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THE DEVELOPMENT OF FISH EPITHELIAL CELL LINES AND PRIMARY CULTURES AS DIAGNOSTIC TOOLS IN ENVIRONMENTAL RISK ASSESSMENT

Submitted by

Kevin J. Dowling B.A. Mod.

For the degree of Philosophiae Doctor to Dublin University, Trinity College

Department of Biochemistry,

Trinity College Dublin

And

Radiation Science Centre,

Dublin Institute of Technology

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DECLARATION

I declare that the work presented in this thesis is entirely my own work and has not been submitted to this or any other university as an exercise for a degree. I give permission to the library to lend or copy this thesis.

Kevin J. Dowling

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I express deep gratitude to Maria for all the advice given to me during my postgrad. I may not have always listened but it was much appreciated. Thanks also to Robert Cummins for all the help and advice given throughout my time in the lab. Orla Howe, what would I have done without you? Thanks also to Ciara for all the help in France.

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To all my fellow students, Natasha, Jenny, Michael and Brian – Keep the faith.

Finally I would like to thank Pat Redmond for creating Paddy Blues and giving me a place to relax and unwind that is equal to none.

SUMMARY

The objective of the this study was to attempt and develop fish epithelial cellular markers as diagnostic tools for effects assessment in ecotoxicolgy using established cell lines and primary cultures. As a target group in the environment teleost fish were selected. The project comprised of three major tasks: 1) Research on cultured cells in order to establish the basic characteristics of the link between the exposure and inducible response with three chemicals, nonoxynol, prochloraz and copper. 2) The development of specific cellular markers of exposure and stress using fish epithelial cell cultures. 3) Research on intact animals in the laboratory in order to clarify a) whether the biological response detected in the isolated cell in an *in vitro* environment can be detected again in the intact *in vivo* environment and b) if the biological response has potential for use as a suitable biomarker of effect or exposure. The assay endpoints studied were measurement of growth rate, measurement of cell death (necrosis and apoptosis), changes in mucous cell secretion or behaviour and activation of heat shock proteins. The following chemicals were used as test substances i) Nonylphenoldiethoxylate (Nonoxynol-2) – a non-ionic surfactant ii) Prochloraz – an imidazole-based fungicide and iii) Copper – a heavy metal.

The first piece of work carried out in this study involved the use of the immortalised cell lines, EPC and CHSE. The major advantage of their use was that it is possible to study the effects of a chemical in future generations. This property was used to investigate whether or not the chemicals caused delayed cell death in the CHSE. All three chemicals caused this phenomenon. However, the appearance of this cell death appeared to differ between the three chemicals studied.

on the surface of the cultures increased in relation to the concentration of the pollutant the parent fish were exposed to. The levels of cells expressing positivity for mucus which were obtained, were very similar to those obtained in the *in vitro* studies. It was also found that cell cultures cultured from fish exposed to the highest dose of the chemical for the full exposure period did not survive and grow at the same rate as cultures grown from control fish.

As a result of the above *in vivo* experiment with prochloraz it was decided to repeat this experiment using nonoxynol and also to treat the fish in river water as well as dechlorinated tap water in an attempt to get nearer real *in vivo* conditions. It was discovered that a similar response was obtained in cultures taken from fish exposed to the high dose of the chemical. It was also discovered that the growth rate of the cultures was directly related to the amount of mucus contained on the surface of their cells. These results demonstrate that the use of mucus production in rainbow trout primary epithelial cell cultures and the survival of these cell cultures have potential for use as a suitable biomarker of stress.

The use of short-term cytotoxicity assays for the initial screening of chemicals not only aids in establishing priorities for the selection of chemicals that should be tested further *in vivo* but also accelerates the time during which potential toxicants can be evaluated. The results of this study demonstrate that the rainbow trout primary epithelial cell culture system used in this study is a useful tool for such assays. It has been demonstrated that the *in vitro* primary cultures do not lose the differential properties of the skin in an *in vivo* situation. One can observe effects on both the pavement cells and goblet mucus cells contained within the cultures.

This thesis was the first work to explore in detail the use of fish skin cell cultures for toxicological studies. Many different markers of cellular toxicity, not explored in this study, could be developed for use with the cell system such as proliferation proteins, stress proteins and

oncogenes, The cultures could also be infected with viruses, and may therefore provide excellent opportunities for research into fish diseases.

DEDICATION

I would like to dedicate this thesis to Jimmie Doran and Padraig Keogh, two friends who I miss dearly. This is for you.

CHAPTER 1

GENERAL INTRODUCTION

1.1 XENOBIOTICS AND THE NEED FOR CELLULAR BIOMARKERS OF EXPOSURE

Xenobiotics are chemicals which are foreign to biological systems and include industrial chemicals and pesticides (Nowak, 1997). It is estimated that over 80,000 chemicals are in use in the United States alone; an average of over two thousand new ones are produced annually (NRC, 1984; OTA, 1995). As many of these chemicals enter into the aquatic environments, there is a need to develop rapid cost-effective bioassays to screen this vast number of chemicals for its potential ecotoxicity (Babich *et al.*, 1987). A class of pollutants which represents a considerable threat to the aquatic and ultimately human life includes compounds of low acute toxicity, low water solubility or high lipid affinity and high bioaccumulation capacity. As a general rule it is more difficult to assess biological effects of such compounds because long-term exposure is often required for their toxicity to become apparent (Denizeau *et al.*, 1984). Increasing concern with the potential consequences of human action for the environment has given rise to a broad range of descriptive and analytical methods that aim at predicting the effects of stress on wild populations (Power, 1997).

The emphasis in the development of ecotoxicology assessment methods has to move from the measurement of acute, lethal effects of single chemicals to the early indication and evaluation of chronic, sublethal stress of chemical mixtures. This focus on sublethal effects is based on the premise that chemical concentrations in most aquatic ecosystems are rarely toxic to fish except under certain conditions like point-source discharges or chemical spills (Niimi, 1990). However, long-term exposure to low doses of pollutants

can alter the normal functions of an organism and destroy a population as effectively as a single lethal dose (Freeman *et al.*, 1980; Fox, 1993). Only the ability for the identification of early disturbances will allow the prevention of overt damage (Senger, 1996).

Substantial research and development is needed to establish early indicators of exposure as a tool for the timely detection of a potential threat to biological systems. The exposure marker (i.e. indicator of chemical exposure) has to be both sensitive and it has to be characterised i.e. dose- and time dependency of the biological response has to be known, its sensitivity to specific chemical groups or modes of action, its reversibility and its behaviour under exposure to mixtures of chemicals. Knowledge on these aspects will help to establish links between biological response and specific chemical stressors. Substantial research and development is also needed to establish early indicators of effect as a tool to identify the functional consequences of sublethal contaminant exposure. The effect marker (i.e an indicator of a particular response to a toxic substance).should reveal whether the exposure- induced change a) is part of an adaptive response (i.e. development of tolerance) or of a pathologic response (i.e. impaired function), and b) translates into responses at other levels of biological organisation (Segner, 1996).

A biomarker is defined as 'a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also of toxic effect' (Peakall *et al.*, 1994). The responses may be at different levels of organisation - molecular, cellular or whole organism (Walker, 1995). The first question that biomarkers can used to answer is 'are environmental pollutants present at a sufficiently high concentration to cause an effect?'. If the answer is positive, further investigation to assess the nature and degree of damage and the agent or agents, is justified. If negative, it means that additional resources do not

need to be invested. In this way biomarkers can act as early warning signals (Peakall *et al.*, 1994). Ideally biomarker assays are simple, specific, sensitive, stable and compatible with non-destructive sampling strategies. As yet few assay types are available.

The prudent use of biomarkers could make an important contribution to the difficult task of risk assessment. In the first place they can give measures of characteristic harmful effects of individual chemicals in laboratory studies. Once validated in the laboratory, biomarkers can provide direct measures of actual effects of chemicals upon living organisms in the field, thereby overcoming large areas of uncertainty implicit in normal risk assessment (Walker, 1995). Certain biomarkers can be used in *in vitro* systems as well as *in vivo*. Some may be specific to mammals or other species, whereas others are applicable to all organisms (eg stress proteins) (Timbrell *et al.,* 1994).

The term biomarkers can be sub divided into i) biomarkers of exposure ii) biomarkers of effect and iii) biomarkers of susceptibility (Timbrell *et al.*, 1994). Biomarkers of exposure have been most actively studied (Hugget *et al.*, 1992; Van Welie *et al.*, 1992). In general terms, markers of exposure rely on measurement in body fluids or tissues of either the substance in question or a metabolite(s) or a product of a reaction with a biological molecule (Timbrell *et al.*, 1994). Biomarkers of exposure can be divided into a) markers of internal dose and b) markers of effective dose (Van Welie *et al.*, 1992). The former is an indicator of the occurrence and extent of exposure of the organism, whereas the latter is an indicator of the extent of exposure of what is believed to be the target molecule, structure or cell. There are a very large number of potential markers for determining the biological effect of chemicals (Bayne *et al.,* 1985; Niimi *et al.*, 1990; Hugget *et al.*, 1992; Fox *et al.*, 1993; Rubenchik, 1996). Biomarkers of effect range

from the simple, such as monitoring body weight and population changes to the sophisticated, such as determination of specific isoenzymes by immunochemical techniques (Timbrell *et al.,* 1994). There is now increasing interest in the role of genetic variation in toxic responses and therefore of variation in susceptibility and markers of such susceptibility are of great interest. Of the four phases of disposition of a xenobiotic, the most important source of variability is metabolism and has been the area of most research (Timbrell *et al.*, 1994).

