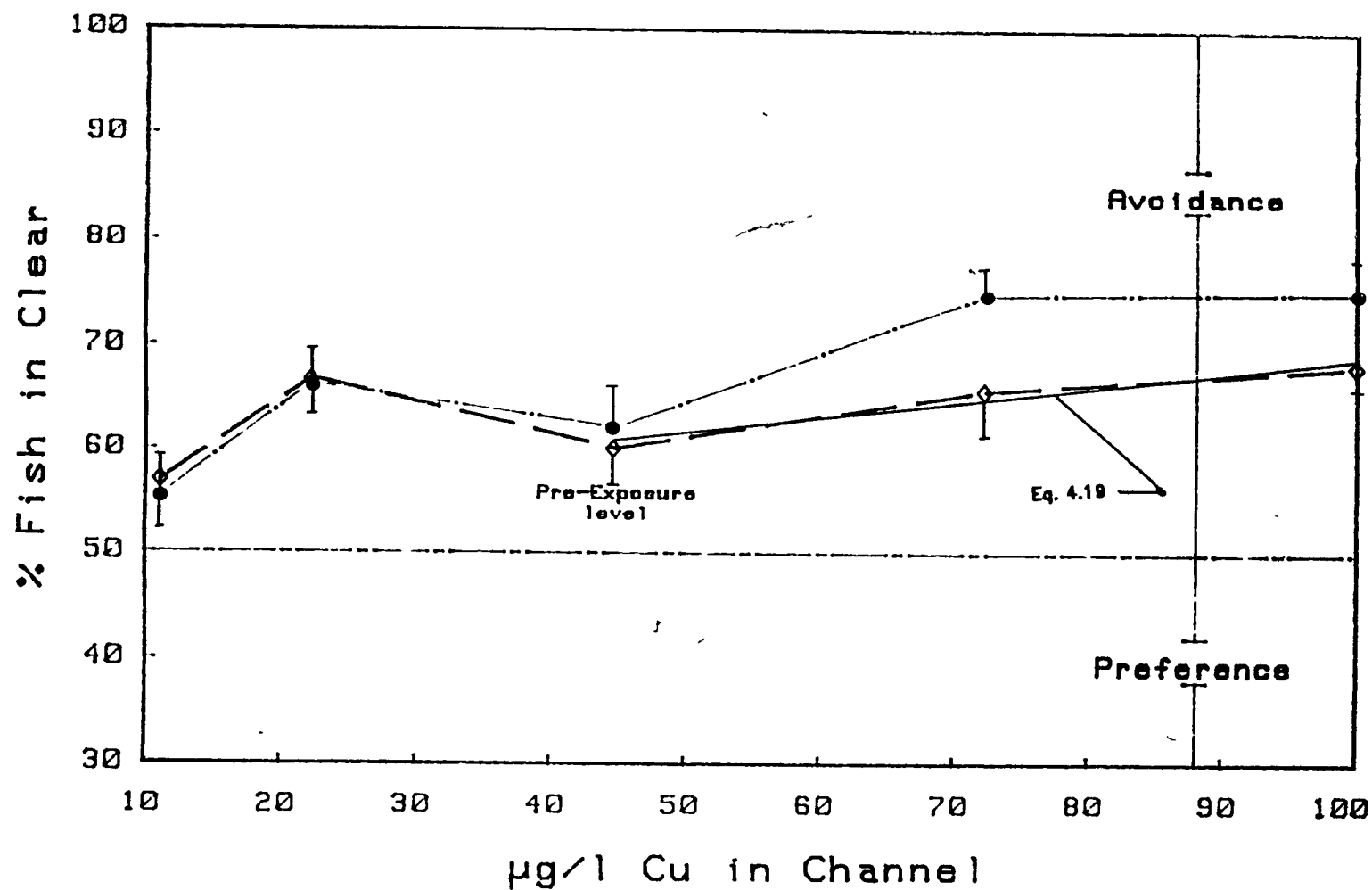


Figure 4.24 Avoidance reaction of rainbow trout pre-exposed to 45.0 µg/l Cu(II) (●) and after 7 days acclimation in clear water (◇).

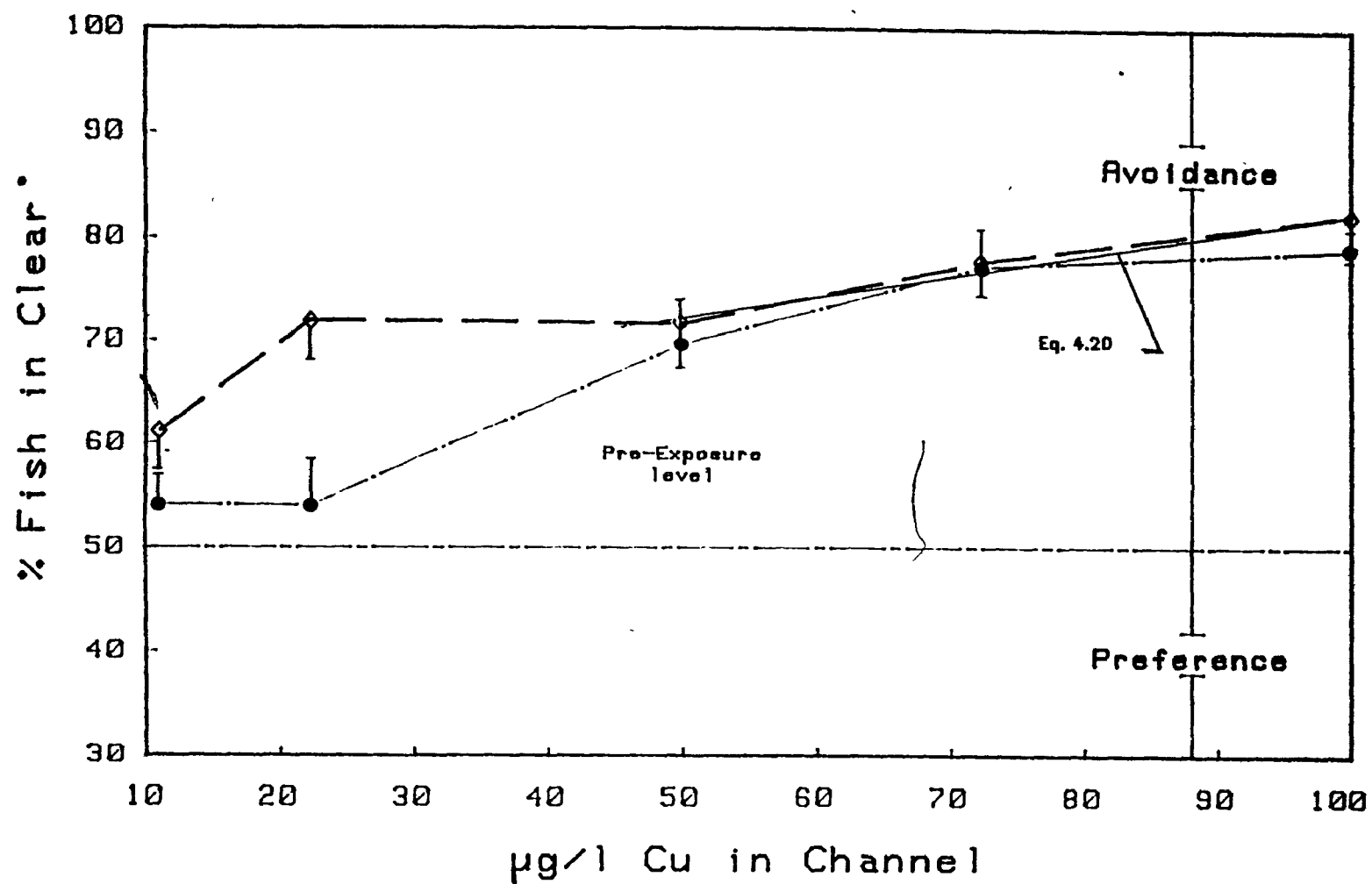


Figure 4.25 Avoidance reaction of rainbow trout pre-exposed to 50.0 µg/l Cu(II) (●) and after 7 days acclimation in clear water (◇).

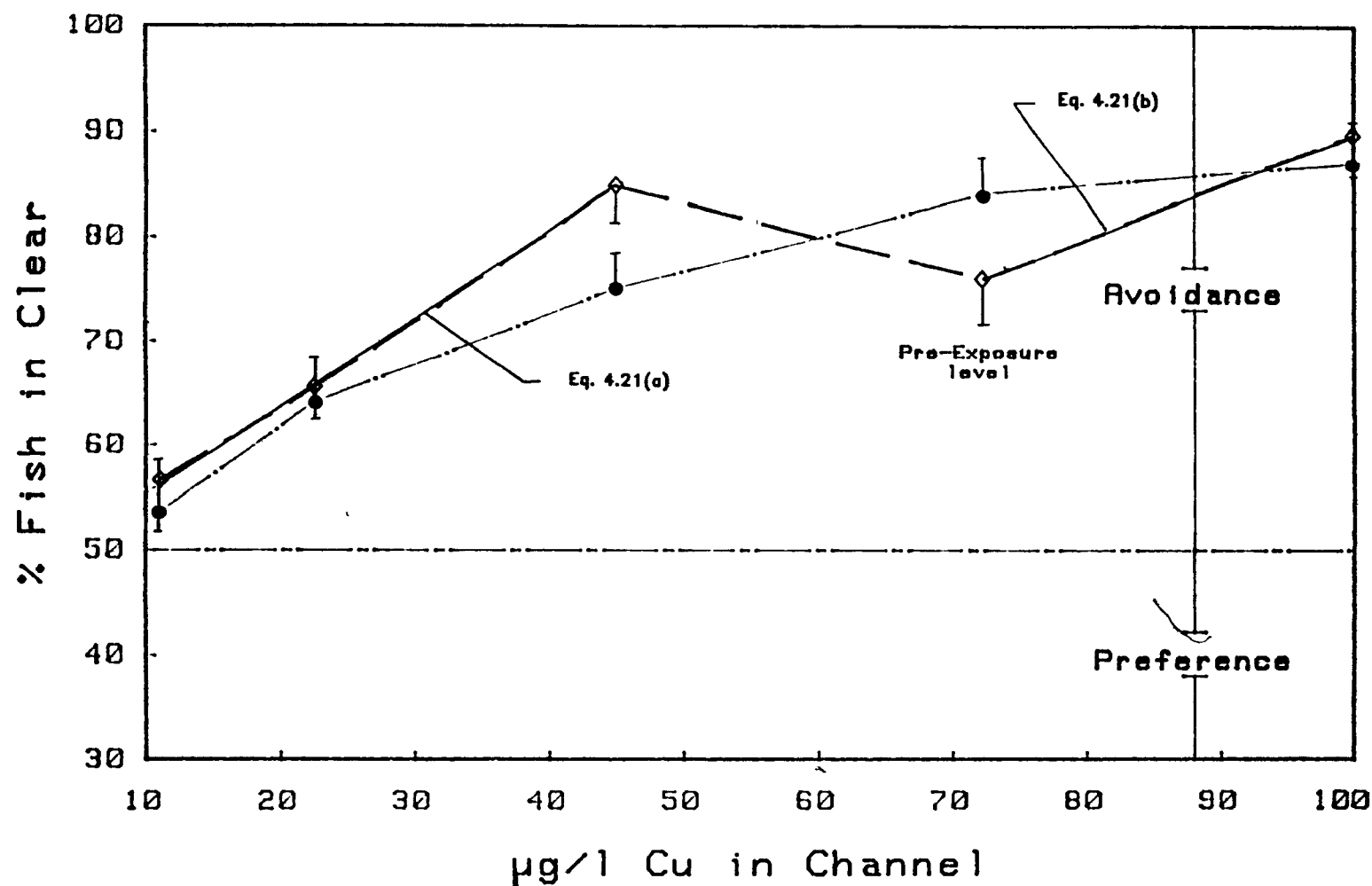


Figure 4.26 Avoidance reaction of rainbow trout pre-exposed to 72.5 $\mu\text{g/l}$ Cu(II) (●) and after 7 days acclimation in clear water (◇).

	Channel Concentrations [ug/l]				
Cu(II) P-E	11.25	22.50	45.00	72.50	100.0
Series (I)	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
1=Control	F=1.35	F=24.94	F=262.9	F=55.56	F=95.97
2=22.5ug/l	PR>F=.261	PR >	F = 0 . 0 0 0 1		
3=45.0ug/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
4=72.5ug/l	A 1 A 3 A 2 A 4	A 3 A 4 A 1 B 2	A 4 C 2 B 1 D 3	A 1 B 2 B 4 C 3	A 1 B 2 A 4 C 3
Series (II)	11.25	30.00	50.00	72.50	100.0
1=Control	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA
2=30.0ug/l	F=5.11	F=198.6	F=251.9	F=67.06	F=56.88
	PR>F=.007	PR >	F = 0 . 0 0 0 1		
3=50.0ug/l	DUNCAN	DUNCAN	DUNCAN	DUNCAN	DUNCAN
	A 3 B 2 BA 1	A 3 C 2 B 1	A 1 B 3 C 2	A 1 B 3 C 2	A 1 B 3 C 2

Table 4.12 Results of ANOVA procedure and DUNCAN tests, on populations subjected to identical channel exposure to Cu(II), with only variable the level of pre-exposure (N=180, $\alpha = 0.05$, $DF_{ANOVA} = 179$, $DF_{DUNCAN} = 176$)

Avoidance reactions decreased, in general, with increasing level of pre-exposure. They reached a minimum at levels in the range of 45 $\mu\text{g/l}$ pre-exposure to Cu(II) . For all pre-exposed populations beyond their pre-exposure level and particularly beyond the 45 $\mu\text{g/l}$ Cu(II) level in the channel, avoidance reaction started increasing again to levels that were higher than those obtained from populations pre-exposed below the 45 $\mu\text{g/l}$ Cu(II) level. This signifies a sensitization of populations pre-exposed beyond the 45 $\mu\text{g/l}$ Cu(II) level. Avoidance reactions for all populations pre-exposed at or beyond the 45 $\mu\text{g/l}$ Cu(II) level were significantly different when comparing reactions from concentrations beyond their respective pre-exposure level (Table 4.12).

Avoidance threshold values for pre-exposure levels below the 45 $\mu\text{g/l}$ Cu(II) are increasing with pre-exposure level. Comparison of threshold values for populations pre-exposed above 45 $\mu\text{g/l}$ Cu(II) , where another slope exists for the avoidance curve, is not straightforward. The only practical consideration would be to establish avoidance thresholds based on the mathematical expression for avoidance behaviour above their respective pre-exposure level (Table 4.11).

For populations pre-exposed below the 45 $\mu\text{g/l}$ level, avoidance reactions after 7 day channel acclimation period were in general higher in mean avoidance values, although not always statistically different (Table 4.13). For populations pre-exposed at or beyond 45 $\mu\text{g/l}$ Cu(II) the avoidance curves after 7 days have a similar trend with the 2 day channel acclimation

Pre-Exposure level [ug/l]	Cu(II) Channel Concentration [ug/l]				
	11.25	22.5	45.0	72.5	100.0
	A	N	O	V	A
22.5	F=20.7 PR>F=.0001	F=14.4 PR>F=.003	F=8.89 PR>F=.0037	F=76.56 PR>F=.0001	F=45.02 PR>F=.0001
	D	U	N	C	A
	A 2 B 1	A 2 B 1	A 1 B 2	A 2 B 1	A 2 B 1
30.0 (Where 22.5ug/l read 30.0ug/l)	A	N	O	V	A
	F=8.91 PR>F=.0037	F=354.6 PR>F=.0001	F=531.6 PR>F=.0001	F=125.9 PR>F=.0001	F=2.51 PR>F=.117
	D	U	N	C	A
	A 2 B 1	A 1 B 2	A 1 B 2	A 1 B 2	A 1 A 2
45.0	A	N	O	V	A
	F=3.98 PR>F=.0491	F=0.24 PR>F=.6249	F=3.27 PR>F=.0738	F=160.86 PR>F=.0001	F=56.39 PR>F=.0001
	D	U	N	C	A
	A 1 B 2	A 1 B 2	A 2 A 1	A 2 B 1	A 2 B 1

Table 4.13 Results of ANOVA procedure and DUNCAN tests, on pre-exposed populations acclimated for 2 days as compared with reactions after 7 day acclimation of the same populations in clear water. (N=90, $\alpha = 0.05$, $DF_{ANOVA}=89$, $DF_{DUNCAN}=88$)

Pre-Exposure level [ug/l]	Cu(II) Channel Concentration [ug/l]				
	11.25	22.5	45.0	72.5	100.0
	A	N	O	V A	
50.0 (Where 45.0ug/l read 50.0ug/l)	F=51.66 PR>F=.0001	F=360.5 PR>F=.0001	F=4.52 PR>F=.0362	F=0.27 PR>F=.606	F=18.29 PR>F=.0001
	D	U	N C	A	N
	A 1 B 2	A 1 B 2	A 1 B 2	A 1 A 2	A 1 B 2
72.5	A	N	O	V A	
	F=5.60 PR>F=.0201	F=2.15 PR>F=.1463	F=137.54 PR>F=.0001	F=104.4 PR>F=.0001	F=18.20 PR>F=.0001
	D	U	N C	A	N
	A 1 B 2	A 1 A 2	A 1 B 2	A 2 B 1	A 1 B 2

Table 4.13 (continued)

curves. Mean values after 7 days channel acclimation for populations pre-exposed beyond the 45 $\mu\text{g/l}$ Cu(II) level are in general, higher in mean values and significantly different from those after 2 days (Table 4.13).