Exposure of biota to foreign chemicals induces a reproducible sequence of changes that starts with compensatory responses and ends with death (Depledge, 1989; Segner *et al.*, 1990) The initial interaction between chemistry and biology takes place at the cellular level (Segner, 1996). The early cellular changes may lead to the development of tolerance, eg. by induction of metabolic detoxification mechanisms (Bayne *et al.*, 1985; Niimi, 1990; Depledge, 1993; Fox, 1993). However, with increasing dose or extended duration of exposure, the health status will be impaired, and (pre) pathological effects and, later on, overt signs of disease will develop. Finally, the biological effects will leave the range of sublethal, reversible damage and irreversible damage will occur (Segner, 1996). Markers at the cellular level are appropriate for the early and sensitive indication of exposure and effect. They respond early in the sequence of exposure-induced events since they provide the most direct link between chemistry and biology. It is at the cellular level where chemical uptake, accumulation and metabolism takes place. It is also at the cellular level where the primary interaction between chemistry and biology takes place. Hence it is, therefore, at the cellular level where the most direct link exists

between exposure and effects and where the further fate and effects of the chemical at higher levels of biological organisation are determined (Segner, 1996).

The development of new ecotoxicological test methods is driven by scientific, social, economic and political factors and advances. Currently assessment of the potential adverse health and environmental effects of chemicals is accomplished largely by tests involving laboratory animals and plants. However, public concern about animal use has resulted into in recent legislative mandates that require scientists to consider, prior to using animals, alternatives that do not require the use of animals, that reduce or eliminate potential pain and distress, and that may reduce the total number of animals required or either replace animals in testing or replace one animal species with another that is lower on the phylogenetic scale. For example, phylogenetically lower organisms such as fish, invertebrate and algal toxicity tests are often conducted to determine the ecotoxicity of single chemicals (NIH, 1995).

Isolated cells that maintain the essential traits of the *in vivo* state during culture *in vitro* provide an excellent experimental approach to establish diagnostic markers. They can be directly exposed to toxicants so that the toxicokinetic phase is easy to model; they allow the establishment of dose and time dependencies of the cellular response to single toxicants and toxicant mixtures without confusing systemic influences; and they are well suited for analysis of mechanisms of disease or mechanisms of tolerance (Segner, 1996). The main advantage of using *in vitro* systems is that experimental conditions can be strictly controlled, providing the opportunity for detailed analysis of toxic mechanisms of action at the cellular level. Results from initial *in vitro* studies can lead to more efficient

experimental design for animal experiments which could result in financial economies and savings in development time (Brady *et al.*, 1995).

To understand the ecotoxicological implications of the cellular indicator response, *in vitro* studies on isolated cells have to be integrated with in vivo studies on the intact animal. There are a few areas where *in vitro* studies have gained acceptance by regulatory authorities. These are essentially limited to mutagenicity data. Testing strategies adopted by regulatory authorities in the mid - 1980s involved a combination of *in vitro* and *in vivo* studies. However it is now widely recognised that the mutagenic potential of a substance can be assessed using *in vitro* studies alone (Fielder, 1994). The use of *in vivo* mutagenicity tests should essentially be limited to ascertaining whether activity seen in vitro can be expressed *in vivo*.

There are considerable difficulties in the development of *in vitro* tests to replace animals in general toxicity studies. This is because by their nature the *in vitro* tests are incapable of mirroring the complexities of all the biological processes involved in the whole animal, where multitude of potential target tissues and biological interactions are possible. A single animal study would need a very large battery of *in vitro* tests to cover the various endpoints that need to be considered (Fielder, 1994).

The study of fish as a monitoring tool for the quality of the aquatic environment is becoming more developed in marine and aquatic pollution monitoring programs (GESAMP, 1991; Vethaak *et al.*, 1992; Wester *et al.*, 1994). The living epidermal cells of fishes are in direct contact with the environment; moreover fish skin composes the initial line of defence against changes in the nature and chemical composition of the surrounding water (Ribeiro *et al.*, 1995). The skin of fish is a metabolically very active

tissue, that quickly responds to stressors (Whitear, 1986; Iger *et al.,* 1992). The epidermal mucus layer constitutes the primary biological interface between fish and the aqueous environment (Ottesen *et al.,* 1997). A number of functions have been ascribed to the mucus layer, such as protection against mechanical injury (Pickering *et al.,* 1980), chemical toxins and friction reducing properties (Pickering *et al.,* 1974). In the broad perspective of environmental toxicology and biohazard assessment, cells of the epithelial type appear as highly significant biological indicators. The importance of the role that the epithelial cells and mucus cells play in the whole, intact, organism as barriers against the penetration of exogenous agents, strengthens the relevance of using this cell type for studying the action of various kinds of toxic xenobiotics (Marion *et al.*, 1983).

1.2 PROJECT OBJECTIVE

The objectives of the proposed project were to develop fish epithelial cellular markers as diagnostic tools for effects assessment in ecotoxicolgy using established cell lines and primary cultures. As a target group in the environment teleost fish were selected. The project comprised of three major tasks:

1) Research on cultured cells in order to establish the basic characteristics of the link between the exposure and inducible response with a set of reference chemicals (see section 1.3).

2) The development of specific cellular markers of exposure and stress using fish epithelial cell cultures.

3) Research on intact animals in the laboratory in order to clarify a) whether the biological response detected in the isolated cell in an *in vitro* environment can be detected again in the intact *in vivo* environment, b) whether the cellular response translates into a single organism response and c) if the biological response has potential for use as a suitable biomarker of effect or exposure.

The assay endpoints to be studied were measurement of growth rate, measurement of cell death (necrosis and apoptosis), changes in mucous cell secretion or behaviour and activation of heat shock proteins.

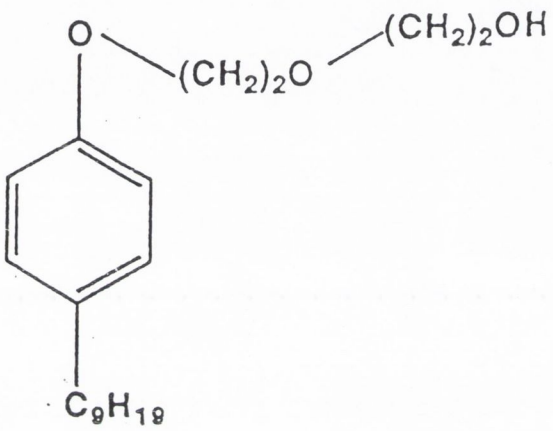
The criteria for the selection of the chemicals tested have been that they belong to different chemical classes, have different modes of toxic action and are chemicals of environmental concern. The chemicals tested were i) Nonylphenoldiethoxylate

(Nonoxynol-2) - a non-ionic surfactant ii) Prochloraz - an imidazole based fungicide and iii) Copper - a heavy metal.

1.3 REFERENCE CHEMICALS TESTED

1.3.1 NONOXYNOL (Nonylphenoldiethoxylate)

Figure 1.1: The chemical structure of nonylphenoldiethoxylate



Hundreds of millions of kilograms of non-ionic surfactants are used annually in U.S. consumer and industrial markets. In 1991, 842 million kg of non-ionic surfactants were produced in the U.S. (USITC, 1991). Alkylphenol ethoxylates (APE's) are non-ionic surfactants that comprise 6% of the total surfactant production and 25% of the total non-ionic surfactant of the U.S. (USITC, 1993). Nonylphenol ethoxylates (NPEs) are the most common of the APEs, constituting 82% of production, and are referred to as nonoxynol, ethononylphenol, polyoxyethylene nonylphenol ether or nonylphenoxypoly(ethyleneoxy)ethanol (Nimrod *et al.*, 1998). Nonoxynol, α-(4-Nonylphenyl)-ω-hydroxypoly-(oxy-1-ethanediyl), is a non-ionic surfactant mixture prepared by reacting nonylphenol with ethylene oxide (Windholz *et al.*, 1983, Naylor, 1998). Nonylphenolpolyethoxylates are used in a variety of products including

institutional cleaning agents, textiles, agricultural chemicals, plastics, paper products, household cleaning agents and personal care products (Talmage, 1994). Nonylphenol polyethoxylates are also used as spermicides in contraceptive applications (Nimrod *et al.*, 1998).

The biodergradation pathway of APEs to short-chain ethoxylate and ethoxy carboxylate metabolites is well established (Giger *et al.*, 1984; Ball *et al.*, 1989). The primary biodegradation of APEs is the hydrolytic removal of ethoxylate groups. This step is relatively rapid and results in the degradation intermediates nonylphenol and nonylphenolmonoethoxylate in the case of nonylphenoldiethoxylate (Naylor, 1992). The ultimate biodegradation of nonylphenol and nonylphenolmonoethoxylate occurs more slowly because of the presence of the benzene ring and the compounds' insolubility (Brunner *et al.*, 1988). These compounds may also be microbially carboxylated to yield APECs with carboxylic acid functional groups at the terminal end of the ethoxylate chain (Ahel *et al.*, 1994). Slow ultimate biodegradation and the fact that these intermediate products are more lipophilic than the parent compounds result in sediment and sewage sludge serving as environmental "sinks". Thus some nonylphenolethoxylates and degradation products can reach the aquatic environment (Nimrod *et al.*, 1998). The most abundant compounds in the water column of the Gatt valley in Switzerland were the carboxylic acid degradation products NP1EC and NP2EC (Ahel *et al.*, 1994).

Humans have the potential to be exposed to APEs and their degradation products through the water supply, sewage sludge used for fertiliser, aquatic flora and fauna serving as food and directly through the use of APE spermicides (Nimrod *et al.*, 1998). The acute toxicity of APEs to mammalian species is quite low; oral LD_{50} values for rats and mice

range from 2 to 4 g/kg. Knaak *et al.* (1966) described the disposition of both NP and nonoxynol. The liver and kidney of female rats were able to clear nonoxynol, labelled with C14 in the ethylene oxide chain, within 48h. However when lactating rats were administered radiolabeled nonoxynol radioactivity appeared in milk and in the feeding pup serum within 2h (Chvapil *et al.*, 1980).

Algae, fish and ducks as well as marine organisms, have all been demonstrated to bioaccumulate nonylphenol, nonylphenolmonoethoxylate and nonylphenoldiethoxylate from freshwater environments (Ahel *et al.*, 1993; Ekelund *et al.*, 1990). In contrast to their effects in mammals, non-ionic surfactants are highly toxic to aquatic organisms. The chemical structure is predictive of a detrimental effects. The toxicity of APEs to aquatic organisms increases with a decreasing number of ethylene oxide units and increasing hydrophobic chain length. Therefore, the toxicity of nonylphenoldiethoxylate is less than the degradation products nonylphenol and nonylphenolmonoethoxylate (Nimrod *et al.,* 1998; Yoshimura *et al.,* 1986).

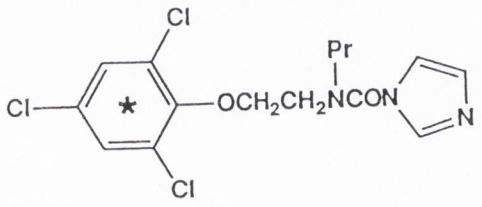
Recently it has been discovered that nonylphenolethoxylates are estogenic (Jobling *et al.*, 1996; Jobling *et al.,* 1993). Oestrogenis activity is associated with the extension of the alkyl side chains in the para-position (EPA, 1997). The chemicals exert their action by binding to the estrogen receptor and regulating the activity of the estrogen responsive genes (Jobling *et al.,* 1996). Nonylphenol-(mono and di)-ethoxylates have been shown to be estrogenic to fish cells *in vitro*, although their relative potencies were approximately 100,000-fold less than that of the natural estrogen 17β-estradiol (Jobling *et al.,* 1993). The role of estrogens in the control of fish reproduction has been well studied in salmonid fishes, particularly in rainbow trout (Billard, 1992). Exposure of wildlife to

environmentally persistent estrogenic chemicals can result in deleterious consequences (Jobling, 1996). A major effect of exposure of male or female teleosts to estrogens is the stimulation of the synthesis and secretion of vitellogin by the liver (Chen, 1983). Although vitellogen is generally a female-specific protein, male fish will produce this protein if they are exposed to estrogens (Jobling *et al.,* 1996). Nonylphenolethoxylates can induce vitellogin production when present in the water at microgram per litre concentrations. Exposure of the male rainbow trout, *Onchorynchus mykiss*, to 30µg/l 4-nonylphenol for 3 weeks significantly induced hepatic vitellogen synthesis and inhibited testicular growth. The threshold concentration for this induction was estimated to be 10µg/l (Jobling *et al.,* 1996). Kline *et al.* (1996) found a clear indication that treatment with surfactant had an effect on the reproduction of fathead minnows at concentrations of 330µg/l and greater. There was some indication of an effect on egg-laying at a concentration of 160µg/l.

The nonylphenolethoxylate used for the purpose of this project was nonylphenoldiethoxylate (Igepal 210). The pollutant shall be referred to as nonoxynol for simplicity in the remaining chapters of the thesis.

1.3.2 PROCHLORAZ

Fig 1.2: The chemical structure of prochloraz



Modern intensive farming depends on a wide variety of pesticides particularly in areas devoted to cereals. Imidazole - based antimycotic compounds have been widely developed for the control of fungal infections (Holt, 1976). Prochloraz (1-[N-propyl-N-2-(2,4,6-trichlorophenoxy)ethylcarbamoyl]imidazole) is an agrochemical fungicide developed for use on cereals and oilseed rape (Wakerly *et al.*, 1985). The compound has been shown to be particularly effective against *Ascomytes* and *Fungi imperfecti* and is the major control agent for eyespot infections (Russell *et al.*, 1988).

Prochloraz ($C_{15}H_{16}Cl_3N_3O_2$), discovered in 1974 by the group FBC Ltd (UK), is a white solid crystal with a molecular weight of 376.5g. It is slightly soluble in water but dissolves readily in organic solvents. The product is stable in organic solvents but stability in water is pH dependent. The product is capable of forming complexes with certain metal salts of manganese, copper and calcium (Saint-Blanquat, 1983).

Prochloraz was found to have an LD50 to the mallard, *Anas platyrhynchos*, of 3132mg/kg. The LC50 to the same species after administration of the product in the food for 5 days was greater than 10,000ppm. The LD50 of prochloraz to the rat, via oral administration, was found to be approximately 1600mg/kg and the LD50 via skin administration was found to be greater than 5000mg/kg. The LC50 for 96 hours of the product to the rainbow trout, *Salmo gairdneri*, and the harlequin, *Rasbora heteromorpha*, were 1mg/l and 2.8mg/l respectively i.e. much less (Saint-Blanquat, 1983).

Studies on the metabolites of prochloraz in plants, using radiolabelled compound, indicated many metabolites. Studies with the rat and dog indicate many metabolites. A single dose administered orally to the rat was eliminated completely within 96 hours. Studies with prochloraz administered via the epidermis indicated that the product does not penetrate the skin as rapidly or in as great quantities (Saint-Blanquat, 1983).

The metabolism and excretion of prochloraz in the rat was studied by Needman *et al.* (1991) (see Fig. 1.3). Following oral administration of prochloraz at 100mg/kg body weight to rats, the compound underwent extensive metabolism, the primary route appearing to be the opening of the imidazole ring followed by hydrolysis of the alkyl chain. The major metabolites were 2,4,6-trichlorophenoxyacetic acid, trichlorophenol and 2-(2,4,6-trichlorophenoxy)ethanol which is present mainly as a glucoronide conjugate. Ring hydroxylation occurred to produce several minor metabolites. No unchanged prochloraz was excreted in the urine. Tissue residues 96 hours after dosing were generally <1mg prochloraz equivalents/kg tissue. The highest residues were found in the liver and kidney, the principle organs of metabolism and excretion. The metabolites were quantitatively excreted within 96 hours. The elimination half-life of

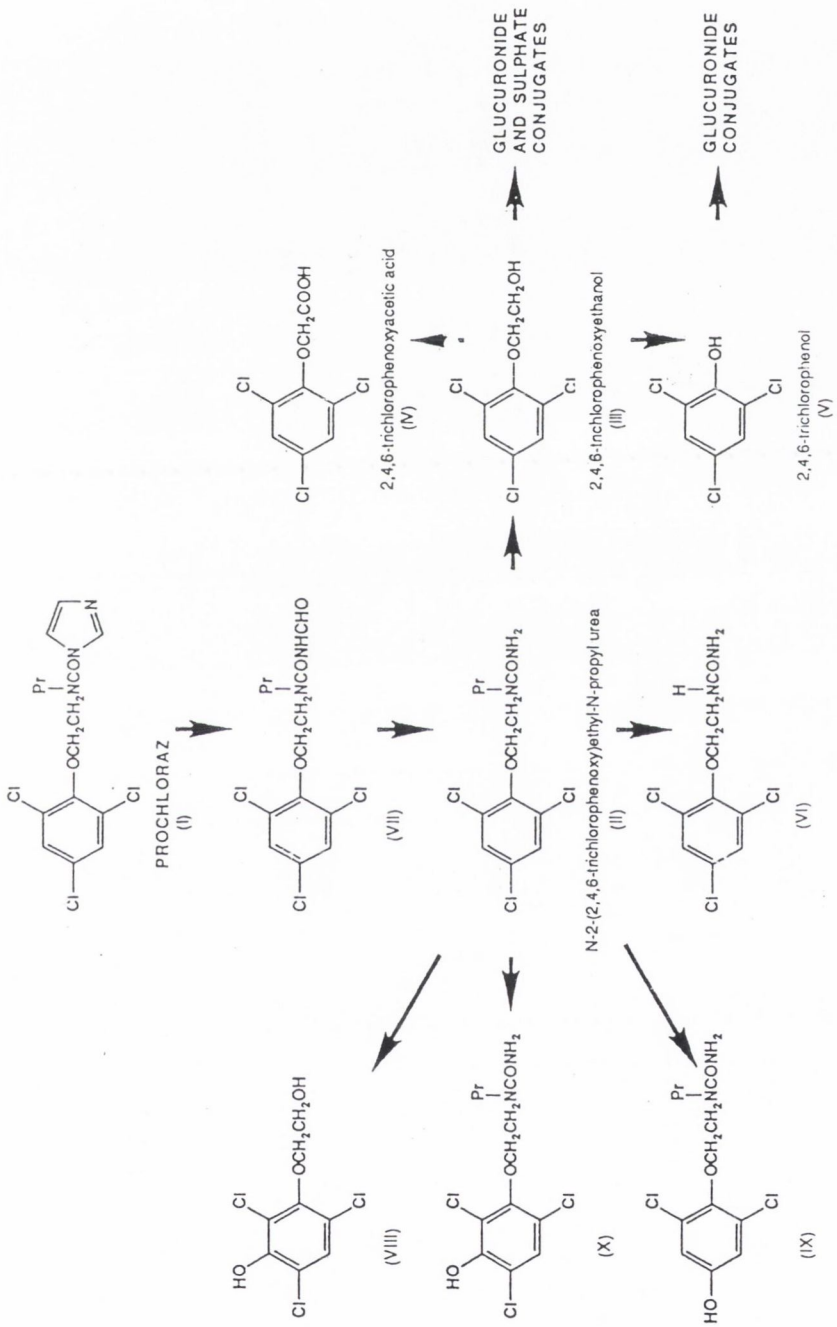
prochloraz in male rats was less than 24 hours whilst for female rats it was between 24 and 48 hours.