Avoidance threshold values increased with increasing levels of pre-exposure up to the critical level of 45 $\mu\text{g/l}$ Cu(II). Further increase in pre-exposure yielded lower avoidance threshold values. The mathematical expression for the avoidance threshold variation is included in Table 4.11.

5.0 DISCUSSION

5.1 Experimental apparatus and procedures

5.1.1 Channel

The hydraulic channel used in this experimental work for avoidance preference reaction testing provided the means of establishing a uniform and stable concentration in the toxicant zone, totally separated from a clear water zone. A well defined concentration and flow regime downstream from the barrier combined both steep and shallow concentration gradient characteristics. In the past, avoidance preference channel designs were either steep or shallow gradient apparatus. Kleerekoper et al. (1972), Ishio (1964), Lubinski (1979) and Westlake et al. (1974) have proposed that fish avoidance as well as locomotor behaviour depend not only on the magnitude but also on the concentration gradient. Lubinski (1979) and Westlake et al. (1974) stipulated that steep gradients may elicit avoidance reactions, while shallow gradients may result in preference towards the toxicant. The present configuration provided fish with the option to choose any concentration ranging from clear water to the concentration in the toxicant discharge zone. A configuration that combines both steep and shallow gradients can be used to eliminate the discrepancies that were encountered in the past, due to the fixed concentration gradient, steep or shallow, within the experimental tank. Results obtained from experiments during which fish were mostly occupying the downstream non-separated region (shallow gradient) were statistically similar to results from experiments during which fish had retreated to the channel separated sec-

tions (steep gradient) (Table 4.4). This suggests that either gradient, steep or shallow, yields similar avoidance results, which is in agreement with Giattina *et al.* (1982). The ratio of 1:2 of non-separated to separated volume in the experimental tank, provided the needed space for the fish to select the appropriate concentration level of preference, without masking behaviour patterns. Actually, the apparatus provided equal volumes for toxicant, clear and varying concentration zones. Also of primary importance is the fact that with such configuration the entire fish population can be accounted for at any time, with clear indication of the preferred level of toxicant concentration. Hence, a proper statistical analysis can be performed. In the past, where a decision area was provided in the experimental channel, fish occupying positions in such areas could not be accounted for in the avoidance evaluation. Thus, either missing values were acknowledged in the statistical analysis or researchers calculated avoidance based on the behaviour of the fish present in the separated sections only. The latter approach was both statistically and practically invalid, since results were not obtained from the same population at all times.

5.1.2 Experimental test procedures

5.1.2.1 Optimum number of fish

When more than one organism is placed in the channel, territorial behaviour may result in a form of aggression, Chiszar *et al.* (1975), masking preference-avoidance reactions. On the other hand, employing more than one fish makes results more represent-

ative of a real-life situation. Results obtained from experiments performed to establish the optimum number of fish, under the standard experimental conditions, indicated the significance of overcrowding in the channel.

The results obtained using the selected optimum number of 18 fish compared with those obtained using 19 fish in the channel, demonstrated the importance of the territoriality load, since the results were different (Table 4.1). Previously, researchers had considered aggressiveness of fish in the experimental channel as a natural expression of social behaviour and therefore they acknowledged it as a non-influencing parameter in their avoidance reactions. Results from the present study indicate that if avoidance reactions are to be evaluated, aggressive behaviour can and must be controlled or even eliminated so that it does not exert masking effects on the actual fish avoidance behaviour. The need to reduce avoidance reaction masking factors led to the concept of territoriality load, which was calculated at 2.09 g/l or for easier reference can be established at the 2 g/l (total weight of organisms per experimental tank volume). This load is proposed as a guideline to establish the maximum capacity of an experimental tank to handle fish when behavioural studies are performed.

5.1.2.2 Single concentration experiments versus step function concentration testing

Two methods were evaluated for running avoidance-preference tests. The first involved exposing the fish to a single toxicant

concentration for the entire testing period. The second employed a sequence of increasing concentrations using the same fish population during the test. Results obtained from the two methods were similar (see 4.1.2.2). Similar responses using the same two procedures were reported by Sprague (1969), Bogardus et al. (1975) and was also proposed by Giattina et al. (1982). The latter authors compared regression lines obtained using the two methods and found no statistically significant differences.

Statistical similarity of results using either method of testing, suggested that previous exposure at lower concentrations had no influence on fish avoidance reaction at subsequent higher concentration levels. Thus it is proposed that the step function concentration testing, in addition to being a far more efficient exploitation of resources, is a valid technique to evaluate fish avoidance-preference reactions. Giattina et al. (1982) suggested that the step function concentration procedure is valid for 20 minute exposure intervals and up to 4 concentrations per experiment, a maximum exposure of 1 hr. and 20 min. The present study demonstrated that a step function concentration testing can yield valid results for intervals of 35 minutes with up to 6 concentrations per experiment, with a maximum exposure of 3.5 hrs.

Since avoidance reaction is an acute response to stress induced by the presence of the toxicant, the critical elements in the step function concentration testing is the length of the intervals of each concentration level and the collection of data at system steady-state rather than the total length of experimentation. This is supported by the repeatability of results,

where experiments were performed with 1 - 6 concentrations per experiment, while the time interval of each exposure level was the same (35 minutes). Therefore it is proposed that any number of concentrations per experiment is expected to yield similar avoidance results as with single concentration experiments provided that the exposure duration and conditions are identical for all experimental runs and avoidance is evaluated at system steady-state. Further, it is proposed that such a procedure be adopted for all avoidance-preference studies, given the significant time and expense savings over the single concentration avoidance experiments.

5.1.2.3 Channel acclimation period prior to testing

In the process of eliminating masking and avoidance influencing factors, the significance of the fish acclimation period in the channel was evaluated. The behaviour of fish in the channel was erratic before the fish recovered from the netting experience and became familiar with the testing chamber. Such erratic behaviour was observed during the 20 minute and the one day acclimation periods described in section 3.1.2.3. Previous researchers had employed acclimation periods ranging from 10 minutes to 2 days prior to subjecting fish to a toxicant concentration field in the channel. The large difference in acclimation periods used by the different researchers may be attributed to differences in sizes of apparatus, species, number of fish in the experimental tank and primarily the time it took for the fish to be distributed evenly in the channel. However, there is one significant

influencing parameter, present in bioassays, which was not considered adequately in the past. The netting process to introduce organisms from the holding tanks to the experimental tank results in a stressful impact on the organism independent of experimental set-up. The recovery period following a stressful experience was proposed by Barton et al. (1985) and Wedemeyer et al. (1979) to be 2 days, based on the time it took for certain biological parameters, used as stress indicators, to stabilize to normal levels. This corroborates what was observed in the present study, repeatability of results after 2 days of channel acclimation and inconsistency of results after shorter channel acclimation periods. A 50-50 fish distribution in the channel prior to performing the experiment is not sufficient proof that fish are acclimated to the new environment. In a 1 day channel acclimation experiment, described in section 4.1.2.3, even though fish were equally distributed in the channel prior to testing, the avoidance results were different from other experiments conducted following 1 or 2 day channel acclimation periods. On the other hand, in 2 day channel acclimation experiments, even when fish were not distributed equally prior to testing, the results were consistent, provided that the pre-testing distribution was considered in the evaluation of fish net avoidance (see section 2.2.5). Evaluating the acquired experience on fish pre-testing acclimation, it is proposed that repeatability of results is the determining factor for ensuring fish acclimation in the channel. This can be achieved only when influence of other stressful factors, such as netting experience, are fully subdued.

5.2 Avoidance-Preference testing with Cr(VI)

5.2.1 Avoidance behaviour of Rainbow Trout to Cr(VI)

Experiments performed on a non-exposed population yielded an avoidance response curve described by Eq. (4.1). Observed fish avoidance reactions increased linearly on a log-normal scale with increasing levels of toxicant in the channel. This is in agreement with all previous reported avoidance reactions, wherever avoidance to the toxicant was observed. A threshold avoidance value of 0.026 mg/l Cr(VI) was established. This is similar to the 0.02 mg/l total chromium proposed in the new draft of the Canadian Water Quality Guidelines (in press). The 0.02 mg/l level reported as maximum concentration for any water use, was established using Daphnia magna as the test organism. To date the existing safe level was set at 0.05 mg/l Chromium (EPS Env. Can. (1980), USEPA (1976)). There is a large range of sensitivity to Cr(VI) among different organisms reported in the literature (App. A.2). Varying water characteristics make results even more difficult to interpret and direct comparisons impossible. The threshold avoidance reaction level is also included in Table 4.5. The exhibited sensitivity of avoidance reactions can classify avoidance testing as an efficient means of predicting safe concentration levels for any toxicant, simply by using the established avoidance threshold level as the proposed safe level for the tested toxicant. In addition, a threshold avoidance level established employing fish bioassays could also provide prediction of safe levels for lower organisms (0.026 mg/l Cr(VI) for fish compared to 0.02 mg/l Cr(VI) for Daphnia magna). Thus avoid-

ance-preference tests can be considered as a potential tool for fast and efficient determination of safe toxicant levels for a wide range of levels of biological integration.

5.2.2 Avoidance behaviour of Rainbow Trout, pre-exposed to Cr(VI)

Populations of rainbow trout pre-exposed to Cr(VI) demonstrated several general avoidance reaction trends. Fish pre-exposed to Cr(VI) exhibited lower avoidance reactions compared to non-exposed fish. This decrease in avoidance reaction was consistent for increasing pre-exposure levels and reached a minimum when fish were pre-exposed at 0.8 mg/l Cr(VI). At the next higher pre-exposure level, 1.0 mg/l Cr(VI), fish avoidance reactions were higher compared to the 0.8 mg/l Cr(VI) pre-exposed population. Further increase in pre-exposure levels yielded again a trend of reduced avoidance reactions with increasing levels of pre-exposure indicating that 0.8 mg/l Cr(VI) can be considered as a critical level for fish pre-exposure to Cr(VI).

This reduction in avoidance reactions suggests an increased tolerance with increasing levels of pre-exposure. This trend is widely accepted as valid in freshwater and marine species and for a variety of toxicants as reported by Dixon and Sprague (1981,1; 1981,2), Luoma (1977), Weis et al. (1981), Saliba and Krzyz (1976), Rahel (1981), Pascoe and Beattie (1979), McKim et al. (1976), Beattie and Pascoe (1978), Dixon and Hilton (1981), Wedemeyer et al. (1979), and Spehar et al. (1978). All research results suggest that fish exposed sublethally to a toxicant at any life stage from eggs to adults become more tolerant

and usually more resistant to the presence of the same toxic agent in the environment.

Sprague (1969) suggested that fish become more tolerant for pre-exposure levels up to 0.6 toxic units where:

$$X \text{ (toxic unit)} = \frac{\text{toxicant concentration}}{96 \text{ hr LC50 value for the same toxicant}}$$

Based on the results of the present experimental work, a critical level of 0.8 mg/l Cr(VI) was supported by two alternative rationales.

On the one hand, a slight change in slope in the avoidance curve of the non-exposed population occurred in the range of 0.8 mg/l Cr(VI) (Fig. 4.5 and further discussed in section 4.6.2) suggesting a change in avoidance behaviour mechanism. Secondly at the 0.8 mg/l Cr pre-exposure level, fish demonstrated the lowest avoidance reaction curve compared to all pre-exposed populations. In fact, fish pre-exposed at the proposed critical level of 0.8 mg/l Cr(VI) exhibited preference for any toxicant concentration up to the threshold avoidance level of 5.8 mg/l.

A wide range of proposed 96 hr LC50 values for Cr(VI) was found in the literature (11-118 mg/l Cr(VI)) (See App. A.2). If 11 mg/l Cr(VI) is accepted as the 96 hr LC50 value, the ratio $0.8/11 = 0.07$ toxic units is almost ten times lower than Sprague's (1969) proposal. Experimental conditions and type of toxicant for Sprague's experiments were different. On the other hand, a level of 0.6 toxic units was established with different considerations for referring to the level as critical. In Sprague's case, tolerance was evaluated at lethal levels through

96 h LC50 values. In the present work, 0.8 mg/l Cr (VI) is proposed as critical because of several indicators such as 1) increased mortality for populations pre-exposed beyond that critical level 2) change in the avoidance behavioural pattern beyond 0.8 mg/l Cr(VI) and 3) return to a normal behaviour pattern compared to non-exposed fish after a short clearance period following pre-exposure at concentrations up to the critical level.

Van der Putte et al. (1981) suggested that fish pre-exposed at 2.0 mg/l Cr (VI) were biologically significantly different than controls, which supports present findings of altered pattern in avoidance reactions beyond the proposed critical level of 0.8 mg/l Cr (VI).

All pre-exposed populations of rainbow trout demonstrated an attraction towards a familiar environment for concentrations that corresponded to their pre-exposure level. This was demonstrated graphically by the inflection points on all avoidance curves (Figs. 4.5 to 4.11).

Despite the fact that fish were acclimated in clear water for 2 days prior to testing, they still demonstrated a distinctive attraction towards their familiar environment. This behaviour has not been reported in the literature. If this behaviour was applicable in natural waters, regulatory standards should be reconsidered in light of this physiological trap, especially since avoidance of pre-exposed populations is lowered with increasing sublethal pre-exposure levels.

All equations included in Table 4.5 were derived to describe

fish avoidance behaviour for concentrations higher than their pre-exposure level. They can serve both as predictive tools for establishing avoidance reactions of pre-exposed fish as well as provide threshold avoidance values for populations under similar pre-exposure conditions.

Avoidance threshold values for all pre-exposed populations increased linearly with increasing levels of pre-exposure. Avoidance threshold values can be correlated to tolerance and as suggested by various researchers, tolerance towards lethal levels was increasing with increasing sublethal levels of pre-exposure. Therefore, the same behaviour patterns in terms of tolerance can be established either through lethal tests or sublethal avoidance reaction testing. This may suggest that avoidance reactions or behaviour in general can be correlated to results at lethal levels. The advantage of avoidance reaction testing is that a relation between pre-exposure level and level of tolerance (avoidance threshold) may be established. In addition, this relation may be used as a predictive tool for safe concentration levels given the pre-exposure level of a particular fish population.

Fish populations pre-exposed up to the critical level of 0.8 mg/l Cr (VI), followed by acclimation for 7 days in clear water, demonstrated a functional recovery of their chemoreceptive capacity. This was evident from the similarity in the respective avoidance curves compared to that of the non-exposed population. While clearance of the toxicant from flesh may be rapid, clearance from other organs and gills is a slow process for a variety

of toxicants. For avoidance reaction testing, it is suggested that flesh toxicant levels play a minimal role in avoidance reactions. On the other hand, gills and olfactory receptors are proposed to play a major role in inducing avoidance reactions. Singh and Ferns (1978) and Hara et al. (1983) suggested a 12 week rehabilitation period for biologically affected fish after pre-exposure that reached steady-state in toxicant content in the organisms. Results for Cr(VI) from the present work suggest that there is a much shorter period for functional recovery from Cr(VI) exposure to behavioural levels comparable with those of non-exposed populations. For populations pre-exposed beyond the 0.8 mg/l Cr (VI), fish have been biologically affected by such pre-exposure levels. This was evident by the difference in the 7 day avoidance curve of a population pre-exposed to 1.0 mg/l Cr (VI) compared to the non-exposed curve (Fig. 4.5 and 4.10). It was also supported by the increased mortality at 1.0 and 3.0 mg/l Cr (VI) compared to controls (respectively 14 and 36% compared to 1.5% in the controls). These observations reinforce the hypothesis that 0.8 mg/l Cr(VI) is a critical level of exposure, beyond which fish cannot recover their avoidance behaviour sensitivity within a short period of time.