Prochloraz has been shown to induce hepatic monooxygenases in Japanese quail (*Couturnix coturnix japonica*), the grey partridge (*Perdix perdix*), pheasant (*Phasianus colchicus*) and the chicken (*Gallus gallus*) (Rivière *et al.*, 1984; Rivière *et al.,* 1985). Prochloraz has been shown to be a strong inducer of hepatic microsomal enzymes in several species of galliformes, as indicated by increases in relative liver weight, microsomal protein and cytochrome P-450 levels and increases in the activities of the microsomal enzymes alderin epoxidase (AE) and 7-etoxyresorufin-o-deethylase (EROD) (Johnston *et al.,* 1989; Johnston, 1991; Rivière *et al.,* 1985). Prochloraz has also been observed to increase the effect of other substances when used in combination. Johnston *et al.*, (1994) found that pre treatment with prochloraz of both the pigeon and partridge increased the effect of the pesticide malathion resulting in enhanced inhibition of serum BuChE (butyrylcholinesterase). This was attributed to the increased activation of malathion following induction of the hepatic monooxygenase system by prochloraz.

The toxicological impact of prochloraz on trout is difficult to evaluate. Snegaroff *et al.* (1989) found that prochloraz interacts with trout liver cytochrome P-450. Prochloraz was found to increase cytochrome P-450 levels in the liver, intestine and pyloric caeca (Bach *et al.,* 1989). Prochloraz *in vitro* inhibited AE by a linear mixed-type-mechanism. Prochloraz was also found to exhibit EROD *in vitro*, the mechanism of which differed from that of AE. The inhibition of EROD was partially non competitive (Snegaroff *et al.*, 1989). Prochloraz *in vivo* produced a more complex response. EROD was found to increase slightly at 4 days exposure and then return to the control level. AE was found to

be strongly inhibited at 4 days exposure and then returned to the control at 21 days exposure.

Fig. 1.3: The metabolism and metabolites of prochloraz (Needham *et al.,* 1991)



1.3.3 COPPER

Copper occurs naturally in the aquatic environment as a result of weathering and land drainage, but the use of various metal-containing pesticides and fungicides and the use of water pipes, antifouling paints and other human devices have introduced additional quantities of copper into the environment (Pilgaard *et al.,* 1994). Copper is considered, at the same time, an essential metal for most, if not all, living organisms (Mauge *et al.,* 1989), a very toxic pollutant (Hellawell, 1988) and a chemotherapeutic agent in aquaculture (Schlotfeldt, 1992). In aquatic biota, copper may become toxic if it exceeds the standard non toxic value which is established at 5-15µg/l (Birge *et al.,* 1979).

Despite its important role in normal body function, toxic effects of high levels of copper have been seen in virtually all physiologic systems studied in fish (Sorensen, 1991). The toxicity of copper among fish species is highly variable with 96 hour LC50 values ranging from 100ppb in brook trout (*Salvelinus fontinalis*), 35ppb total hardness, to 2400ppb in bluegills (*Lepomis macrochirus*) at 45ppm total hardness (Mckim *et al.,* 1971; O'Hara, 1971). In channel catfish, the 96 hour LC50 was found to range from 0.054mg Cul^{-1} (pH 7.3; total hardness 16 ± 1ppm) to 1.13mg Cul^{-1} (pH 8.4; total hardness 161 ± 12ppm) (Straus *et al.,* 1993).

The toxicity of water-borne copper depends on a wide variety of water quality factors, such as pH, alkalinity and total hardness (Lauren *et al.,* 1986). These factors determine the specification of copper, which is crucial to bioavailability. Salinity has also been demonstrated to affect copper toxicity (Hall *et al.*, 1995). A strong trend of copper toxicity at lower salinity was exhibited amongst most of the studies carried out. It has

also been found that copper exposure in acidic water significantly reduced swimming performance. Trout exposed to 0.08 $\mu mol/l^{-1}$ Cu(II) at pH5 achieved a critical swimming speed some 25 to 50 percent slower than control trout (Beaumont *et al.*, 1995).

Many studies have been carried out on the accumulation and acclimation of fish to copper. The accumulation of copper in the long term water-borne exposures is a two step process, a rapid accumulation in the exchangeable pools which should reach a steady-state situation in a few hours or days depending on the concentration of the available copper, followed by a second phase, at a slower rate, in which copper would be only accumulated in the non-exchangeable or slowly exchangeable pools, while the concentration in the exchangeable pools would not be increased (Carbonnell *et al.,* 1994). No relationship between copper accumulation and copper toxicity can be expected after exposures longer than a few days. The accumulation of copper in the whole body or different tissues in medium or long-term exposures will depend on the capability of the organisms to provide and/or synthesis non exchangeable binding sites (Carbonnell *et al.,* 1994). Different detoxifying mechanisms for Cu(II) poisoning have been described including the induction of metallothioneins (Harrison, 1986) allowing copper retention for weeks or months after absorption without producing toxic effects.

It has been shown that copper susceptibility depends on gender (Perkins *et al.*, 1997). Tsai et al. (1981) demonstrated that a size related sexual difference to copper susceptibility exists in the common guppy (*Lepistes reticularus*), with the rate of toxic action being higher in males than in females. A large differential in susceptibility to copper also exists between male and female mosquitofish (*Gambasia affinis*) (Changnon *et al.*, 1989). Accumulation of copper has also been suggested to have gender-dependent

variations in some fish. Miller *et al.* (1992) suggest that gender related differences may exist in basic mechanisms of metal homeostasis as well as in the manner in which these processes respond to insult with metals.

The toxicity of copper can be modified by previous sublethal exposure to the element which sometimes leads to a higher tolerance (Dixon *et al.,* 1981). The enhanced lethal resistance has been shown to be the result of a true acclimation (defined by Prosser, 1973) to copper during prolonged exposure to sublethal concentrations (McDonald et al., 1993), which involves physiological and biochemical modifications that restore the impaired branchial sodium transport (Lauren *et al.,* 1987). Cu acclimation, however, does not seem to involve any reduced branchial copper uptake (McCarter *et al.*, 1984). Previous copper exposure has been found to lead to a change in the tissue distribution of the copper entering the fish (Groswell *et al.*, 1996).

Data suggests a rapid distribution of copper from plasma to the extracellular fluids, with a half-life of a few minutes. The final elimination half-life is slightly more than 3 hours (Carbonnell *et al.*, 1994). The second phase is a real elimination of copper, not a binding to non-exchangeable ligands (Eisler, 1979). Evidence suggests that this elimination is by multiple elimination routes, hepatic and renal (Grosell *et al.,* 1997), fecal (Grepus *et al.*, 1986) and branchial (Grosell *et al.,* 1997).

Various studies have demonstrated that Copper accumulation in fish can be associated with adverse physiological responses (Marr *et al.*, 1995). Copper has been shown to cause reduced growth. This reduction in growth has been attributed to metabolic demands associated with metal detoxification involving metal-specific detoxifying proteins (ie metallothioneins) (Dixon *et al.,* 1981; Marr *et al.,* 1995). Such increased

metabolic demands divert resources from normal growth processes (Hogstrans *et al.*, 1995; Marr *et al.,* 1995). Reduced growth caused by waterborne copper exposures has also been associated with suppressed feeding (Collvin, 1984). Waiwood *et al.* (1978) observed reduced growth rates in response to copper in rainbow trout juveniles which exhibited depressed appetite and decreased food consumption. Marr *et al.* (1996) observed that rainbow trout exposed to copper concentrations as low as 4.6 μgl^{-1} had a significant reduction in both weight and length compared with control fish.

Epithelial cellular responses of teleosts to copper exposure include increased necrosis (accidental cell death) and apoptosis (programmed cell death) (Pelgrom, 1995). Bury *et al.* (1998) demonstrated that incubation of gill filaments with 50μM and 100μM $CuSO_4$ caused an approximate 5- and 16-fold increase, respectively, in chloride cell necrosis when compared to controls, but no significant effect on apoptosis. In contrast Julliard *et al.* (1993) observed that after 15 days exposure with a high level of copper, degenerating rainbow trout olfactory receptors did not exhibit signs of necrosis but rather typical features of cell apoptosis. Doucette *et al.* (1983) demonstrated that neuronal death by apoptosis could be found with neuron necrosis in rat olfactory epithelium.

Copper has been shown to cause mutagenic effects. Copper shows a high specific binding to DNA producing DNA strand breaks in several biological systems (Sagripanti et al., 1991). It has also been demonstrated that copper markedly enhances the formation of DNA strand breaks in the presence of hydroquinone (Li *et al.,* 1993). Copper has also been shown to depurinate DNA by releasing adenine. It was speculated that the mechanism of depurination involves oxygen free radicals. Cu (II) was found to react with hydrogen peroxide to yield high concentrations of hydroxyl radicals. Depurination occurs

concomitant with DNA strand scission (Schaaper *et al.,* 1987). Copper has been shown to act as a catalyst in the formation of reactive oxygen species and catalyses peroxidation of membrane lipids (Chan *et al.,* 1982). Enhancement of lipid peroxidation *in vitro* and *in vivo* has been observed for Cu(II). Lipid peroxidation products were found to mediate the formation of 8-oxodG (7,8-dihydro-8-oxo-2'-deoxyguanosine) and strand breaks in DNA (Kasprazk *et al.*, 1995). A Cu (II) complex was found to react with hydrogen peroxide to yield high concentrations of hydroxyl radicals which may be directly responsible for DNA damage (Ozawa *et al.,* 1993). It has also been shown that the enhancement of oxygen-activating enzymes or mimicking such enzymes can enhance oxidative damage by Cu(II) (Frenkelet *et al.*, 1986).

Copper has also been found to enhance the mutagenicity of other products. Benzoylperoxide, a commercial oxidant widely used for industrial household and medical purposes, was found to decompose and produce promutagenic DNA damage in the presence of Cu(I) (Akman *et al.,* 1992).

Copper has been demonstrated to cause a reduction in EROD activity *in vitro* and *in vivo* in the sea bass (*Dicentrarchus labrax*). *In vitro* experiments showed a competitive inhibition of NADPH-cytochrome reductase and a non competitive inhibition of GYPIA by copper, and the appearance of cytochrome P420 as a function of copper added to the medium and accompanied by a destruction of cytochrome P450 (Stein *et al.,* 1997).

Copper has also been demonstrated to have a detrimental effect on the immune system. In coho salmon, increasing levels of copper interfered with vaccination to *Vibro anguillarum*, resulting in an increased mortality due to branchial infection (Stevens, 1977). An increased susceptibility to IHN and IPN viruses was observed in rainbow trout

and other fish species exposed to sublethal concentrations of copper (Roales *et al.*, 1977; Hetrich *et al.,* 1979).

CHAPTER 2

THE TOXICITY OF NONOXYNOL, PROCHLORAZ AND COPPER TO ESTABLISHED CELL LINES

2.1 INTRODUCTION

Acute LD50 tests with fish are very costly and time-consuming, requiring specially designated aquatic laboratory facilities and appropriately trained staff. Furthermore, there is much concern to develop and establish *in vitro* research methods to replace live animals, especially those used in acute toxicity testing. One *in vitro* approach that is applicable to the testing of aquatic contaminants is the use of cultured fish cells in a variety of cytotoxic and genototoxic assays (Babich *et al.*, 1991). The use of short-term assays for the initial screening of chemicals not only aids in establishing priorities for the selection of chemicals that should be tested further *in vivo*, but also accelerates the time in which potential toxicants can be evaluated (Babich *et al.,* 1987).

The aim of the first part of this chapter was to screen the three tests pollutants on the EPC cell line (*Epithelium Papulosum Cyprini*) using the clonogenic assay technique established by Puck and Marcus (1955).

In the last few years evidence has accumulated that certain conditions of radiation exposure can induce a form of delayed death that results in the appearance of lethal and non-lethal mutations and chromosomal instability in large numbers of progeny of the surviving cells (Seymour *et al.,* 1986; Born *et al.*, 1988; Gorgojo *et al.*, 1989; Chang *et al.*, 1992; Kadhim *et al.,* 1992; Mendonca *et al.*, 1993; O'Reilly *et al.*, 1994; Kadhim *et al.*, 1995; O'Reilly *et al.*, 1997; Mothersill *et al.,* 1997). The unique characteristics of this delayed death effect are that it occurs at a high frequency and is non-clonal (Kadhim *et al.,* 1996, Mothersill *et al.*, 1998). The effect appears to increase the probability that

damage or mutations will be expressed during normal cellular functions of surviving cells (Mothersill *et al.*, 1998). Delayed expression of cell death is now considered by many scientists to be one manifestation of genomic instability (Mothersill *et al.*, 1997). Division failure in clonal progeny and chromosomal instability can lead to the production of monoclonal chromosomal mutations in clonal progeny, microsatellite instability and gene amplification (Tlsty *et al.*, 1989, Branch *et al.,* 1993). As a result surviving cells which appear normal and which can divide, have latent damage which can appear at any time in near or distant progeny to induce an abnormal event (Mothersill *et al.,* 1997).

Mothersill *et al.* (1998) demonstrated that genomic instability effects can be induced in surviving progeny by a wide range of genotoxic agents and other substances which cause oxidative stress, including heavy metals. Recent work by O'Reilly *et al.* (1997) and Mothersill *et al.* (1998) have demonstrated that the effect can be seen not only in mammalian cell lines but also in teleost cell lines, and with environmental stressors other than radiation. As a result of these finding this project aimed to investigate whether aquatic pollutants from different pollutant classes can cause delayed death in progeny of treated CHSE (*chinook salmon embryo*) cells and the characteristic of any resulting delayed cell death.

2.2 MATERIALS & METHODS

2.2.1 Cell Lines Used

EPC: (Epithelium papulosum cyprini)

The EPC cells originated from carp epidermal herpes-virus induced hyperplastic lesions (Fijan *et al.*, 1983) and have epithelial-like morphology. They were maintained as a monolayer in BHK-21 medium (Glasgow MEM) supplemented with 10% foetal calf serum (Gibco), Hepes buffer solution 0.025mol/l (Gibco), 5ml L-glutamine(200mM) (Gibco), 5ml penicillin/streptomycin 5000IU/ml (Gibco). The flasks were incubated at 30^0C. The EPC cells treated with nonoxynol and copper were grown in 5%CO_2. Those treated with prochoraz were grown without CO_2. This was due to the lack of availability of a CO_2 incubator when carrying out the test with prochloraz.

CHSE: (Chinook salmon embryo cells)

THE CHSE-214 cell line was established in 1964 from chinook salmon embryo cells. Cell growth is supported at a wide range of temperatures with an optimum growth temperature of 21^0C. They were maintained as a monolayer in 500ml Dulbecco's Modified Eagle Medium (Gibco) supplemented with foetal calf serum (Gibco) 12.5%, L-glutamine 200mM(Gibco) 1mM, 5ml penicillin/streptomycin 5000IU/ml (Gibco), , hepes buffer solution 0.025mol/l(Gibco). The flasks were incubated at 18^oC.

2.2.2 Chemical Assays

Nonoxynol (Igepal C210) and prochloraz (Sigma) were dissolved in a known volume of dimethylsulfoxide (DMSO). Sequential dilutions of this stock were made prior to addition to the cultures in volumes of 50µl per flask - the control contained 50µl of DMSO.

Cupric copper chloride (Sigma) solution was prepared as an aqueous stock solution. Sequential dilutions of this stock were made prior to addition to the cultures in volumes of 50µl per flask. The control contained 50µl of water.

2.2.3 Clonogenic Assay

Reduction in colony-forming ability was assessed using the clonogenic assay established by (Puck *et. al.,* 1955). Appropriate numbers of cells were seeded into 25cm^2 flasks with 5 ml of the appropriate medium such that about 100 viable colonies could be expected to form after about 7 days. After 4h, different concentrations of nonoxynol were added in volumes of 50µl per flasks (1%w/v). EPC Colonies were stained with 15% carbol fuschin after 20 days incubation, CHSE cells were stained after 35 days incubation. The toxic effects of the test substances were assessed by determining the colony forming efficiency (CE) of these cells during exposure and relating the percentage of colonies to untreated control figures.

The survival fraction was calculated using the following formula

$$\% \text{ Colony Forming Efficiency} = \frac{\text{no of colonies counted}}{\text{no. of cells plated}} \times \frac{100}{\text{Control Plating Efficiency.}}$$

(C.F.E.)

2.2.4 Delayed Cell Death

Cells were seeded into six $25cm^2$ flasks per dose point of the test substance. The cells were allowed to attach to the surface of the flask and treated with the appropriate doses of the test substance. When the cells had produced macroscopic colonies, three of the original six flasks were stained with carbol fuschin, and the colonies counted. The medium from the remaining three flasks was removed and replaced with 5ml of fresh culture medium. Upon reaching confluence the cells were trypsinised and the cell suspension counted to determine the number of cells per ml. 400 cells from each flask were then reseeded in three extra flasks and the resulting microscopic colonies counted. To determine the residual survival fraction (R.S.F.) the following equation was used:

$$\% \text{ Residual Colony Forming Efficiency} = \frac{\text{Initial \% C.F.E. X Progeny \% C.F.E.}}{100}$$

The residual colony forming efficinecy is a measure of the level of survival at times distant from the initially treated cells. When the initial C.F.E.and the R.C.F.E. are plotted versus dose a reduction in the initial colony forming efficiency as compared to the residual forming efficiency, would indicate the presence of delayed death.

versus dose a reduction in the initial colony forming efficiency as compared to the residual forming efficiency, would indicate the presence of delayed death.