5.3 Avoidance-Preference testing with Cr(III)

5.3.1 Avoidance-Preference testing with Rainbow Trout not previously exposed to Cr(III).

Avoidance-preference experiments performed on trout using Cr(III) as the toxicant yielded an avoidance response curve

described by equations presented in Table 4.8. It was evident that no single log-linear mathematical expression could fit the Cr (III) data. Therefore two separate log-linear expressions are proposed depending on concentration ranges. The change in avoidance reaction occurs around 3.0 mg/l Cr(III). Avoidance reactions increased with increasing Cr(III) concentrations, although results were not significantly different over a wide range of concentrations up to 3.0 mg/l Cr(III). This behaviour is in general agreement with observations by Mearns (1985), Pickering and Henderson (1966), and Clarke (1974), where they proposed that Cr(VI) is more effective in eliciting toxic effects at low chromium concentrations, while Cr(III) yields higher toxicity at high concentration levels (Fig. 4.5 and 4.13). This is further evidence that avoidance behaviour can be directly correlated with toxicity results based on lethal bioassays.

A threshold avoidance value of 0.0026 mg/l Cr(III) was determined from the experimental data, which is 10 times smaller than the Cr(VI) respective threshold. This is also 8 times smaller than the 0.02 mg/l total chromium, proposed by the Canadian Council of Resource and Environment Ministers (in press) and significantly lower than the existing standard of 0.05 mg/l total chromium reported by EPS Env. Can. (1979), and USEPA (1976). Threshold avoidance values and mathematical expressions are summarized in Table 4.8.

5.3.2 Avoidance-Preference testing with Rainbow Trout pre-exposed to Cr(III)

Fish avoidance reactions following 0.01 and 0.1 mg/l Cr(III) pre-exposure were not different compared to the behaviour of non-exposed fish. This suggests that the impact of such levels of pre-exposure on the fish was not significant. Considering the strength of Cr(III) bonding and adsorption efficiency, the experimental data can support two possible explanations. Strongly bonded Cr(III) resulting from pre-exposure is not mobilized within the two days acclimation period in the channel, whereas non-exposed fish readily bind Cr(III) on gills and olfactory membranes to levels similar to the pre-exposed populations. Although not supported by quantifiable data, this proposal is plausible since Cr(III) binds readily and bonding is extremely strong on biological membranes (Ferguson 1982, Tobin 1986). Alternatively, pre-exposure levels up to and including 0.1 mg/l Cr(III) do not affect fish. This proposal is supported by Clarke (1974), where the lowest concentration of Cr(III) for toxic sublethal effects was established at 0.33 mg/l.

Populations pre-exposed at 0.3 mg/l Cr(III) and beyond, when tested at concentrations beyond their pre-exposure level, demonstrated reduced avoidance reactions with increasing levels of pre-exposure. Reactions reached a minimum at the 0.8 mg/l Cr(III) pre-exposure level similar to the case of Cr(VI) pre-exposure. Overall avoidance reactions of populations pre-exposed at 1.0 mg/l Cr(III) level were higher compared to those obtained for populations pre-exposed at 0.8 mg/l Cr(III). At concentra-

tions beyond the 0.8 mg/l Cr(III) level of pre-exposure, avoidance reactions started decreasing with increasing levels of pre-exposure (see Figures 4.18 and 4.19). Comparing results between Cr(VI) and Cr(III) pre-exposures, the similarity in behaviour at the 0.8 mg/l Cr(III) pre-exposure level, which was suggested from Cr(VI) tests as a critical level of chromium pre-exposure or in appropriate terminology as a maximum allowable toxicant concentration, is not coincidental.

Fish exposed to any form of chromium, if given sufficient time, eventually accumulate chromium in its trivalent form (Singh and Ferns¹ (1978)). The fact that similar overall avoidance behaviour was observed between the several pre-exposed populations to two different chromium compounds can be attributed to the assumption that long term exposure of the population negates the effect of initial differences in ionic speciation. After several days, Cr(VI) will be eventually transformed to Cr(III) and further accumulation at steady-state of pre-exposure would yield biologically similar populations, even if such populations were initially exposed to different ionic species of the same toxicant.

The differences in mortality of populations exposed at 1.0 mg/l and 3.0 mg/l between Cr(VI) which exhibited mortality, and Cr(III) with no mortality, can be attributed to higher acute toxicity of hexavalent chromium, over the trivalent form (Clarke (1974)). Additional supportive evidence of the range where Cr(III) becomes an acutely effective toxicant can be drawn from the avoidance reaction curves of non-exposed populations (Fig.

4.5 and 4.13) subjected to Cr(III) and Cr(VI). The actual change in slope of the avoidance reaction curve for Cr(III) occurred at 3.0 mg/l Cr(III) with a steeper slope, compared to Cr(VI). This suggests that Cr(III) becomes a more effective toxicant beyond the 3.0 mg/l Cr(III) level for cases of acute exposure compared to Cr(VI). Similarly, the actual avoidance reaction levels are in general higher for Cr(VI) at low concentrations up to the range of 3.0 mg/l and for all pre-exposed populations up to the critical pre-exposure level of 0.8 mg/l. Beyond the proposed critical level, avoidance reactions of pre-exposed populations to Cr(III) are higher compared to respective Cr(VI) pre-exposed populations. This evidence further supports the idea of a direct correlation between avoidance data and toxicity effects.

Consideration of similarities and differences in avoidance reactions of populations exposed at the same net chromium levels to two different chromium compounds, indicate that Cr(VI) is of increased toxic potential compared to the Cr(III) form for acute exposure, within a range of sublethal concentrations below the 3.0 mg/l level. Cr(VI) is also proposed to be more toxic than Cr(III) for short term continuous exposure beyond the suggested maximum allowable toxicant concentration (MATC) of 0.8 mg/l as total chromium, as suggested by the increased mortality of the Cr(VI) pre-exposed populations. On the other hand Cr(III) is the form that fish eventually accumulate and store in their tissues. This was stated by Singh and Ferns (1978) and also suggested by the present results since fish populations pre-exposed to either

Cr(VI) or Cr(III) exhibited a similarity in avoidance response trends over the entire range of pre-exposure levels. Results obtained using either chromium compound yielded the same critical level for long term pre-exposure, based on the similar response of populations pre-exposed at 0.8 mg/l of chromium. (Fig. 4.9 and 4.17). Slight differences in levels of actual avoidance can be attributed to the bioassay.

A comparison of avoidance curves obtained from non-exposed populations subjected to Cr(III) and Cr(VI) in the channel, suggests that at chromium concentrations beyond the 10.0mg/l level, Cr(III) is more effective in eliciting stronger avoidance reactions compared to Cr(VI) (Fig. 4.5 and 4.13). Similar results were observed from a comparison of results from all populations pre-exposed to Cr(III) and Cr(VI). Cr(III) was more effective in eliciting avoidance responses beyond the 3.0 mg/l level. This reduction in the level that causes stronger avoidance reaction for Cr(III) compared to Cr(VI), between non-exposed and pre-exposed populations, may be due to the accumulation of chromium in organisms in its trivalent form (Singh and Ferns (1978)).

The observed higher avoidance reactions due to Cr(III) compared to those due to Cr(VI) at high chromium levels (> 3.0 mg/l), can also correlate indirectly avoidance behaviour, with toxicity, since Clarke (1974) reported higher Cr(III) toxicity compared to Cr(VI) at high nominal chromium concentrations.

One apparent inconsistency between avoidance reaction trends for the two forms of chromium as stated above, occurred at the 0.3 mg/l pre-exposure level (Fig. 4.8 and 4.16). Avoidance

curves for Cr(III) and Cr(VI) at the 0.3 mg/l pre-exposure level follow identical trends with avoidance reactions to Cr(III) being consistently lower.

The first pre-exposure level where fish-avoidance reactions differed from the non-exposed populations occurred at 0.3 mg/l Cr(III). Therefore 0.3 mg/l Cr(III) can be proposed as the effective concentration to yield significant avoidance reactions. This is also corroborated by Clarke (1974), who reported 0.33 mg/l Cr(III) as the lowest level to produce toxic effects. This suggests an additional correlation between avoidance-preference data and levels established using other lethal bioassay techniques.

Threshold avoidance levels are summarized in Table 4.8. Threshold avoidance values were consistently higher with increasing levels of pre-exposure, indicating improved tolerance with increasing levels of pre-exposure. This is in line with observations of previous researchers as stated in section 5.2.2.

For all populations pre-exposed below the 0.8 mg/l critical level, avoidance reactions after a 7 day channel acclimation period were generally lower compared to those after the standard 2 day channel acclimation period.

It appears that avoidance-preference reactions are mediated through chemoreceptors on nose, palatal and gill membranes. (See section 5.6.2). On the other hand, the bulk of the accumulated toxicant is cleared through a tissue-plasma-gill pathway. Since gills are the last step in the clearance process and a key sensory organ to contribute in avoidance reactions, any accumulation

of toxicant due to clearance on the gill epithelium may cause a change in avoidance level. Therefore, a reduction of avoidance reaction due to the different clearance period (7 days compared to 2 days) may be attributed to an increased Cr(III) concentration on the gill especially considering the bonding strength and the affinity of Cr(III) for biological membranes (Tobin (1986)).

A probable mechanism to explain the change in avoidance reaction would involve a comparison of the test concentration to that of the epithelial level. If the epithelium concentration is higher than the ambient water concentration of the toxicant then a neutral or mild reaction might be expected as long as the epithelium remains below saturation. Therefore for two fish populations tested under the same channel conditions, the one with higher initial gill toxicant concentration, due to a longer clearance period, is expected to react at a lower level, as was the case for populations pre-exposed below the 0.8 mg/l Cr(III) critical level.

Populations pre-exposed at or beyond the 0.8 mg/l Cr(III) exhibited higher avoidance reactions after a 7 day channel acclimation period compared to those after the standard 2 day channel acclimation period, as long as channel concentrations were beyond the respective pre-exposure level. Fish avoidance reactions due to toxicant impact are concentration range specific. Reactions in general follow a three stage pattern, alarm - resistance - exhaustion. This is also demonstrated graphically in Fig. 2.2 and 2.4. Avoidance results can establish a similar 3 range concentration pattern to describe the toxicant impact on organisms.

The first range consists of the safe zone of exposure or no effect zone. It expands from non-detectable levels of the toxicant and is upper bound by the avoidance threshold level of the population considered. This is further proposed as the maximum toxicant concentration for safe water use. The second range extends from the threshold value to the critical toxicant level, as established in the present study. This zone will be further referred to as the elastic exposure zone, because fish exposed to toxicant concentrations within that range will be biologically stressed in an elastic or reversible fashion. Fish pre-exposed within this zone maintain the capacity to recover to their initial or normal state after the removal of the pollution source (Fig. 2.4).

The proposed critical level is established using the available avoidance curves of non-exposed and pre-exposed populations. It is the minimum of the two levels established from the range where a change in slope of the avoidance reaction curve occurs and from the pre-exposure level that yielded a curve with the minimum intensity in avoidance reaction.

The critical level can be proposed as a M.A.T.C. This is the concentration where fish start being biologically affected and long term exposure beyond that level will produce increased population mortality. The third range of toxicant concentration ranges from this critical level to beyond the LC50 value. Fish exposed within this range will be irreversibly biologically affected to such a degree that even if there is no immediate mortality observed, recovery to a normal state of health is not

guaranteed, especially within a short time period.

Avoidance reactions are stress induced and the intensity of the stress is related to the detection capacity of the fish. The detection capacity can be altered by the clearance process following a long term exposure of fish to the toxicant. In fish bioassays the gills are the last step in a clearance process and at the same time, a major toxicant detection centre. If a toxic substance such as Cr(III), which binds strongly on biological membranes such as gills, is cleared from all other tissues and organs, it will accumulate on the gills before it will eventually be cleared from the organism. The longer the clearance period, the more likely for an increased level of Cr(III) to exist on gill membranes, and therefore altered avoidance behaviour may be observed. On the other hand, fish of the same species and same overall characteristics of age and sex are expected to have similar capacities of binding and clearing Cr(III) on gills when subjected to the same test conditions and before reaching saturation. It appears, therefore, probable that the level of Cr(III) on the gill surface, should be increasing with the level of pre-exposure for the same clearance period until the gill membrane becomes saturated with Cr(III). For low pre-exposure levels, below the 0.8 mg/l level, avoidance reactions after 7 days channel acclimation were lower than the equivalent 2 day channel acclimation period and were in general decreasing with increasing pre-exposure levels. The observed behaviour is in agreement with the assumption of higher concentration of Cr(III) on gill epithelia for the same clearance period, as long as gill surface

concentration had not reached saturation. Therefore higher Cr(III) gill concentration can be correlated to decreased avoidance reaction if gill epithelium concentration is below saturation level. If during clearance the gill membrane became saturated, fish would no longer be able to handle an external Cr(III) burden on the gills. The subsequent avoidance reaction is expected to be stronger compared to fish subjected to a shorter clearance period as long as their gill concentration was below saturation. This mechanism also provides a plausible explanation for the behaviour of fish after 7 days channel acclimation, which were pre-exposed at and beyond the 0.8 mg/l Cr(III) level. Those populations demonstrated an increased avoidance reaction compared to their respective 2 day channel acclimation results.

It can be generally suggested that the effectiveness of a toxic compound in eliciting avoidance, and indirectly its toxicity, depends on two characteristic parameters of the toxicant:

- 1) the range of concentrations that the toxicant is encountered in nature and
- 2) the affinity and binding strength of the toxicant on biological membranes.

An avoidance preference test can directly and sensitively provide qualitative and quantitative information on both aspects.

5.4 Avoidance preference testing with Cu(II)

Contrary to the limited information on chromium toxicity in the literature, a plethora of papers has been published on copper toxicity, since Cu is respected as an extremely toxic agent. The

purpose of testing fish avoidance reactions to copper, using the established methodology, was to determine if fish react similarly to the response observed with chromium when exposed to a different toxic agent.

Similar experiments were performed using fish populations with the same characteristics as with chromium, and following the same experimental procedures. Pre-exposure levels were chosen to cover a range between 0.05-0.30 toxic units. LC50 values for rainbow trout exposed to Cu(II) using water with the same quality characteristics were provided by Dr. P. Anderson (personal communication) and was established at 256 - 270 $\mu\text{g/l}$ as Cu(II).

5.4.1 Avoidance-Preference testing with Rainbow Trout which have not been previously exposed to Cu(II)

Two distinct avoidance reaction expressions representing two slopes in the avoidance curve can be established for trout tested with Cu(II). The expressions are presented in Table 4.11. The initial steep slope of the avoidance curve yields an avoidance threshold of 2.1 $\mu\text{g/l}$. This concentration is among the lowest reported values based on avoidance or other methods of establishing water quality standards. Only Folmar (1976) in Giattina and Garton (1983) reported a lower avoidance threshold of 0.1 $\mu\text{g/l}$ Cu(II) for rainbow trout in water with 89.5 mg/l hardness. Folmar's 0.1 $\mu\text{g/l}$ Cu(II) level is the lowest reported concentration to affect any species in any bioassay technique. This is an indication of the sensitivity of the avoidance-preference method. USEPA (1980) suggests 5.6 $\mu\text{g/l}$ as a 24h average in the water

quality criteria for copper. This level is proposed as safe to protect any form of aquatic life. Canada accepts a 5 µg/l level for the protection of fresh water aquatic life (EPS, En. Can. (1980)). The level for protecting fresh water aquatic life is the lowest suggested compared to criteria for any other water use. The background concentration of Cu(II) in the source water for the present experiments was undetectable. Therefore total Cu(II) in the channel to yield threshold avoidance was in fact 2.1 µg/l. Unfortunately, researchers do not always report background Cu(II) concentration levels, which for low test concentrations may introduce a high percentage of discrepancy between results obtained on different experimental set-ups. A threshold avoidance level of 2.4 µg/l Cu(II) was previously reported by Sprague (1964) for Atlantic salmon (Salmo salar) with a water of total hardness of 18 mg/l compared to the 100 mg/l total hardness as CaCO₃ in the present study. It is well established that toxicity increases with decreasing hardness because of less potential complexation of the toxicant. A field concentration of 17-21 µg/l Cu(II) has been suggested to cause downstream movement of Atlantic salmon while a level of 38 µg/l Cu(II) prevented upstream migration of the species (Sprague et al. (1965)). Westlake et al. (1974) suggested a 5 µg/l threshold avoidance for goldfish in water with total hardness of 5.4 mg/l. Similarly D.G. Stevens (in Giattina et al. (1982)) proposed a threshold value of 14 µg/l Cu(II) for rainbow trout in water with 28 mg/l total hardness. Finally, Hara et al. (1976) reported that a threshold concentration of copper required to cause a minimal

depression in rainbow trout bulbar response when combined with the olfactory stimulant L-serine was 8 $\mu\text{g/l}$. All reported cases for avoidance thresholds in terms of absolute values are not far from the 2.1 $\mu\text{g/l}$ Cu(II) set by this study, if the background Cu(II) concentration of laboratory water of previous studies (2-3 $\mu\text{g/l}$) is disregarded. Considering the lower total water hardness as CaCO_3 for all reported cases (ranges: 5.4 - 28.4 mg/l) compared to the present study 100 mg/l , the sensitivity of determining effective thresholds using the proposed methodology appears to be an improvement over previous methods. Rainbow trout avoid copper at concentrations within the range of the proposed water quality standards for safe water use and definitely below all chronic toxicity values reported for copper in EPS Env. Can. (1980) and USEPA (1980).

The overall behaviour of the Cu(II) avoidance curve for the non-exposed population of rainbow trout presents characteristic similarities as in the case of Cr(III), where two distinct slopes were evident. The upper limit of the first range for Cu(II) was 45 $\mu\text{g/l}$. The level, where the avoidance curve changes slope, was proposed in the case of Cr(III) to be considered as a MATC. Winner and Farrell (1976) suggested a level $> 40 \mu\text{g/l}$ Cu(II) as critical for reduction in survival and growth rate in 4 species of Daphnia. Similarly, Williams and Anderson (1986) (private communication) have established MATC for zebra fish between 30-76 $\mu\text{g/l}$ Cu(II) using a water source with the same water quality characteristics as in the recent study. In addition, Hodson et al. (1979) mentioned respiration and osmoregulation problems for

rainbow trout in the range of 40 - 60 $\mu\text{g/l}$. Sprague (1964) measured avoidance reactions of Atlantic salmon (Salmo salar). In light of the results of the recent study, a re-examination of Sprague's data (Fig. 5.1) indicate that a change in slope occurs in the range of 40 $\mu\text{g/l}$ Cu(II). These findings support the proposal of 45 $\mu\text{g/l}$ Cu(II) as a probable MATC level for rainbow trout, established through avoidance testing. Further it supports the method as capable of establishing MATC's as well as safe concentration levels through threshold avoidance values. (See also discussion in section 5.3.2). McKim and Benoit (1971) established MATC for brook trout exposed over a long term period to Cu(II) between 17.4 and 9.5 $\mu\text{g/l}$ as Cu(II) based on results from survival, growth and reproduction. The discrepancy between their MATC values and the one proposed by the present study can be attributed to the difference in total water hardness, 45 mg/l as CaCO_3 for McKim and Benoit (1971) compared to 100 mg/l as CaCO_3 for the present study, and the use of different test species. To emphasize the importance of the effect of hardness on Cu(II) toxicity, data presented by Bell (1976) in Förstner and Prosi (1978) demonstrated that the lethal threshold for Cu(II) and rainbow trout increased over 120% (from 80 $\mu\text{g/l}$ Cu to 180 $\mu\text{g/l}$ Cu) with an increase in CaCO_3 hardness from 45 to 100 mg/l . Giattina et al. (1982) correlated their avoidance threshold value with MATC values suggested by McKim and Benoit (1971) and Drummond et al. (1973) obtained on brook trout. The present work clearly distinguishes and establishes a threshold avoidance value that correlates to safe levels of water use, while MATC is corre-

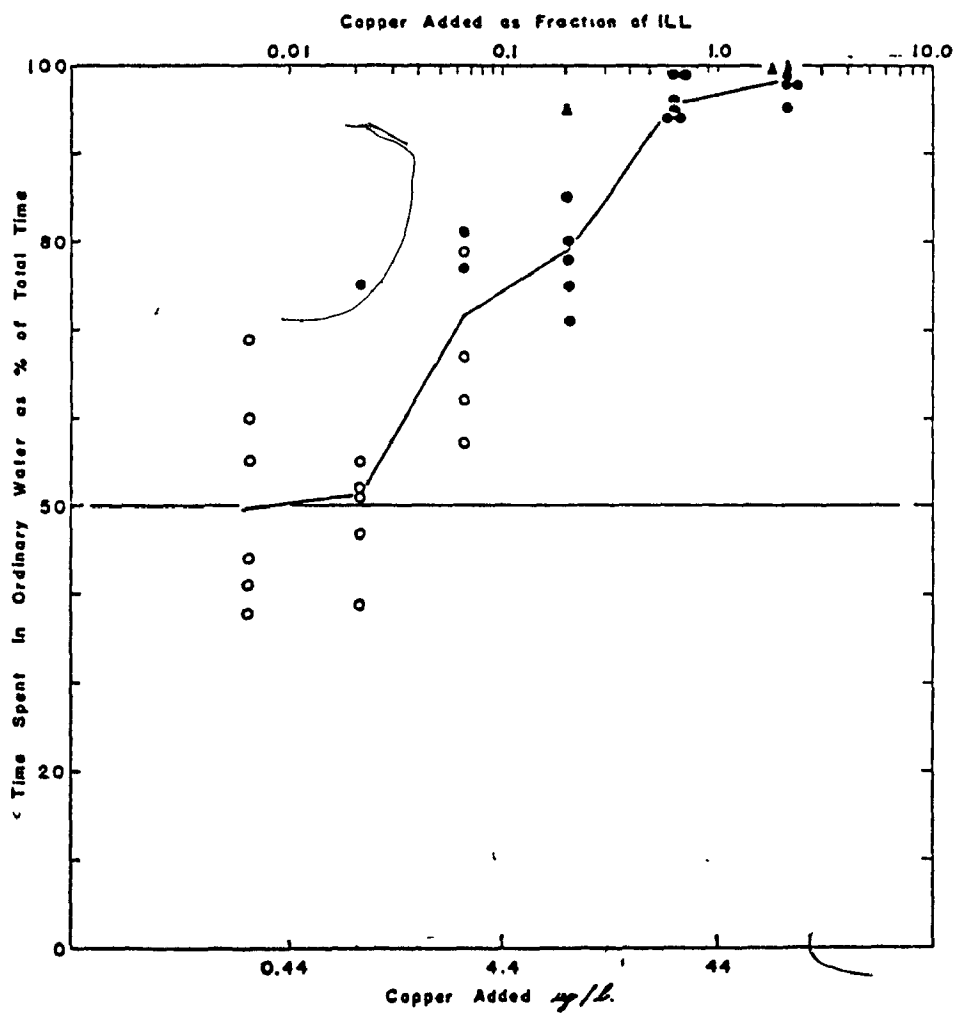


Figure 5.1 Avoidance response of young salmon to solutions of copper sulfate (after Sprague, 1964).

lated with the suggested critical level of the toxicant as established in the present study.

Avoidance reactions tend to increase with increased Cu(II) levels in the channel for concentrations beyond 45 $\mu\text{g/l}$ Cu(II). The apparent difference in slope and the linearity of response for both ranges of concentrations (up to 45 $\mu\text{g/l}$ and beyond 45 $\mu\text{g/l}$), suggests that avoidance reactions are not triggered by the same biological mechanism in the two ranges. Hara (1976) suggested that depression of the bulbar response increased with increasing concentration of Cu(II), while irreversible damage to the olfactory chemoreceptors of rainbow trout occurred at 50 $\mu\text{g/l}$ Cu(II). Therefore a very strong correlation can be established between olfactory chemoreception and the upper limit of the lower range of avoidance reactions of rainbow trout, since within the same range, when fish finally lose their chemoreceptive capacity (at 50 $\mu\text{g/l}$ Cu(II) according to Hara (1976)), the avoidance reaction curve changes slope (at 45 $\mu\text{g/l}$ Cu(II)). Similar results were obtained for two estuarine species, by Gardner and LaRoche (1973), who found physical damage on olfactory chemoreceptors at 50 $\mu\text{g/l}$ Cu(II), which was the lowest concentration they tested. It was reported by various authors on different species (Black and Birge (1980); Maciorowski et al. (1977), Hara and Sherer (unpublished data) in Giattina et al. (1982)), that organisms exhibited a strong attraction to high copper concentrations following initial avoidance at lower levels. What was not reported in most cases was the relationship of the high levels to the lethal levels for the species tested. In all cases where organ-

isms were attracted to copper, concentrations of Cu(II) in the channel were 10 - 100 times the lethal levels for the species tested. From the present study and existing relevant information, it appears that fish exposed to copper up to one toxic unit will eventually avoid at 100% lethal concentrations of the metal (Sprague and Drury (1969), Giattina et al. (1982)). If on the other hand, concentrations of copper increase to extremely high lethal levels (10-100 toxic units), attraction may result due to narcotic effects (Jones 1947, 1948) or changes in sensitivity of chemoreceptors.

Results demonstrating attraction at extremely high levels are not supported by evidence in natural systems. Once concentrations have greatly exceeded lethal levels (10-100 toxic units), the population is biologically affected to such an extent that any consideration of avoidance reactions and attempts to compare results between biologically different populations, is meaningless. The use of a sensitive and effective method at sublethal levels to predict effects on fish at extremely lethal levels is an attempt to conduct an analysis outside of the useful range of the methodology.

Fish exposed at highly toxic concentrations, being on the verge of death, are not expected to respond to the toxicant following the same mechanism as for sublethal concentrations.

Available data on fish avoidance reactions in nature indicate that fish do react in the presence of low Cu(II) concentrations, by avoiding the polluted area, therefore indicating that avoidance can be an important influencing factor on the migra-

tion, distribution and survival of fish (Sprague et al. (1965); Sutterlin and Gray (1973); Geckler et al. (1976)).

Fish kills by extremely lethal (10-100 toxic units) levels of Cu(II) are non-existent or rare, suggesting that avoidance data obtained at these levels are of limited practical significance. Due to the mechanics of toxicant transport in natural waters, and the response of fish to changes in ambient water chemistry, avoidance preference tests should be performed within a range of concentrations with LC50 as the upper limit.

5.4.2 Avoidance-Preference testing with Rainbow Trout pre-exposed to Cu(II)

Pre-exposed fish avoidance reactions to Cu(II) after 2 days channel acclimation were in general significantly different compared to non-exposed populations. All pre-exposed populations demonstrated several general avoidance reaction trends.

Pre-exposed fish avoidance reactions decreased with increasing levels of pre-exposure yielding a minimum avoidance reaction curve when fish were pre-exposed at 45 $\mu\text{g/l}$ Cu(II). At the next higher pre-exposure level, 50 $\mu\text{g/l}$ Cu(II), fish avoidance reactions were higher compared to the 45 $\mu\text{g/l}$ Cu(II) pre-exposed population. Further increase in pre-exposure levels yielded again a trend of generally reduced avoidance reactions with increasing levels of pre-exposure. This overall Cu(II) avoidance behaviour is in agreement with both chromium cases. The observed reduction in avoidance reactions suggests an increased tolerance to Cu(II) with increasing levels of pre-exposure. This is in agreement

with evidence presented by various investigators (see 5.2.2) and specifically for Cu(II) and trout by Dixon and Sprague (1981,1). Avoidance reaction curves obtained from pre-exposed as well as non-exposed populations demonstrated a change in slope that occurred always in the range of 45 $\mu\text{g/l}$. This suggests that 45 $\mu\text{g/l}$ is a critical level for Cu(II) exposure. In addition, it implies that avoidance driving mechanisms are independent of pre-exposure level.

For Cu(II), 45 $\mu\text{g/l}$ can be established as a critical level of pre-exposure and be proposed as the MATC for Cu(II) especially in light of the information presented in 5.4.1 about the minimum levels, where biological alterations or toxic effects start occurring.

All pre-exposed populations of rainbow trout demonstrated an attraction towards a familiar environment for concentrations corresponding to their pre-exposure level. This attraction can be depicted from the inflection point of the avoidance curves for all pre-exposed populations (Fig. 4.21 - 4.26). Based on the observed behaviour it may be proposed that fish can recognize a familiar environment in terms of water quality and return there if given the option. Fish can also distinguish a familiar sensation when faced with subtle changes in toxicant concentrations. The observed behaviour may link avoidance reactions directly to homing and may demonstrate the significance of avoidance-preference at least for migratory fish. This attraction towards a familiar environment was independent of toxicant (same for Cr and Cu) and independent of level of pre-exposure.

Threshold avoidance values for all pre-exposed populations along with mathematical expressions for avoidance curves to predict avoidance reactions beyond appropriate pre-exposure levels and variation of avoidance thresholds with pre-exposure level are summarized on Table 4.11. These expressions can give an estimate of avoidance reactions given the appropriate pre-exposure conditions.

Rainbow trout pre-exposed to the critical level of 45 $\mu\text{g/l}$ after 7 days channel acclimation, demonstrated a generally higher avoidance reaction compared to results from the respective 2 day channel acclimation tests. For fish pre-exposed beyond the critical level, avoidance reactions after 7 days channel acclimation were in general lower than the respective 2 day channel acclimation avoidance response. This observation further supports the proposal of 45 $\mu\text{g/l}$ of Cu(II) as a critical level for rainbow trout exposed to copper, "since recovery from pre-exposure was not evidenced within a short period for populations pre-exposed beyond the 45 $\mu\text{g/l}$ Cu(II) level.

The above information suggests that clearance is concentration dependent and is not driven by the same mechanism over the entire concentration range of the toxicant. It was proposed by Buhler et al. (1977) that the clearance rate of Cu(II) is faster at lower concentrations compared to high Cu(II) levels for both natural and laboratory reared populations. Therefore it is expected that a higher rate of Cu(II) losses will occur for fish that were pre-exposed at low pre-exposure levels. This will result in lower concentrations on the gill epithelia compared to

populations pre-exposed at high pre-exposure levels (beyond the 45 $\mu\text{g/l}$).

Toxicant elimination in organisms occurs mainly through a tissue-plasma-gills clearance model. In addition Cu(II) has a high binding strength and low saturation uptake (Tobin (1986)). Considering the difference in clearance rate as suggested by Buhler et al. (1977), it is expected that populations pre-exposed at low pre-exposure levels will have a low gill epithelium concentration of Cu(II) after the 7 day channel acclimation period. On the other hand, population pre-exposure at high Cu(II) sublethal levels will lead to fish loaded with Cu(II) on their gills after the 7 day acclimation period. The above rationale can explain the observed avoidance reactions of all pre-exposed populations to Cu(II), after a 7 day channel acclimation period.

5.5 Similarities in avoidance reactions of Rainbow Trout when exposed to chromium and copper

Examining results obtained during the course of the present work, several similarities can be established in terms of fish avoidance reactions when exposed to different ionic species and toxicants. Although levels of avoidance reactions were, in general different, depending on the toxicant and its concentration in the test channel and during the pre-exposure period, there were several general trends that can be established independent of the toxicant involved.

5.5.1 Non-Exposed populations

In all instances, the impact of the toxicant on the fish behaviour was immediate, yielding a stable level of reaction within a very short period of time (20 minutes for the present study's experimental conditions) with an excellent degree of reproducibility of results. Avoidance reactions were stabilized within the same period of time independent of the toxicant used, thus classifying the method as eligible for use with any other toxic substance, with the possible exception of highly volatile toxicants, where a modification of the apparatus configuration may be required.

Results obtained, following the procedure established in the present work, yielded threshold avoidance values that were either below or very close to the lowest existing water quality standards, independent of the method used to establish such safe levels (microorganisms, invertebrates or algae).

The inflection point, where the avoidance curve changes slope for non-exposed populations or the critical level of pre-exposure coincides with the range of concentrations that can be proposed as MATC. Concentrations of the toxicants beyond this point of inflection were either accompanied by higher mortality or fish were significantly impaired in different biological functions as suggested by biological information.

5.5.2 Pre-Exposed populations

Pre-exposed fish could distinguish and were attracted to the presence of the toxicant at the level of the pre-exposure concen-

tration. This concept suggests that familiar sensations, even if harmful to the organisms, may be preferred to a neighbouring lower risk environment, especially if fish have associated such sensations with feeding or breeding grounds.

In general, an increased tolerance of pre-exposed fish populations to the toxicant was observed, witnessed through lowered avoidance reactions compared to non-exposed fish.

In all cases, the minimum avoidance reaction curve occurred in the same concentration range where the non-exposed population avoidance reaction curve changed slope. This concentration was considered as a critical level for toxicant pre-exposure. At this critical level, fish have exhausted their capacity to adjust and compensate biologically to adverse conditions caused by the presence of the toxicant (Lloyd, 1972). The estimated critical level can be used to predict a MATC for the particular toxicant.

It was observed that threshold avoidance values for pre-exposed fish were in general increasing with increasing levels of pre-exposure. This observation can link avoidance-preference tests with results obtained from lethal bioassays (LC50's, I.L.L.'s), since similar behaviour was observed by previous researchers for the influence of pre-exposure on fish tolerance at lethal levels.

Results obtained after 7 days clearance during the channel acclimation period demonstrated the role of the chemical form and speciation of the toxicant used for pre-exposure. There are three essential characteristics that determine the differences between toxicants following a pre-exposure period: the saturation

uptake of the toxicant on any particular biological membrane and especially for avoidance-preference tests on the gill epithelia; the binding strength of the toxicant used; and the adsorption constant.