2.3 RESULTS

2.3.1 EPC CELL LINE

2.3.1.1 NONOXYNOL

Table 2.1: The % colony forming efficiency of the EPC cell line exposed to nonoxynol.

Dose Applied (µmol/L)	% Colony Forming Efficiency
0	100
0.5	95.5 ± 3.0
1	89.8 ± 1.5
5	62.3 ± 1.7
7	48.4 ± 1.6
8	13.1 ± 1.3
9	1.3 ± 0.5

Table 2.1 shows the % colony forming efficiency of the EPC cell line post treatment with nonoxynol. Each figure represents the mean ± SEM for n = 9.

Figure 2.1: The % colony forming efficiency of the EPC cell line post treatment with nonoxynol

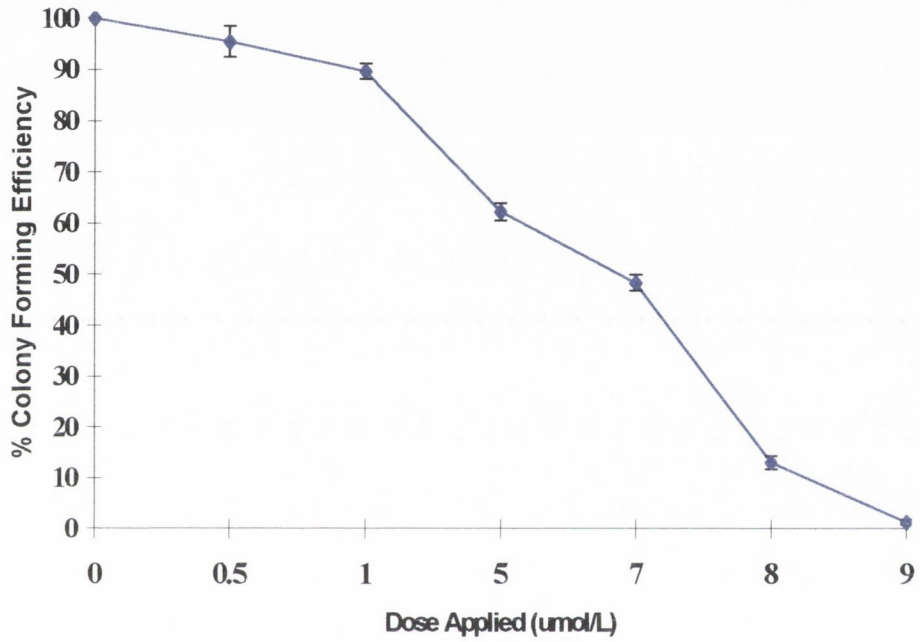


Figure 2.1 shows the percentage colony forming efficiency of the EPC cell line post treatment with nonoxynol. These results are expressed as mean ± SEM for n = 9. The lethal dose (that required to cause 100% cell death) of the pollutant on the cell line was 10μmol/l.

2.3.1.2 PROCHLORAZ

Table 2.2: The % colony forming efficiency of the EPC cell line exposed to Prochloraz.

Dose Applied (µmol/L)	% Colony Forming Efficiency
0	100
0.5	83.3 ± 1.9
1	67.8 ± 1.5
5	46.4 ± 1.3
6	40.9 ± 1.1
7	32.5 ± 0.9
8	27.0 ± 1.0
9	23.1 ± 0.8
10	15.1 ± 0.6
11	10.9 ± 0.4
12	3.5 ± 0.5

Table 2.2 shows the % colony forming efficiency of the EPC cell line post treatment with prochloraz. Each figure represents the mean ± SEM for n = 9.

Figure 2.2: The % colony forming efficiency of the EPC cell line post treatment with prochloraz

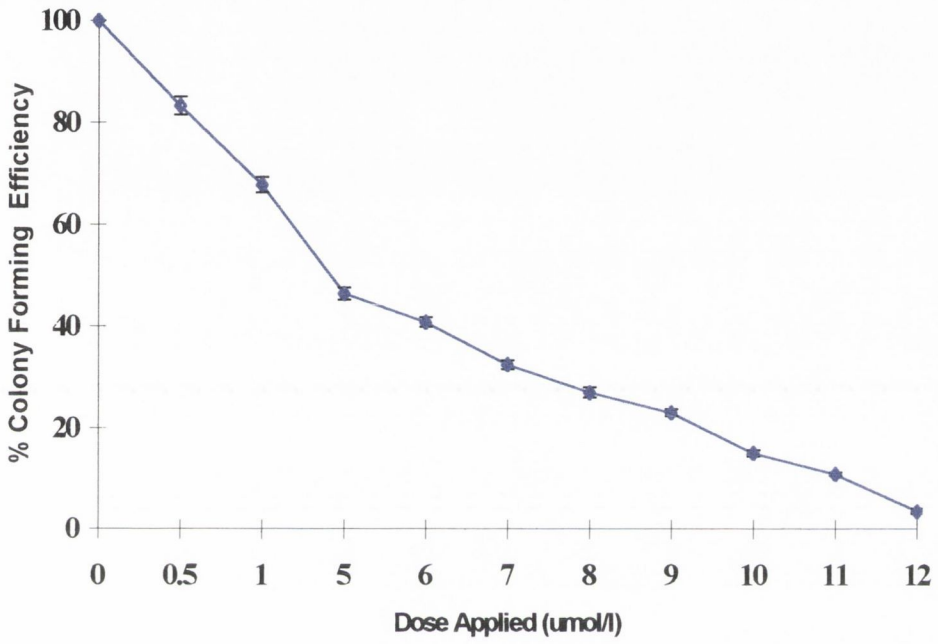


Figure 2.2 shows the percentage colony forming efficiency of the EPC cell line post treatment with prochloraz. These results are expressed as mean ± SEM for n = 9. The lethal dose (that required to cause 100% cell death) of the pollutant on the cell line was greater than 12μmol/l..

2.3.1.3 Copper

Table 2.3: The % colony forming efficiency of the EPC cell line exposed to copper.

Dose Applied (μmol/l)	% Colony Forming Efficiency
0	100
5	92.3 ± 1.9
10	83.1 ± 1.7
15	63.4 ± 2.3
30	51.6 ± 1.9
40	14.9 ± 0.9
50	8.5 ± 0.9
60	6.7 ± 0.6

Table 2.3 shows the % colony forming efficiency of the EPC cell line post treatment with copper. Each figure represents the mean ± SEM for n = 9.

Figure 2.3: The % colony forming efficiency of the EPC cell line post treatment with copper.

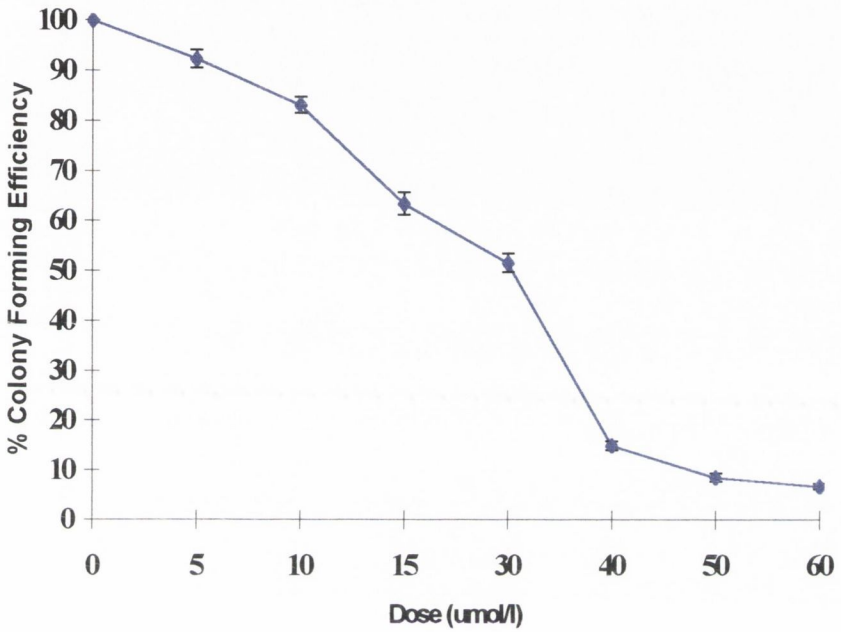


Figure 2.3 shows the percentage colony forming efficiency of the EPC cell line post treatment with Prochloraz. These results are expressed as mean ± SEM for n = 9. The lethal dose (that required to cause 100% cell death) of the pollutant on the cell line was greater than 60μmol/l.

2.3.2 DELAYED CELL DEATH (CHSE CELL LINE)

2.3.2.1 NONOXYNOL

Table 2.4: The % colony forming efficiency and residual colony forming efficiency of the CHSE cell line treated with nonoxynol

Dose Aplied(µmol/L)	Initial Colony Forming Efficiency f(0)	Progeny Colony Forming Efficiency f(1)	Residual Colony Forming Efficiency f(0) X f(1)
0	100	100	100
0.5	85.3 ± 4.3	85.2 ± 3.8	72.6 ± 2.5
1	86.5 ± 4.0	71.1 ± 2.3	61.4 ± 1.3
5	66.3 ± 5.7	56.3 ± 2.2	37.3 ± 1.2
6	50.8 ± 4.0	56.8 ± 2.7	28.9 ± 1.1
7	43.5 ± 5.3	40.9 ± 3.0	17.8 ± 1.4
8	31.8 ± 2.5	26.7 ± 2.2	8.5 ± 0.6

Table 2.4 shows the Initial % colony forming efficiency f(0), the Progeny % colony forming efficiency f(1) and the residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with nonoxynol. Each f(0) figure represents the mean ± SEM for n = 9.