The uptake-clearance process can be described by an adsorption isotherm of the Langmuir form

$$q = q_L b C_f / (1 + b C_f) \quad \text{Eq. (5.1)}$$

where: q = metal uptake $[M(\text{toxicant}) / M(\text{organism membrane})]$

C_f = final solution concentration of toxicant in $[M/L^3]$

q_L = saturation uptake $[M(\text{toxicant}) / M(\text{organism})]$

b = adsorption constant $[L^3/M(\text{toxicant})]$

It is proposed by Tobin (1986) and O'Connor (1980) that the level of uptake of cations increases directly with molecular weight or alternatively with the ionic radius. The greater the ionic radius, the greater the number of functional groups which may participate in binding the ion on the membrane. On the other hand, the decreased strength of hydration of large ions may contribute to their increased biomass binding strength. The affinity that the solute (toxicant) had for the solid (membrane) is due to a combination of ionic, physical (Van der Waals) and chemical forces. O'Connor (1980) proposed that for short term contact of toxicant to the membrane, the rate limiting step for adsorption (or clearance) is the transport from (to) the liquid film to (from) the surface of the membrane. O'Connor (1980) also suggested that diffusion of the toxicant in the membrane pores and fixation of the metal on interior pores or capillary surfaces appears to play no significant role for conditions similar to

those of the present work.

A multiplicity of non-equivalent uptake sites with differing affinities for various ions is present in any biomass. Adsorption sites may consist of 2 or more different functional groups participating to various degrees in binding the ions. The strength of binding (b in Eq.5.1) depends on the type, the number and the spacing of all functional groups involved as binding sites. Further, metallic anion uptake involves mainly electrostatic binding to positively charged functional groups involving amine groups of hexosamines and proteins, (Tobin 1986, Strik et al. 1978) while cations form bonds with phosphate and carboxyl groups.

Sulphydryl groups of the protein fraction provide another metal binding site. They are a small fraction of the proteins and unlikely to be a major factor in the metal uptake process (Tobin 1986), but they may be involved in producing toxic effects (see 2.1.4.1).

Since in the present study the only difference between experiments was the toxicant used and the levels of exposure, the characteristics which possibly lead to differences in results were the atomic weight, different charge of the prominent ionic species, maximum metal adsorbed on membranes and binding strength of each toxicant. Table 5.1 includes characteristics for Cr(III) and Cu(II).

According to the data in Table 5.1, it is suggested that Cu(II) has higher affinity and binding strength compared to Cr(III) ($b_{Cr} = 1.28 < b_{Cu} = 7.2$). It is also expected that a biological membrane can absorb larger quantities of chromium com-

Table 5.1 Absorption parameters of metal cations with respect to biological membranes (after Tobin, 1986).

Metal Ion	Ionic Radius (A)	q_L mmol/g	95% Confidence Interval	b L/mmol	95% Confidence Interval
Cr ³⁺	0.69	0.59	0.09	1.28	0.29
La ³⁺	1.15	0.35	0.04	14.5	5.4
Mn ²⁺	0.80	0.22	0.04	13.5	5.3
Cu ²⁺	0.69	0.25	0.02	7.2	1.13
Zn ²⁺	0.74	0.30	0.06	2.72	0.67
Cd ²⁺	0.97	0.27	0.05	6.3	2.4
Ba ²⁺	1.35	0.41	0.07	6.32	2.04
Hg ²⁺	1.10	0.29	0.12	3.5	2.8
Pb ²⁺	1.20	0.44	0.06	12.9	13.9

pared to copper ($q_L^{\text{Cu}} = 0.25 < q_L^{\text{Cr}} = 0.59$). The latter implies that fish will be affected by copper at a much lower concentration compared to chromium. Therefore, toxic effects will start to appear faster on copper exposed fish since copper binds strongly (b) and membranes are quickly saturated (q_L). In fact, observed avoidance reactions to copper were higher in mean values and observed at much lower toxicant concentrations compared with the respective chromium levels. Similarly at lethal concentrations, the literature suggests that copper is significantly more toxic than chromium when the same biological organisms are involved.

For Cr(VI), data for (b) and (q_L) are not available, but if the same concept is applied, it is expected that $q_L^{\text{Cr(III)}} < q_L^{\text{Cr(VI)}}$ and $b_{\text{Cr(VI)}} < b_{\text{Cr(III)}}$. This hypothesis is corroborated qualitatively by Strik *et al.* (1978), and supported by the present study's avoidance response of fish to chromium, for low concentrations, since avoidance reactions were lower for Cr(III) compared to Cr(VI). Lamb and Tollefson (1973) suggested that, toxic effects on biological oxidation were in the order of $\text{Cu}^{2+} > \text{Cr}^{6+} > \text{Cr}^{3+}$. A relationship for q_L 's and b's between the three ionic species, of the form $q_L^{\text{Cu(III)}} < q_L^{\text{Cr(III)}} < q_L^{\text{Cr(VI)}}$ and $b_{\text{Cr(VI)}} < b_{\text{Cr(III)}} < b_{\text{Cu(II)}}$ could support the interpretation of all results obtained from pre-exposed populations for 2 and 7 days channel acclimation periods.

Wherever avoidance reactions were reduced after 7 days acclimation in clear water, strong binding and high saturation capacity on gills during clearance is suggested. Practically, if saturation has not been reached, there are more sites available

on the biological membrane (gill) for additional toxicant to bind, if it binds strongly and rapidly. Increased avoidance reaction after 7 days channel acclimation compared to the respective 2 day channel acclimation response indicates low binding strength and low saturation capacity during clearance. Due to low binding strength, the toxicant that reaches the gill surface from the plasma readily passes to the clear water while toxicant in the ambient water cannot bind on contact. In addition, if the biological membrane has low saturation capacity for the toxicant, then any available increase in ambient water toxicant concentration cannot be handled by the fish, because there are no additional sites available for binding, and fish tend to avoid the polluted zone.

5.6 Mechanisms to support the observed avoidance-preference reactions

5.6.1 Single versus two mechanism avoidance-preference model

To-date, avoidance preference reactions were thought of as being driven, independent of toxicant concentration, by one single biological mechanism. Therefore, results were always presented with an effort to linearize the fish avoidance preference behavioural pattern. This task was not difficult, considering the wide scatter of data points, obtained by previous investigators, about the proposed mean values.

Initially in this study, the individual results obtained for Cr(VI) from different populations, pre-exposed or not, were considered using the one mechanism rationale for driving fish avoid-

ance reactions. In that respect, one single mathematical expression was proposed as a tool to predict fish avoidance reactions to Cr(VI) for concentrations beyond the level of their pre-exposure (Anestis and Neufeld, 1986).

Combining the information of all avoidance reactions due to different conditions of toxicant testing, a schematic representation of the present study findings is illustrated in Figures 5.2 - 5.4, where all data for reactions beyond the respective pre-exposure level, is combined on one graph for each toxicant used (Cr(VI), Cr(III), Cu(II)).

The figures suggest a two mechanism avoidance-preference model, independent of toxicant used. It is also suggested that the same set of mechanisms apply to all pre-exposure levels, since the point where a change in slope occurs, referred further as the avoidance break point, is evident for any pre-exposed population in the same concentration range. In addition, the slope of the avoidance reaction curves for all pre-exposure levels, below or above the avoidance break point exhibit a distinct similarity in slope. This similarity in slope implies that fish pre-exposure at any sublethal concentration does not cause a dramatic impairment in the biological or physiological mechanisms dictating avoidance-preference reactions.

A closer examination of the available data in the literature (Sprague (1964), Sprague and Drury (1969), Ishio (1964), Black and Birge (1980), Giattina et al. (1982), Larrick et al. (1978), Scherer (1975)), in light of the results of the present study reveals that in most cases, where net avoidance is proposed, two

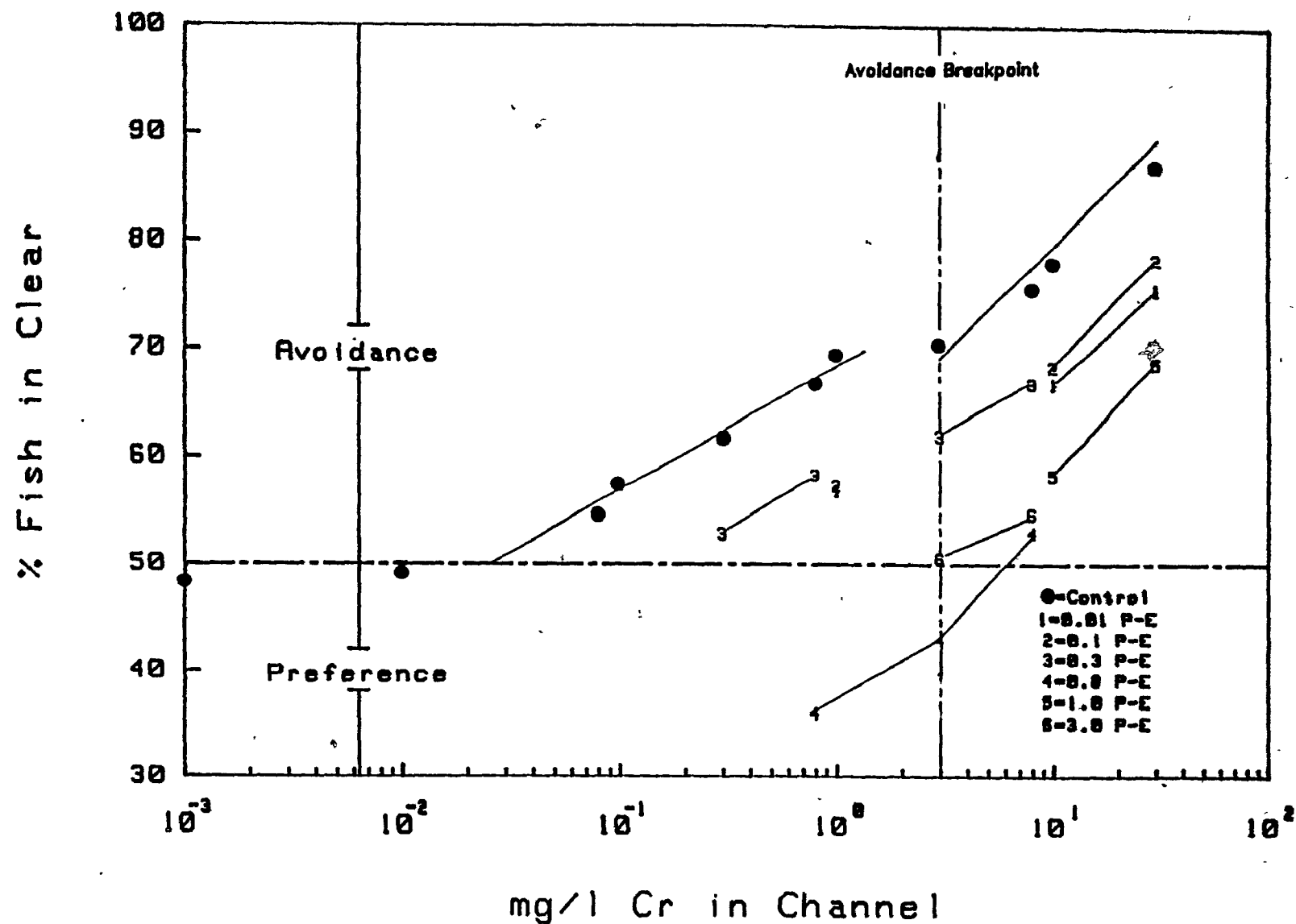


Figure 5.2 Avoidance reaction of all populations of fish tested with Cr(VI) considering data for reactions beyond fish pre-exposure levels.

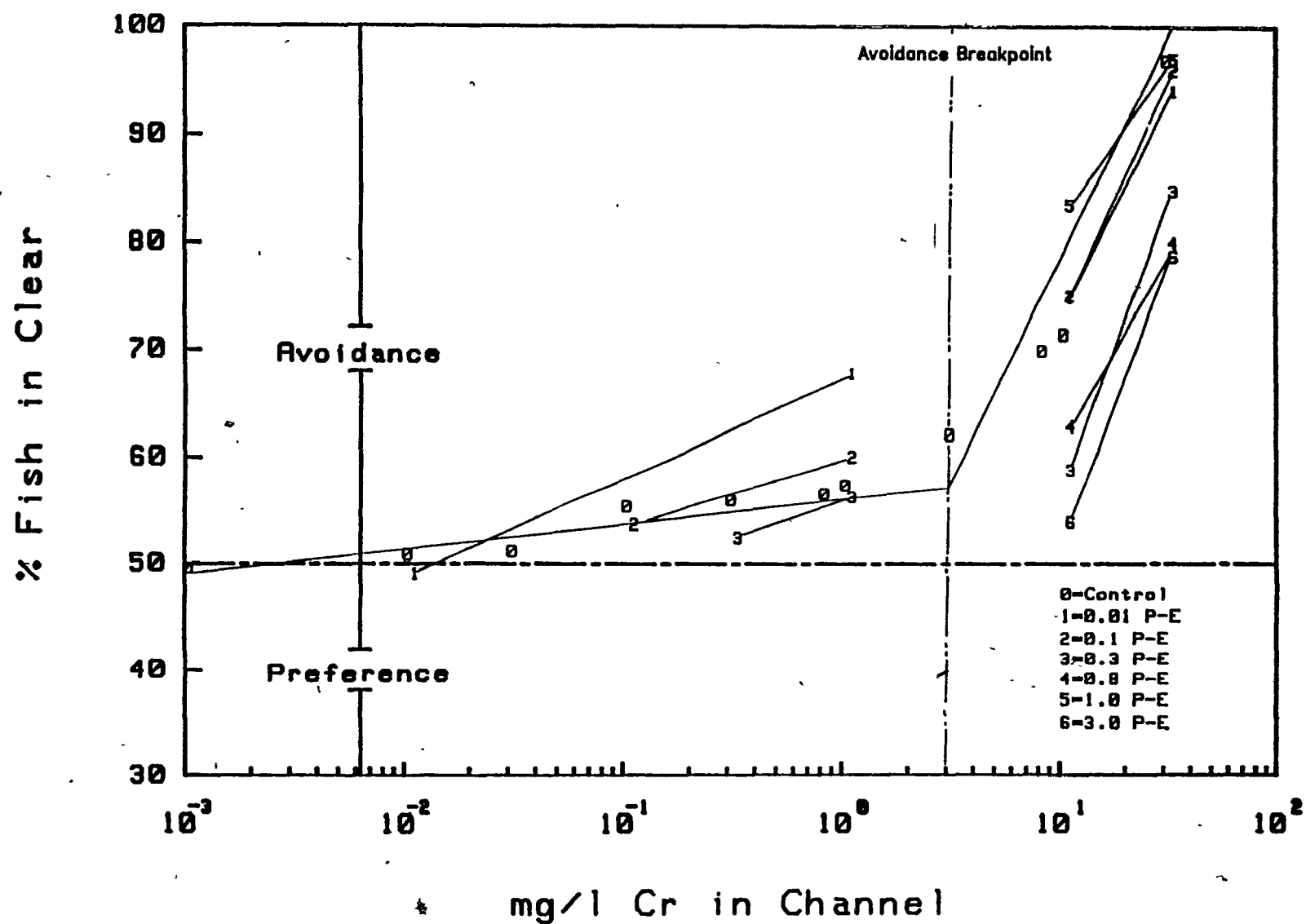


Figure 5.3 Avoidance reactions of all populations of fish tested with Cr(III) considering data for reactions beyond fish pre-exposure levels

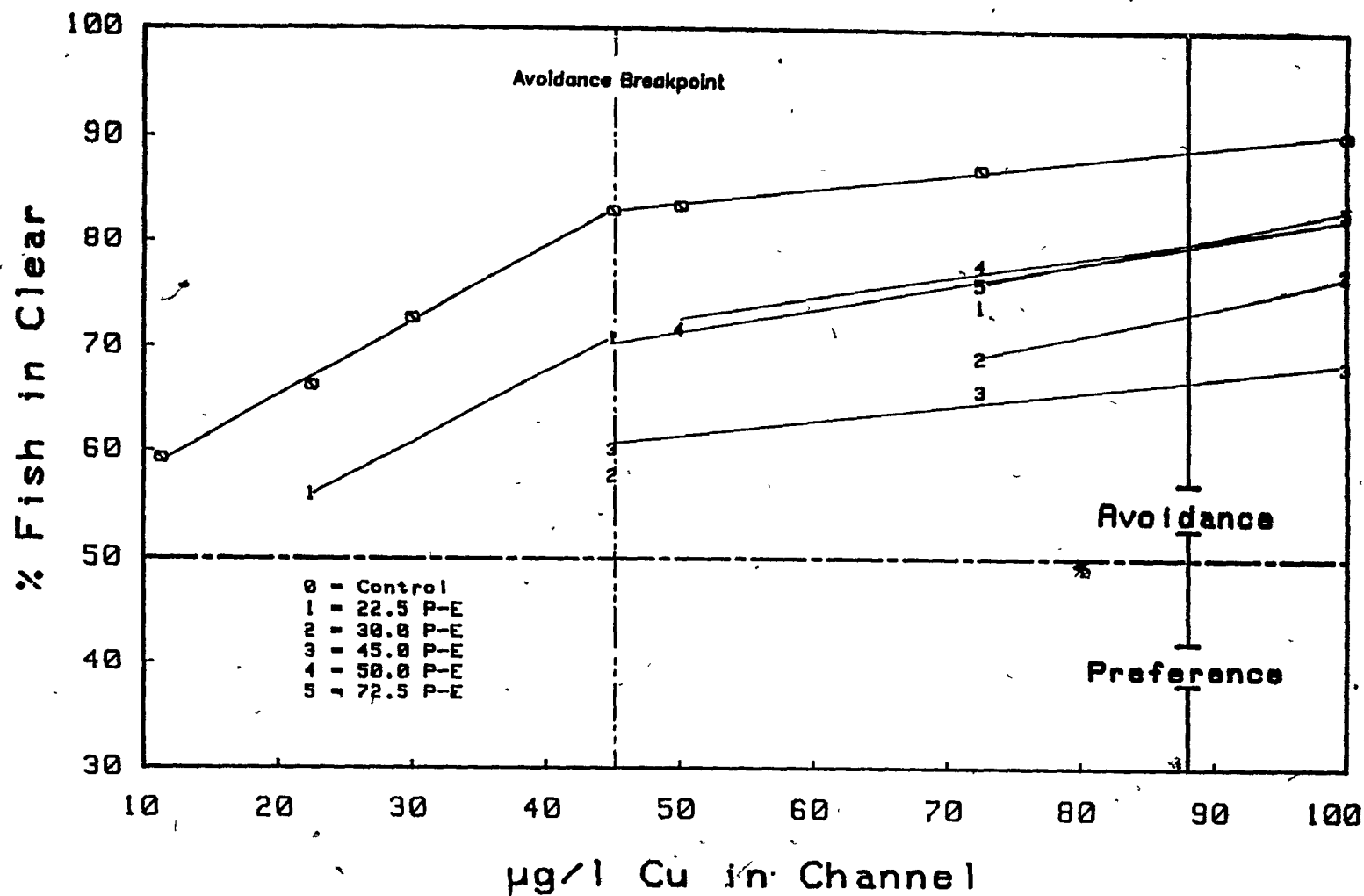


Figure 5.4 Avoidance reactions of all populations of fish tested with Cu(II), considering data for reactions beyond fish pre-exposure levels

discrete slopes could be detected with the change in slope occurring at an intermediate concentration (Fig. 5.5 and 5.6). Such a change in slope on the avoidance curve was observed in all tests involving a variety of pollutants causing stress to the organisms, such as pulp and paper effluents, detergents, pesticides, and metals.

In most cases, whenever an avoidance break point was not evident, the range of concentrations examined was either narrow or in the lethal region. Additionally, in a multiparametric problem involving numerous critical parameters, as is the case of avoidance reactions (Table 2.2), there is the probability of an inadequate consideration of any essential parameter. This may mask fish behaviour and change the outcome of the test.

In light of this re-evaluation of the existing information, it appears that avoidance reaction follows a two-slope reaction curve and the evidence of an avoidance break point resulting from a wide variety of toxicants, suggests the existence of a two mechanism avoidance-preference model for fish exposed to sub-lethal concentration levels. Each mechanism is manifested by one discrete slope on the avoidance-preference curve. The two discrete slopes, based on the previously presented as well as in data Van der Putte *et al.* (1982), Lett *et al.* (1976), and Scherer (1975) extend over two concentration ranges. The first slope at lower concentrations corresponds to toxic exposure that permits recovery to normal reaction levels after removal of the source of the pollutant, without evidence of impairment in any biological function, such as growth and reproduction (Van der

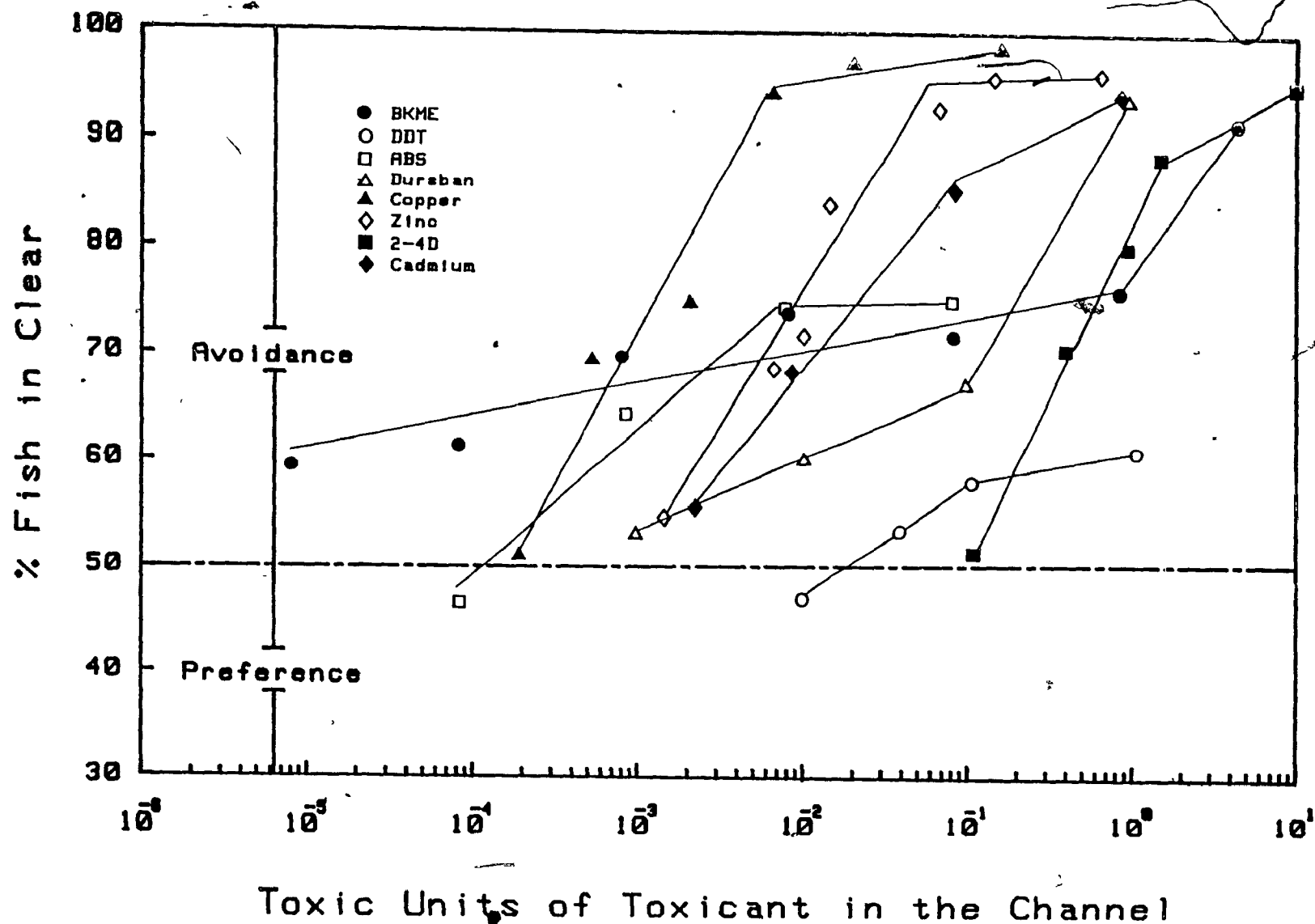


Figure 5.5 Avoidance reactions of different species to different toxicants obtained from different sources in literature, indicating a 2-slope mechanism (X-axis in toxic units).

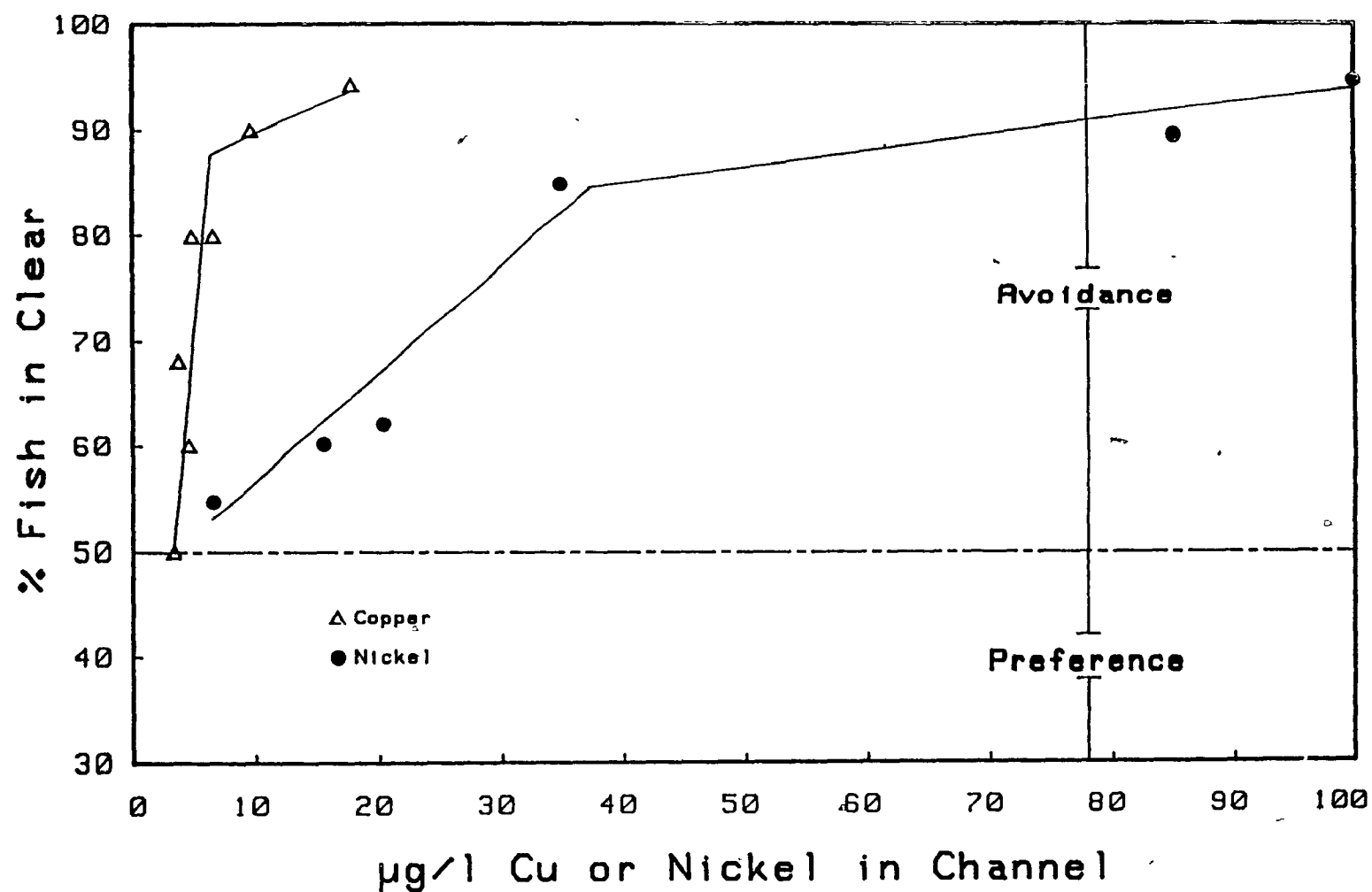


Figure 5.6 Avoidance reactions to nickel and copper indicating a 2-slope avoidance mechanism (Replotted from Giattina *et al.*, 1983)

Putte et al. (1982), Hara (1983), and Spehar (1976). The concentration range, beyond the avoidance break point on the avoidance curve, coincides with the range of proposed unsafe levels of exposure. This was substantiated by increased mortality and impairment of biological functions such as reproduction, hatchability and affected organs (Van der Putte et al. (1982), Spehar (1976)).

An equivalent two stage toxic action in biological oxidation was reported by Lamb and Tollefson (1975), for cupric, chromate and chromic ions. They suggested that during the first stage a fast adsorption takes place, with the second stage consisting of a slower rate determining step resulting in the toxic effect. They proposed that the order in producing toxic effects was $\text{Cu}^{2+} > \text{Cr}^{6+} > \text{Cr}^{3+}$, which is consistent with the results of the present study and correlates toxicity with avoidance reactions.

Another indication of two concentration dependent stages for toxic action was reported by Palachek and Tomasso (1984). In their study, methemoglobinemia was considered to be the major mechanism for nitrite toxic action on large mouth bass (Micropterus salmoides). The rate of methemoglobin conversion changed, only past a discrete concentration of nitrite (48.7 $\mu\text{g/l}$).

An equivalent physical model would be the case of a load-deformation curve (Fig 5.7). On such a curve two deformation ranges exist, the elastic and the plastic regions. When a deformation corresponds to a load in the elastic region, by removing the load, the deformation vanishes with time (self-curing process). On the other hand, once the load produces a deformation

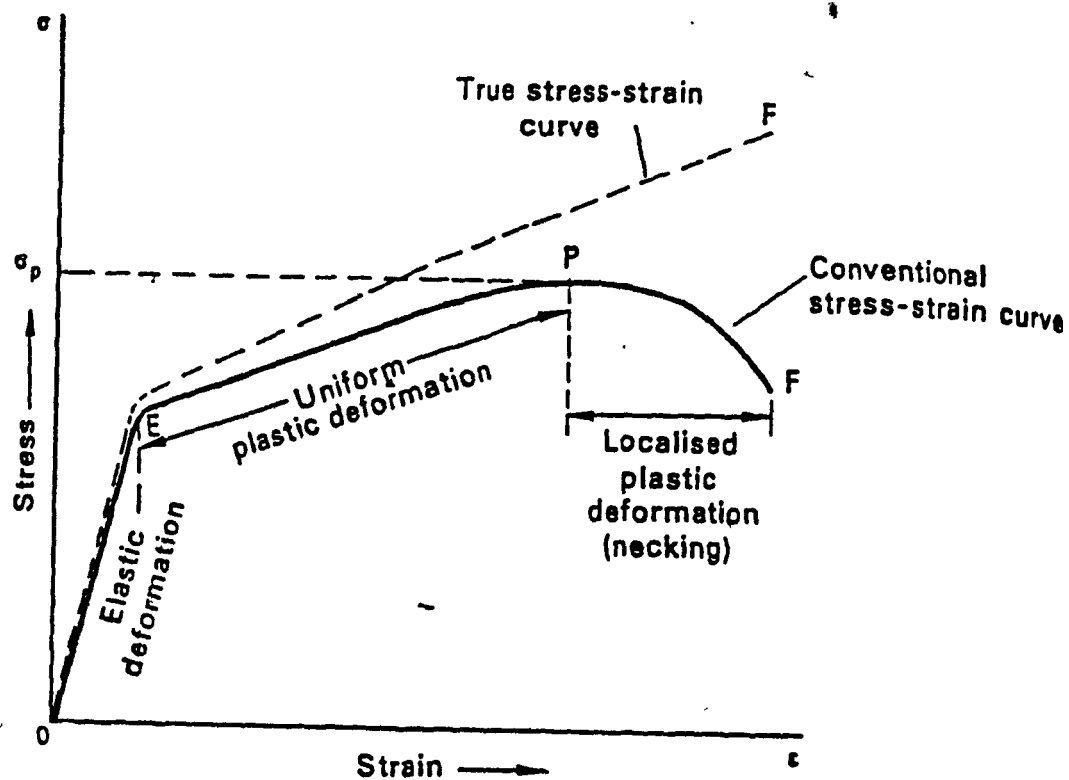


Figure 5.7 True and conventional stress-strain curves for a metal. E is the elastic limit and F the point of fracture (after Ilston et al., 1979)

in the plastic region, the effect is irreversible.

5.6.2 Actual mechanisms that can dictate avoidance-preference reactions

It was established in the past that pre-exposure renders fish more tolerant to the pre-exposure toxicant (Beattie and Pascoe(1978), Spehar (1976), Chapman (1978), Paul (1952)). This effect can be observed from the lowering of the avoidance curve in terms of, % fish in clear water for the same concentrations of the toxicant (Fig.5.3 - 5.5) depending on the pre-exposure level. This suggests that a mechanism of exclusion (reduction-excretion) is present to explain the higher tolerance of the populations or equivalently that the rate of excretion or detoxification inside the fish has changed.