Figure 2.4: The % initial colony forming efficiency and residual colony forming efficiency of the CHSE cell line exposed to nonoxynol.

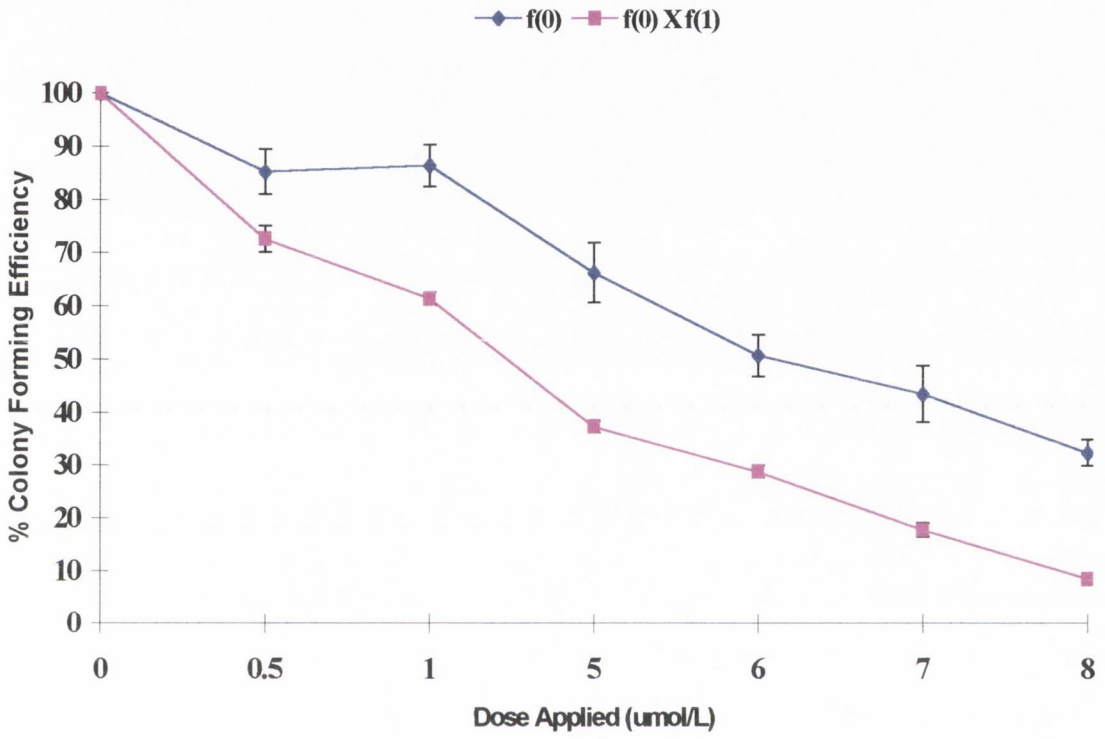


Figure 2.4 shows the Initial % colony forming efficiency f(0), and the residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with nonoxynol. Each f(0) figure represents the mean ± SEM for n = 9. Each f(1) figure represents the mean ± SEM for n = 27.

2.3.2.2 PROCHLORAZ

Table 2.5: The % colony forming efficiency and residual colony forming efficiency of the CHSE cell line treated with prochloraz

Dose Applied (µmol/L)	Initial Colony Forming Efficiency f(0)	Progeny Colony Forming Efficiency f(1)	Residual Colony Forming Efficiency f(0) X f(1)
0	100	100	100
1	88.3 ± 1.3	101.7 ± 2.0	89.7 ± 2.0
10	43.8 ± 0.5	86.4 ± 1.6	37.8 ± 0.7
11	40.2 ± 0.7	81.5 ± 1.7	32.7 ± 0.7
12	33.9 ± 0.6	75.4 ± 1.5	25.6 ± 0.4
13	29.5 ± 0.8	68.0 ± 1.2	20.0 ± 0.3

Table 2.5 shows the initial % colony forming efficiency f(0), the progeny % colony forming efficiency f(1) and the residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with prochloraz. Each f(0) figure represents the mean ± SEM for n = 9.

Figure 2.5: The % initial colony forming efficiency and residual colony forming efficiency of the CHSE cell line exposed to prochloraz.

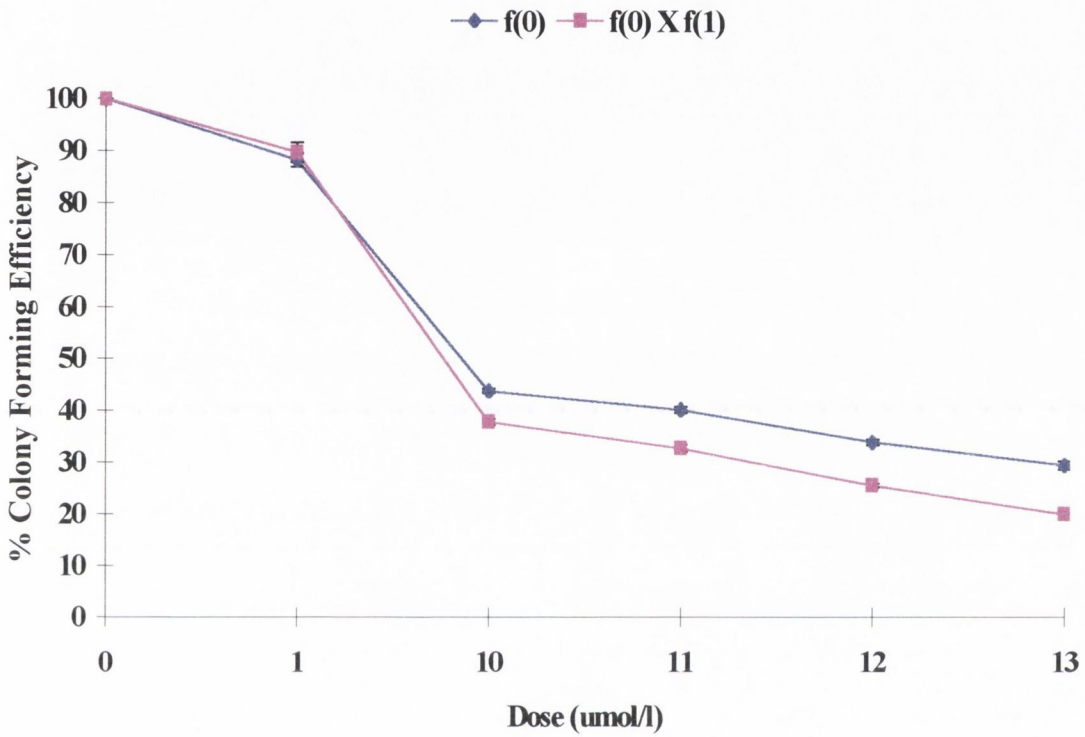


Figure 2.5 shows the Initial % colony forming efficiency f(0), and the Residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with prochloraz. Each f(0) figure represents the mean ± SEM for n = 9. Each f(1) figure represents the mean ± SEM for n = 27.

2.3.2.3 COPPER

Table 2.6: The % colony forming efficiency and residual colony forming efficiency of the CHSE cell line treated with copper.

Dose Applied (µmol/L)	Initial Colony Forming Efficiency f(0)	Progeny Colony Forming Efficiency f(1)	Residual Colony Forming Efficiency f(0) X f(1)
0	100	100	100
1	83.4 ± 3.1	101.7 ± 1.9	84.8 ± 2.1
5	69.8 ± 2.1	98.4 ± 1.6	68.7 ± 1.5
10	51.8 ± 1.5	63.3 ± 1.2	32.8 ± 2.3
15	45.9 ± 2.0	60.6 ± 1.1	27.8 ± 1.0
20	34.3 ± 1.0	66.3 ± 2.1	22.8 ± 1.3
30	29.0 ± 1.0	64.7 ± 1.9	18.7 ± 0.9
40	26.6 ± 1.0	62.5 ± 1.5	16.7 ± 0.8
50	22.0 ± 0.7	64.5 ± 1.4	14.2 ± 0.5

Table 2.6 shows the initial % colony forming efficiency f(0), the progeny % colony forming efficiency f(1) and the residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with copper. Each f(0) figure represents the mean ± SEM for n = 9.

Figure 2.6: The % initial colony forming efficiency and residual colony forming efficiency of the CHSE cell line exposed to copper.