Several plausible biological mechanisms can be proposed to explain the avoidance reaction of Rainbow Trout to different metals and probably applicable to other species and toxicants.

At low concentrations, below the avoidance break point, avoidance can be attributed to the fish nervous system, which is one of the most susceptible and vulnerable parts of the animal body, particularly to olfactory response. The olfactory system of the fish can efficiently model aspects of neural interaction with the environment. Olfactory responses mediate such diverse phenomena as feeding, recognition of predator and prey, sexual and social behaviour, orientation and migration (Hara, 1979). Some of these factors are directly related to avoidance behaviour. The olfactory receptor membranes are unprotected by external bar-

riers and therefore any external modification of the ambient water quality interferes immediately with their function. This results in impaired communication between fish and the environment (Bardach et al., (1965); Atema et al., 1973; Sutterlin, 1971; Gardner and LaRoche, 1973).

It was suggested (Lamb and Tollefson, 1973) that the first step in metal toxic action is a fast adsorption on biological membranes. The first membranes to contact the toxicant are the olfactory and palatal membranes. Thus, it appears logical that such membranes mediate an immediate neural signal which is translated into a modified avoidance behaviour compared to the previous fish position. An additional evidence of instant reaction of fish to any modification of toxicant concentration in the ambient water, was manifested during all tests performed for this study, by the immediate avoidance response of the individual facing the toxicant cloud, the moving interface of clear water/toxicant solution. Fish which remained in the toxicant discharge zone, move downstream at the same speed as the velocity of the propagation of the toxicant cloud in the channel. This demonstrated that sensitization was centered mainly around the mouth, nose area and the gill operculum. Fish did not react significantly, once in the toxicant cloud indicating that lateral line does not play an important role in fish avoidance behaviour.

The primary mechanism driving fish avoidance for all concentrations below the avoidance break point, appears to be an integrated nervous system response triggered by chemoreceptors on olfactory and probably palatal membranes.

The aspect of avoidance reactions driven by olfactory chemoreception, for concentrations up to the level of the avoidance break point is further supported by the work of T.J. Hara on olfaction. The avoidance break point for Cu(II) in the present study was established at 45 $\mu\text{g/l}$, while Hara (1976, 1979) suggested that a level of 50 $\mu\text{g/l}$, yielded 100% depression on rainbow trout olfactory bulb. Therefore avoidance reaction can be correlated to olfaction through the curve of depression of bulbar response. An avoidance break point can be referred to as the level of toxicant concentration, where the olfactory response becomes highly depressed and consequently no longer plays the prominent role in directing fish avoidance reactions.

Three mechanisms may be proposed to explain the difference in slope for avoidance reactions beyond the avoidance break-point.

- a) Hypoxic stress
- b) Metallothioneins production and availability, and
- c) Osmo- and iono-regulatory stress.

During actual experiments, a much higher opercular rate of movement was observed with fish remaining in the toxicant zone at higher toxicant concentrations.

It is well established that hypoxic conditions, whatever the source, can be a major stressor in fish (Pickering (1968), Stott and Cross (1977), Wedemeyer et al. (1979), Hodson (1975), Skidmore (1970) in Hodson (1975)). Since avoidance reaction is a stress related behaviour, reduced oxygenation capacity can be one of the potential driving mechanisms for avoidance at higher toxi-

cant concentrations.

Longer periods of exposure to higher levels of toxicants have been shown to affect and reduce gill efficiency causing different physiological alterations on gill epithelium, lamellae and chloride cells (Van der Putte (1982); Strik et al. (1975); Wedemeyer et al. (1979); Mallat (1985)) reducing fish oxygenation capacity. Strik et al. (1975) reported increased hemoglobin and hematocrit values for pre-exposed fish beyond the 1 mg/l Cr(VI). This can be attributed to an increased number of red cells in the blood stream compared to non-exposed populations. This increase can only be explained by an increase in $p\text{CO}_2$ and decrease in $p\text{O}_2$ in blood, which is a result of hypoxic conditions. Similar observations were reported by Neville (1979) for rainbow trout exposed to acidic conditions, once more indicating similarities between the effects of acidic stress and toxic stress on fish and a possibility of similar mechanisms in action.

The role of hypoxic stress and similarity of mechanisms driving avoidance reactions is also supported from the findings of Stott and Cross (1973). A reduction in dissolved O_2 concentrations yielded immediate avoidance behaviour to Roach (Rutilus rutilus). In their study they pre-exposed fish to reduced O_2 levels. The subsequent Roach avoidance reaction, was similar to that observed in the present study with a two slope avoidance curve. In addition Roach returned to familiar conditions, when given the option, and exhibited increased tolerance when exposed to the same stressor (reduced O_2 levels).

Branchial alterations, such as hypertrophy, hyperplasia and

lifting were confirmed for cases of sublethal toxicant exposure similar to those employed in the present study (Skidmore and Tovell, 1972, Burton et al. 1972, Strik et al. (1975). Those alterations serve to slow entry of the toxicant to the bloodstream, but have the undesirable side effect of reducing the oxygenation capacity of the organism threatening to suffocate the fish. Avoidance preference is a stress related phenomenon and it is expected that any stressor (e.g. temp, pH, other toxicants) would produce similar avoidance preference curves or equivalently, it can be hypothesized that avoidance behaviour is driven by the same mechanisms, independent of stressor involved.

Therefore hypoxic stress could be proposed as a potential candidate mechanism to drive avoidance reactions at higher toxicant concentrations.

The second mechanism proposed to explain changes in avoidance behaviour pattern, could be a change in detoxification rate, which is proposed to be driven by metallothioneins (MTN) when such proteins bind the available metal ions modifying or neutralizing their toxic action (Klaverkamp et al. 1985). MTN synthesis was correlated to metal toxicity (Brown and Parsons, 1978) where Hg pathology became evident after liver MTN became saturated and Hg started affecting fish enzymes. They suggested the 'spill over' hypothesis which was first introduced by Winge et al. (1973) (in Klaverkamp et al. 1985)) for mammalian MTN's. Brown and Parsons (1978) suggested that metal toxicity or modification in toxic effects result as soon as MTN production can no longer sequester metal toxicants. The change in behaviour past the avoidance

break-point especially for pre-exposed fish can be better understood considering the work of McCarter et al. (1982). They proposed that the rate of MTN synthesis rather than the actual concentration of the protein is the critical factor to determine the ability of fish to acclimate to metals and withstand their adverse impact.

Independent of the actual MTN mechanism, a strong correlation can be established from the literature between metal acclimation and the presence of metallothioneins, with evidence that wherever fish accumulate the highest quantities of metals is the locus of highest MTN production (e.g. gills, liver, kidneys, muscle, gastrointestinal tract, spleen and bile (I. Van der Putte et al. (1982), Klaverkamp et al. (1983))). Observed differences in fish potential to metal acclimation was attributed to differences in metal ion binding affinities. One probable mechanism to explain why fish change their behaviour at the avoidance break-point is a drastic change in the equilibrium between ambient toxicant concentration and MTN production rate and/or availability.

MTN's can be hypothesized as contributors for the increased tolerance of pre-exposed fish populations compared to controls and the relatively flat response of populations pre-exposed beyond the avoidance break-point. It can be seen on Fig. 4.11, that changes in behaviour due to toxicant concentration increases are not so drastic for such populations.

The role of MTN in the change in rate of detoxification can be used to explain the increased tolerance of pre-exposed fish assuming higher MTN production compared to previously non-exposed

populations. The flat avoidance response of fish populations exposed at concentrations beyond the avoidance break-point is due to higher detoxification capacity. It is expected that higher rates of MTN's production yield lower levels of reactional changes to variations of the toxicant.

The third mechanism suspected to be responsible for changing the slope of the avoidance curve is a combination of osmoregulatory stress. In the past, researchers reported no evidence of impaired renal function after fish pre-exposure to different stressors, while the only mechanism for fish to balance their net water gain is through urinary excretion. It was reported that acid exposure stimulated activity of fish interrenal cells and increased size of nuclei after 4 days (Barton et al. 1985), while shorter exposure (3-24 h) to acid stress (Midge et al. 1977), produced decreased cell and nuclear size suggesting that activity of interrenal tissue decreased after short term acid exposure. In either case, no renal impairment was proposed. An increase in urine production can be the result of an increased net ambient water uptake or loss of body fluids to subsidize the observed higher urinary losses, resulting in excessive stress on the fish. Both cases are driven by the same physiological mechanism, osmoregulation. The second case could be proposed as the major factor for higher water ambient concentrations compared to inner body liquid concentrations, (e.g. for marine species acclimated in fresh water or vice-versa). Actual concentration differences between ambient water and freshwater fish inner body fluids in sublethal tests are only subtle.

The most probable reason to explain the increased renal production is a net gain in water uptake. The only way fish can handle net water gain is by attempting to establish a compensatory mechanism for renal liquid losses. Since 70 - 90% of the net osmotic gain of water in freshwater fish occurs through the gill epithelium (Giles et al. (1984)) such a compensatory mechanism would involve increased gill permeability and/or activity (Smissaert et al. 1975) and could be also partly attributed to an increased drinking rate. The increase in drinking rate is not supported by any evidence from the literature.

The fish are faced with the stress of higher losses of water and electrolytes from their body and attempt to compensate such losses with a modified gill transport mechanism. Giles (1984) and Van der Putte et al. (1982), suggest no adverse effect on the efficiency of electrolyte transport mechanisms at the gill epithelium since gill ATPase levels were elevated, indicating a good branchial transport for electrolytes. Blood electrolyte regulation in fresh water fish is the result of two interacting processes, absorption of electrolytes from water by active transport mechanisms (predominantly at the gill surface) and selective reabsorption of electrolytes from urine when osmotically gained water is excreted. Any stress that impairs one or both processes will result in a plasma electrolyte imbalance, which further stresses the animal.

Data from the literature for fish exposed to metals (Giles (1984), Van der Putte (1982)), and other stressors or irritants such as O₃ (Wedemeyer et al., 1975), lowered pH values (Giles et

al., 1984 and Passino, 1979), indicate plasma Na^+ , K^+ , Ca^{2+} concentrations are lowered in all cases and such imbalances can be the result of impairment of net electrolyte influx at the gill, dilution of plasma by increased rates of water influx and a redistribution of electrolytes between intracellular (IC) and extracellular (EC) compartments. No adequate documentation exists to support or subdue the role of electrolyte redistribution between (IC) and (EC) compartments. From the above considerations for water and electrolyte losses, it seems logical to suggest that fish should gain more water osmotically through alterations at the gill epithelium in an effort to compensate for the loss of water and electrolytes from the animal's system.

Supportive evidence for the validity of such a hypothesis is the fact that fish exposed acutely or in chronic toxicant-irritant studies (Mallatt (1985); Giles (1984); Wedemeyer (1979); Van der Putte et al. (1982)), exhibited epithelial lifting, hypertrophy and hyperplasia which were largely non specific in nature, since they were detected under many different stressful conditions. At the same time the most frequently reported lesion, the lifting of branchial epithelium was reported more often in fresh water than in marine species, suggesting that osmolarity of the ambient water influences this lesion.

Table 5.2 suggests that epithelial alterations were present in most cases of fish exposed to irritants. Mallatt (1985) suggested that it is highly probable that fish exposed to stressors exhibit gill alterations as the result of a defense response, supported by the non-specificity of branchial alterations. The

Table 5.2 Epithelial alterations with toxicant-irritant pre-exposure (after Mallatt, 1985).

	Acute Exposure (up to 95h)		Chronic lethal (>95h)		Chronic sublethal	
	Present	Absent	Present	Absent	Present	Absent
1. Epithelial lifting	92.9%	7.1%	91.7%	8.3%	90.3%	9.7%
2. Gill hypertrophy	100. %	0%	87.5%	12.5%	90%	10%
3. Gill hyperphasia	70.8%	29.2%	90.9%	9.1%	88%	12%

latter along with the observations from Table 5.2 that gill epithelial lifting and hypertrophy were present in most cases of acute as well as in chronic sublethal exposure, which apply in the present study, support further the hypothesis that osmo- and iono-regulation mechanisms are responsible for fish avoidance reactions at higher concentration levels by exerting additional stress on the organisms. It is speculated that fish avoidance reactions at higher metal concentrations is regulated by the following sequence of events.

Fish find themselves in a higher concentration of the irritant and osmoregulation is imbalanced. Fish subsequently start losing higher volumes of water and react in an adaptive manner to compensate for the increased rate of loss, with a gill alteration (lifting and hypertrophy) that would allow for higher volumes of water to be perfused in fish bodies. At the same time, epithelial alterations result in reduction of the rate of irritant uptake by increasing the distance the substance has to travel through the gills (Mallatt, (1985)). Both stages create a large ionic imbalance in fish blood and plasma since higher urine discharge yields higher ion losses and stress to the organism. Fish attempt to compensate such imbalance by taking in water, high in metal ionic species, thus creating a further impact in fish ionic balance and consequently further stress on the animal.

Another gill alteration, hypersecretion of branchial mucous cells, was also associated with metals (Mallatt 1985). Mucins, which often are polyanions, may be especially effective at trapping metal cations. In our study, metal ionic species were not

always cationic, while the avoidance behaviour was consistent for all compounds used. Therefore the effect of hypersecretion in dictating avoidance reactions, if any, is proposed not to be considered significant.

Concluding, it is proposed that an osmo-iono regulation mechanism is the driving force behind fish avoidance behaviour beyond the avoidance break point. All observed fish gill alterations can be considered as a defense response to compensate for osmotic and ionic imbalances and should be considered as stereotyped physiological reactions of gills to stress.

Gill alterations such as lifting, swelling and hyperplasia of the epithelium, could serve as a defense mechanism since these alterations increase the distance across which waterborne irritants must diffuse to reach the bloodstream while at the same time creating an additional hypoxic stress to the fish (Mallatt 1985). This can also explain the behaviour of the pre-exposed populations of fish, where such lesions were present (Van der Putte, 1982; Strik *et al.*, 1978). Fish did react with abrupt changes (Fig.4.11) and exhibited a higher tolerance compared with controls (Fig.4.5), since within the same time frame and given the same ambient conditions, fish with modified gill epithelia probably accepted less toxicant in their bloodstream per unit time, and were under lower stress.

Roberts (1978) and Scott and Rogers (1980) viewed irritant induced gill alterations as part of a general systemic response to stress (general adaptation syndrome), since they encountered gill lesions produced by stressors such as fish handling, dietary

vitamin deficiency etc. They proposed that gill alterations are controlled by the pituitary-adrenal axis, or by the central and autonomic nervous system. The latter statement suggests that generally, fish avoidance preference reactions which are stress related are mediated by the central and autonomic nervous system hypothesized to be triggered through probably three concentration dependent mechanisms. One for lower concentrations to the level of the avoidance breakpoint and two co-operating mechanisms for concentrations beyond the range of the avoidance breakpoint. Chemoreception through olfactory and gill membranes is the avoidance driving mechanism for concentrations below the avoidance breakpoint, which occurs when olfactory responses are highly depressed.

Of the three hypothesized candidates as mechanisms to dictate avoidance responses for concentrations beyond the avoidance breakpoint, one is dropped based on the probability of not contributing significantly in all cases documented in this study. The significance of the rate of MTN's production and availability in driving avoidance reaction seems quite obscure in the case of non-exposed populations. Non pre-exposed fish did react and finally retired in the clear water zone for high channel-toxicant concentrations. This behaviour was independent of the method used i.e. continuous exposure to increasing levels of the toxicant or one single concentration per experiment, since no significant statistical differences between results obtained using either method were recorded. This in turn suggests that populations which were not previously exposed to the toxicant and

therefore had no chance of absorbing the substance for the production of MTN's to be triggered, reacted similarly and in a manner consistent compared to populations which had the chance to absorb the material and start producing the MTN's. That observation by itself implies a subdued significance of the role of MTN's in avoidance reactions in general and directs towards the other two proposals which seem to operate in conjunction to produce the observed avoidance-preference reactions for toxicant concentrations beyond the avoidance break point.

It is hypothesized, therefore, that the mechanisms involved in driving avoidance reactions beyond the avoidance break point are

- i) Reduced capacity of the fish for oxygenation leading to hypoxic stress and
- ii) Osmo and iono-regulatory stress that starts as osmoregulation imbalance and proceeds as a combination of osmo-iono regulatory stress combined with hypoxic stress at the gill level.

As a concluding remark, this study proposes the concept of a stress related two mechanism avoidance-preference model applicable for all stressors and irritants independent of previous exposure of the species. This principle is equivalent to the physical model of load-deformation relationship. These mechanisms are concentration dependent with an avoidance break point determining the range of application of each mechanism. The range of concentrations below the break point specifies a domain of concentrations where effects of the toxicant on the animal are reversible, while concentrations beyond the avoidance break point fall in a

domain where toxicant concentrations are harmful in the long run for the fish populations. Thus avoidance preference reaction tests can be used as a fast, accurate screening test to establish ranges of toxicant concentrations that produce irreversible results after long term exposure as well as an in-plant monitoring tool to detect subtle changes in effluent quality. The hypothesized biological mechanisms directing avoidance reactions are all related to fish nervous system. In the lower concentration range, up to the level of the avoidance break point, olfactory responses to toxicant induced stress are responsible for fish avoidance reactions while in the higher range, hypoxic stress induced by the toxicant's presence and osmo-iono regulatory stress due to presence of the irritation agent, give rise to avoidance behaviour.

6.0 Conclusions

1. A methodical approach was applied to the development of a standard methodology for avoidance-preference testing.
2. Avoidance threshold values, which can be associated with safe levels for the toxicant, were established at:
 - a) 26.0 $\mu\text{g/l}$ for Cr(VI)
 - b) 2.6 $\mu\text{g/l}$ for Cr(III), and
 - c) 2.1 $\mu\text{g/l}$ for Cu(II)
3. Avoidance reactions increased, for all toxicants used, with increasing levels of toxicant concentration at test concentrations beyond the population pre-exposure level.
4. Rainbow trout pre-exposed to the toxicants demonstrated a preference towards their familiar environment, that is for concentrations similar to the pre-exposure level.
5. Threshold avoidance values for all toxicants increased in general linearly with increasing levels of pre-exposure.
6. Rainbow trout pre-exposed to the test toxicant appeared more tolerant to the toxicant compared to the non-exposed populations.

7. A critical level of exposure to the particular toxicant was established which is referred to as avoidance breakpoint. The breakpoint was determined from a change in the slope on the avoidance curves and through an overall minimum avoidance reaction level at all test concentrations compared with populations exposed at lower or higher pre-exposure levels. The avoidance breakpoint was 3.0 mg/l for chromium and 45 μ g/l for copper. These levels can be used as MATC values for the toxicants.
8. At concentrations below the 0.8 mg/l as chromium, the hexavalent form resulted in higher avoidance reactions. For concentrations above the 3.0 mg/l level for chromium, the Cr(III) form was more effective in producing stronger avoidance reactions compared to the hexavalent form.
9. After 7 days of acclimation in clear water, fish pre-exposed to Cr(VI) below the critical level of 0.8 mg/l, behaved similarly to the nonexposed population, indicating a rapid clearance of the toxicant and functional recovery of chemoreceptive capacity. Fish pre-exposed to Cr(III) and Cu(II) did not recover their chemoreceptive capacity within a 7 day clearance period, indicating a stronger bonding of the toxicant to fish biological membranes.

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10. General behavioural similarities for all toxicants used, suggested a two mechanism avoidance model independent of toxicant and pre-exposure level. Those two mechanisms are only concentration dependent.
11. Avoidance reaction curves can provide information on safe levels and MATC's for a toxicant through the avoidance threshold value and the minimum value of the range of the avoidance breakpoint respectively. Concentrations between these two levels can induce biological alterations which are reversible if the toxicant is removed. Concentrations beyond the avoidance breakpoint are eventually lethal to the fish.
12. Avoidance is a stress related reaction and the two observed mechanisms were biologically related to olfaction for lower concentrations. For concentrations beyond the avoidance breakpoint a combination of hypoxic stress in conjunction with osmo-iono regulatory stress is proposed to give rise to fish avoidance reactions.

7.0 Contribution of this study

1. A standard method was established for performing avoidance preference tests yielding consistent and repeatable results independent of fish population used.
2. Avoidance threshold values were established for rainbow trout exposed to copper and trivalent and hexavalent chromium. The threshold avoidance values can be used as safe concentrations for water quality criteria.
3. The use of trout pre-exposed to the toxicant provided insight into the changes of fish behaviour following sublethal pre-exposure. It established a recognition and attraction towards a familiar environment independent of toxicant and pre-exposure level. It also helped establish a critical level of pre-exposure, which was correlated to the MATC.
4. A two-mechanism concentration dependent model for fish avoidance was proposed based on results from all toxicants used. The mechanisms were correlated to plausible biological mechanisms.

Suggestions for future research

Due to the consistency and repeatability of results using the methodology established for the present study, it is proposed that further investigation be performed as follows.

1. Different single toxicants of inorganic and organic nature should be examined.
2. Actual effluents bearing substances considered as toxic may be assayed. These experiments combined with information for the individual toxic substances may indicate possible synergistic and antagonistic effects between the chemical species involved in the effluent.
3. The methodology should be applied on different designs of apparatus to examine its potential use as a standard method for performing avoidance tests.

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APPENDICES

Appendix A1

EXPERIMENTAL DESIGNS OF CHEMICAL AVOIDANCE STUDIES
 IN CHRONOLOGICAL ORDER (modified from Hadjinicolaou, 1983)

	<u>Author</u>	<u>Year</u>	<u>Type of Design</u>
1.	Shelford and Allee	1913-14	1-DES A
2.	Shelford and Powers	1915	1-DES A
3.	Wells	1915	1-DES A
4.	Jones	1947	2-DES E
5.	Jones	1948	2-DES E
6.	Jones	1951-52	2-DES E
7.	Hoglund	1951-53	3-DES I
8.	Lindahl and Marcstrom	1958	-
9.	Whitmore et al	1960	2-DES E
10.	Hoglund	1961	3-DES I
11.	Bishai	1962	2-DES E
12.	Behread and Betherman	1963	-
13.	Kleerekoper and Mogertsen	1963	3-DES J
14.	Ishio	1964	1-DES B
15.	Sprague	1964	2-DES E
16.	Sprague et al	1965	2-DES E
17.	Warner et al	1966	-
18.	Costa	1966	2-DES D
19.	Summerfelt and Lewis	1967	1-DES B
20.	Hill	1968	2-DES E
21.	Sprague	1968	2-DES E
22.	Hanson	1969	2-DES D
23.	Sprague and Drury	1969	2-DES E
24.	Hoglund and Hardig	1969	3-DES J
25.	Anderson and Prins	1970	-
26.	Rehwooldt and Bida	1970	2-DES E
27.	Kleerekoper et al	1970	2-DES F
28.	Weir and Hine	1970	-

Appendix A1 (continued)

	<u>Author</u>	<u>Year</u>	<u>Type of Design</u>
29.	Hansen	1972	2-DES D
30.	Harfield and Johansen	1972	-
31.	Kleerekoper	1973	2-DES F
32.	Hansen et al	1973	2-DES D
33.	Scherer and Novak	1973	2-DES E
34.	Wilson	1973	3-DES K
35.	Kynard	1974	2-DES E
36.	Scherer	1975	2-DES E
37.	Westlake and Lubinski	1976	2-DES F
38.	Muller and Fry	1976	2-DES J
39.	Fava and Isai	1976	-
40.	Folmar	1976	2-DES D
41.	Maciorowski and Benfield	1976	-
42.	Lewis and Livingston	1977	2-DES
43.	Scherer	1977	2-DES E
44.	Birtwell	1977	1-DES C
45.	Reynolds, W.	1977	-
46.	Greer and Kosakoski	1978	2-DES G
47.	Black and Birge	1980	3-DES K
48.	Lubinski	1980	2-DES F
49.	Spraggs, Gehr and Hadjinicolaou	1981	2-DES H
50.	Giattina et al.	1982	2-DES E
51.	Anestis and Neufeld	1986	2-DES H

Key words:

SH.GR. = Shallow gradient = 1
 ST.GR = Steep gradient = 2
 FL.SY. = Fluvial system = 3
 DES = Design

POLLUTANT	SPECIES	RESULTS	System*	Temp °C	Test** Conditions	References
Chromium (VI)	Fathead minnow juvenile juvenile life cycle	96h-LC50 36.2 mg/l 96h-LC50 36.9 mg/l MATC 1.0-4.0 mg/l	S F F	25 25 16-29	pH 7.7 HD 209	Pickering (1980)
Chromium trioxide	<u>Collisa fasciatus</u>	96h-LC50 40 mg/l	S	25	pH 7.3 HD 120	Shrivastava (1979)
Chromium (III) (potassium chromate)	Goldfish Goldfish shiner Bluegill Channel catfish Rainbow trout	24h-LC50 109-354 mg/l 24h-LC50 104-151 mg/l 24h-LC50 214-280 mg/l 24h-LC50 50-72 mg/l 24h-LC50 59-141 mg/l	S S S S S	5-30 5-30 5-30 5-30 5-18	DO 6-11 HD 36 pH 5.8	Cairns J.Jr. (1978)
Chromium	Rainbow trout	96h-LC50 11.2 mg/l 96h-LC50 9.0 mg/l 96h-LC50 7.05 mg/l	- - -	- - -	- PCB previous exposure 0.01 mg/l PCB pr. exp. 0.1 mg/l	Bills T.D. et al (1977)
Chromium (III) (chromium nitrate)	Rainbow trout	96h-LC50 24.09 mg/l	F		ALK 82-132 DO 4.8-9.0 pH 6.4-8.3	Hale, J.G. (1977)

Appendix A2. Results Collected from the Literature for Lethal and Sublethal Toxicity of Chromium

* F = flow through system
S = static tests

** ALK - alkalinity
HD - hardness
DO - dissolved oxygen
A - acidity

POLLUTANT	SPECIES	RESULTS	System *	Temp °C	Test** Conditions	References
Chromium (VI) (sodium dichromate)	Rainbow trout	60-D-MATC 51-105 mg/l	F	10	A-3.3, ALK-30.1 DO-9.1, HD-33.4, pH-6.7-7.0	Sauter, S. et al (1976)
	Lake trout	60-D-MATC 105-194 mg/l	F	10	A-3.6, ALK-31.5 DO-9.5, HD-34.0 pH-6.8-7.1	
	Channel catfish	60-D MATC 150-305 mg/l	F	22	A-4.0, ALK-33.7 DO-8.1, HD-36.2 pH-7.0-7.4	
	Bluegill	60-D MATC 599-1122 mg/l	F	25	A-6.6, ALK-33.0 DO-6.6, HD-38.3 pH-6.7-7.1	
	White sucker	60-D MATC 990-538 mg/l	F	17	A-3.2, ALK-34.6 DO-8.3, HD-38.8 pH-6.9-7.2	
Chromium	Rainbow Trout	96h-LC50 100 mg/l	S			EPA E.R.S. Water Quality Criteria (1973)
Chromium (+6)	Variety of aquatic life Chinook salmon	MATC 0.01 mg/l	S			Cherry K.F. (1982)
		sublethal threshold 0.002 mg/l	S			

POLLUTANT	SPECIES	RESULTS	SYSTEM	TEMP °C	TEST CONDITIONS	REFERENCES
Chromium(VI)	Fathead minnow	96h-LC50 33mg/l S.C. 1mg/l	S	—	Hardwater	Pickering(1971) ○ (unpublished)
Chromium(VI)	Brook Trout Rainbow Trout	96h-LC50 33 mg/l S.C. 0.6 mg/l 96h-LC50 69 mg/l S.C. 0.3 mg/l	S	---	Soft water	Benoit(1971) (unpublished)
Chromium (III)	Fathead Minnow	96h-LC50 27 mg/l S.C. 1.0 mg/l	S	—	Hard water	Pickering(1979) (unpublished)
Chromium (VI)	Rainbow Trout	Inconclusive	F(avoidance)	15°C	-----	Hadjinicolaou(1983)
CrO ₃	Bluegill	6-84h-LC50 104 mg/l	S	—	—	Cairns(1956)
Chromium (VI)	4 Species	96h-LC50 17-118 mg/l	S	—	Range of hardness	Pickering & Henderson (1966)

Appendix A2 (continued)

Cr(VI) Concentration in Channel [mg/l]			
Expected	Measured	Range	%Difference
0.001	0.00105	0.00092-0.0011	-8 to +10
0.01	0.0097	0.0095-0.0113	-5 to +13
0.03	0.031	0.028-0.033	-6 to +10
0.1	0.0105	0.102-0.108	+2 to +8
0.3	0.29	0.28-0.32	-7 to +7
0.8	0.81	0.78-0.83	-2 to +4
1.0	1.02	0.92-1.11	-8 to +11
3.0	3.17	2.73-3.24	-9 to +8
8.0	8.28	7.79-8.51	-3 to +6
10.0	9.84	9.28-10.66	-7 to +9
30.0	28.92	28.17-31.42	-6 to +5

Appendix B1. Sample concentrations in the channel.

(Average of 30 samples)

Cr(III) Concentration in Channel [mg/l]			
Expected	Measured	Range	%Difference
0.001	0.00096	0.00092-0.0011	-8 to +10
0.01	0.0095	0.0093-0.011	-7 to +10
0.03	0.029	0.028-0.032	-6 to +7
0.1	0.0108	0.098-0.11	-2 to +10
0.3	0.31	0.28-0.34	-7 to +13
0.8	0.82	0.76-0.84	-5 to +5
1.0	1.04	0.97-1.09	-3 to +9
3.0	3.06	2.87-3.33	-6 to +11
8.0	8.39	7.54-8.81	-6 to +10
10.0	9.92	9.17-11.2	-8 to +12
30.0	29.21	28.28-32.1	-6 to +7

Appendix B1 Sample concentrations in the channel.
(Average of 30 samples)

Cu(II) Concentration in Channel [µg/l]			
Expected	Measured	Range	%Difference
11.25	10.86	10.45-11.42	-7 to +1.5
22.5	22.35	21.9-23.2	-3 to +3
30.0	31.3	28.4 -32.3	-5 to +8
45.0	45.87	44.2-47.8	-2 to +6
50.0	52.4	47.3-54.2	-6 to +8
72.5	74.82	69.8-77.3	-4 to +7
100.0	98.35	93.2-106.7	-7 to +7

Appendix B1. Sample concentrations in the channel.
(Average of 21 samples)

Cr(VI) Concentration in Pre-Exposure Tanks [mg/l]				
Expected	Measured	Range	%Difference	max Drop after 1 day
0.01	0.011	0.0092-0.013	-8 to +13	22 %
0.1	0.096	0.094-0.107	-6 to +7	17 %
0.3	0.31	0.29-0.33	-3 to +10	24 %
0.8	0.81	0.78-0.84	-4 to +5	12 %
1.0	1.06	0.92-1.12	-8 to +12	18 %
3.0	2.93	2.64-3.35	-12 to +12	29 %

Appendix B2. Sample concentrations in the channel.
(Average of 30 samples)

Cr(III) Concentration in Pre-Exposure Tanks [mg/l]				
Expected	Measured	Range	%Difference	max Drop after 1 day
0.01	0.0096	0.0091-0.012	-9 to +12	12 %
0.1	0.107	0.095-0.112	-5 to +12	18 %
0.3	0.32	0.27-0.34	-10 to +13	14 %
0.8	0.86	0.72-0.89	-10 to +11	26 %
1.0	1.07	0.89-1.08	-11 to +8	22 %
3.0	3.12	2.72-3.24	-10 to +8	31 %

Appendix B2. Sample concentrations in the channel.
(Average of 30 samples)

Cu(II) Concentration in Pre-Exposure Tanks [ug/l]				
Expected	Measured	Range	%Difference	max Drop after 1 day
22.50	21.4	21.0-23.2	-7 to +3	9 %
30.0	31.2	29.2-32.0	-3 to +7	12 %
45.0	44.88	43.4-47.8	-4 to +8	25 %
50.0	51.3	48.2-56.4	-4 to +13	21 %
72.5	71.85	70.3-76.8	-3 to +8	24 %

Appendix B2. Sample concentrations in the channel.

(Average of 21 samples)

Appendix B3Routine Water Quality AnalysisA. Water Quality Control of the Water
entering the System (before treatment)

	<u>PPM</u>
Sodium	14.2
Calcium	23.5
Magnesium	8.0
Aluminium	<0.1
Iron	<0.1
Chromium	<0.1
Copper	<0.1
Nickel	<0.1
Lead	<0.1
Cadmium	<0.1
Zinc	<0.1
Potassium	-
Manganese	<0.1
TOC	8.0
TIC	17.0
Phenols	-
Total coliforms	0
Fecal coliforms	0

Appendix B3B. Water Quality Control before and after
the ultra violet lights

	PPM	
	Before Ultra Violet Lights	After Ultra Violet Lights
Phosphorus inorganic	<0.05	<0.05
Phosphorus total	0.02	0.01
Nitrite/Nitrate	0.39	0.39
N. Total KHELDAHL	<2.5	<2.5
N. Ammonium	0.6	0.2
Cyanide	-	-
Fluoride	112 CaCo ₃	95 CaCo ₃
Chromium	<0.1	<0.1
Copper	<0.1	<0.1
Nickel	<0.2	<0.1
Lead	<0.1	<0.1
Cadmium	<0.1	<0.1
Zinc	<0.1	<0.1