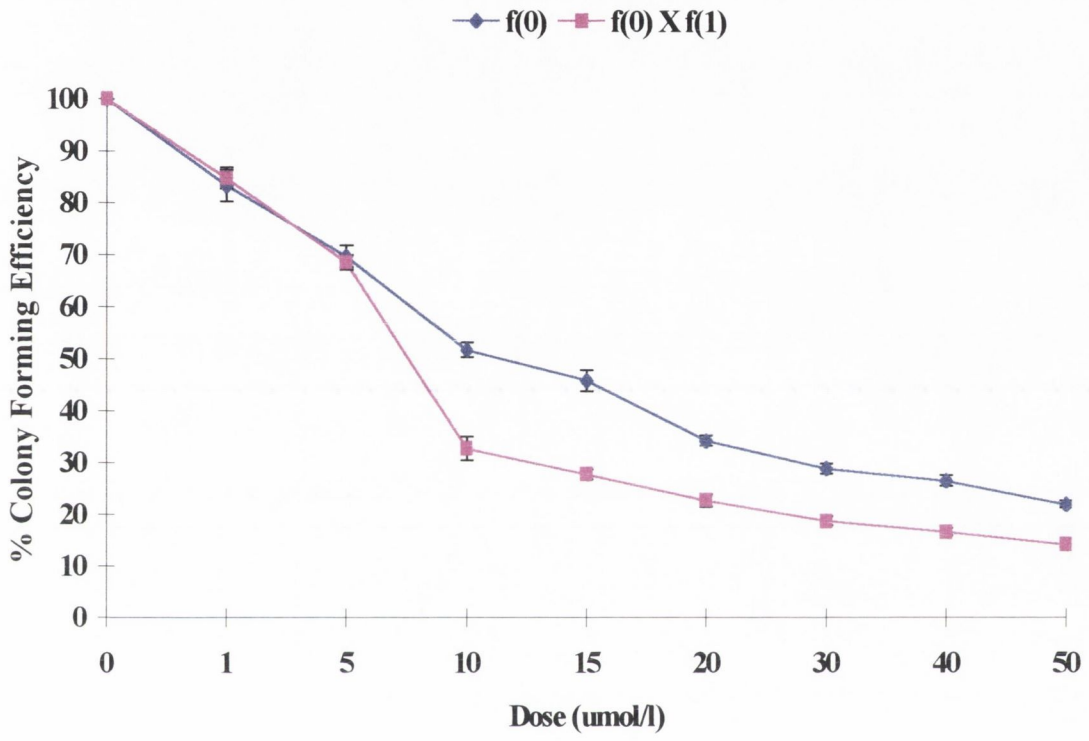


Figure 2.6 shows the Initial % colony forming efficiency f(0), and the Residual colony forming efficiency f(0) X f(1) of the CHSE cell line post treatment with copper. Each f(0) figure represents the mean ± SEM for n = 9. Each f(1) figure represents the mean ± SEM for n = 27.

2.3.1 EPC CELL LINE

2.3.1.1 Nonoxynol (table 2.1; figure 2.1)

The lethal dose (that required to cause 100% cell death) of the pollutant on the cell line was 10μmol/L. The mean plating efficiency of the control cells was 21.4% ± 0.8.

2.3.1.2 Prochloraz (table 2.2; figure 2.2)

The lethal dose of the pollutant on the cell line was greater than 12μmol/L. The mean plating efficiency of the control cells was 28.0% ± 0.6.

2.3.1.3 Copper (table 2.3; figure 2.3)

The lethal dose of the pollutant on the cell line was greater than 60μmol/L. The mean plating efficiency of the control cells was 22.4% ± 0.4.

2.3.2 DELAYED CELL DEATH (CHSE CELL LINE)

2.3.2.1 Nonoxynol (table 2.4, figure 2.4)

The mean plating efficiency of the control cells was 23.6 ± 0.4. The pollutant was found to cause delayed cell death at a dose of 0.5μM. The initial colony forming efficiency at this dose was 85.25%. The delayed response was a linear response ie. the % colony forming efficiency of the progeny appeared to fall at a constant rate. The effect appeared to stop between 5μM to 6μM (table 2.4, f(1)) and then resume to fall.

2.3.2.2 Prochloraz (table 2.5, figure 2.5)

The mean plating efficiency of the control cells was 26.8 ± 0.4. The pollutant was found to cause delayed death at a dose of 10μM. The initial colony froming efficiency at this threshold dose was 43.8%. A linear response of delayed death was observed from this point.

2.3.2.3 Copper (table 2.6, figure 2.6)

The mean plating efficiency of the control cells was 26.8 ± 0.4. The pollutant was found to cause delayed cell death at 5μM. The initial colony forming efficiency at this dose was 83.4%. The f(1) generation was found to decrease in colony formation to 63.3% with increasing chemical concentration up to 10μmol/l. As the chemical concentration increased from 10μmol/l, with decreasing intitial colony froming efficiency, the progeny forming efficiency was found to remain constant at above 60% (table 2.6, f(1)). The initial survival at 10μM was 51.2%.

2.4 DISCUSSION

Nonoxynol was found to be the most toxic of all the chemicals tested to the EPC cell line. Copper was found to be the least toxic. This is as one might expect as organic pollutants have been found in the past to be more toxic to fish cell lines than heavy metals. Babich *et al.* (1990) found that organic mercury compounds were more toxic to BG/F cells than mercury itself. The toxicity of copper was observed to be less to the EPC cell line than that of cadmium, observed by Lyons-Alcantara *et al.* (1996), and more toxic than that of nickel, observed by McSweeney (1998). Babich *et al.* (1986) observed a similar relationship between these three metals when tests the *in vitro* cytotoxicity of metals to bluegill BF-2 cells.

The EPC cell line was not used to study the ability of the pollutants to cause delayed cell death. Previous studies have indicated that it is not possible to observe this phenomenon in EPC cells (O'Reilly *et* al., 1997; McSweeney 1998) because the cell line is a highly malignant cell line (Fijan *et al.*, 1983), as it is derived from a hyperplastic lesion. The CHSE cell line does not share this characteristic and has been shown to demostrate delayed cell death (Mothersill *et al.*, 1998).

All three chemicals were found to induce delayed cell death in surviving progeny of the CHSE cell line. However, the appearance of this cell death appears to differ between the chemicals tested. Prochloraz was not found to cause delayed cell death until the initial toxicity was reduced to 43.80% (table 2.5). The percentage colony forming efficiency of the f(1) progeny at this point was 86.4%. The amount of delayed cell death appeared to be constant irrespective of the level of the initial toxicity once the

threshold was exceeded. A similar response was observed when HaCaT cells were treated with ionising radiation and bleomycin (Mothersill *et al.*, 1998). In this study the threshold initial toxicity was found to be 60%. However in the study carried out with prochloraz no dose levels were investigated between 1 and 10μM. The fact that the percentage survival at the threshold concentration of response, 43.8%, was as high as 86.4% suggests that the threshold concentration necessary to initiate the delayed cell death effect was only slightly less than 10μM.

Nonoxynol is found to cause delayed cell death. In contrast with prochloraz the increase in yield of delayed damaged accompanied an increase in initial damage. A similar result was observed with HaCaT cells treated with UV radiation and hydrogen peroxide (Mothersill *et al.,* 1998; O'Reilly *et al.,* 1997). The amount of delayed cell death caused by nonoxynol was found, however, to remain constant between 66% and 50% initial survival at approximately 56% (table 2.4). At initial colony forming values less than those just stated delayed cell death was found to occur at the same rate as that of the values above 66%. There are a number of reasons as to why this may be happening. The prochloraz data (table 2.5, figure 2.5) suggests that a certain concentration of chemical exposure is required in order to see delayed death occurring. This suggests that a degree of damage is tolerated by the cell by repair mechanisms but once this level is exceeded delayed damage occurs. It is possible that the initial effect may be caused by a metabolite of nonoxynol which like UV radiation causes delayed cell death once initial colony forming efficiency decreases, but like cadmium (Mothersill *et al.,* 1998) and copper (please see below) the amount of delayed death remains constant once a certain initial colony forming efficiency is reached. Then when a greater amount of

nonoxynol is added ie 7μM, the levels of another metabolite of the chemical may rise and cause the delayed cell death which occurs at lower initial colony forming efficiency values. This metabolite may be tolerated by the cell up to a certain concentration. It is also possible that the initial cell death observed at high initial colony forming efficiency values is due to a detergent effect and that the cells can tolerate this after a certain quantity of cell death has occurred but if the concentration of the chemical exceeds a certain amount the cells are no longer able to tolerate the pollutant. This would explain why there was no increase in delayed death between 66% and 50% initial cell death. The effect observed at initial colony forming efficiency values less than 50% may be caused by the chemical itself, metabolites of the chemical, residues of the chemical or its metablites remaining in the cells or a combination of the chemical effect and the detergent effect.

The delayed cell death caused by treatment with copper shows a different pattern than that observed with nonoxynol and prochloraz. Delayed death is found to decrease with decreasing initial colony forming efficiency to a progeny colony forming efficiency rate of slightly above 60% which remained constant at the further decreasing colony forming efficiency rate. Another heavy metal, cadmium, was found to cause a similar type of effect to both CHO cells (Lyons Alacantara, 1997; Mothersill *et al.,* 1998) although at different critical doses.

These results show that pollutants other than radiation can cause delayed cell death. However there appears to be differing mechanisms involved. It has been suggested that delayed cell death is caused by persistent oxygen radical generation in progeny of treated cells leading to persistent oxidative damage and consequent increases in cell

death, mutations and chromosome aberrations (Clutton *et al.*, 1996). It has been known from radiobiological studies that ·OH reaxts with all components of nuclear chromatin. It can modify DNA bases and deoxyribose and produce DNA-protein crosslinks. It can also cause DNA depurination and strand scission (Kasprzak, 1995). It has been suggested that DNA strand breaks rather than the existence of oxidative stress is the initial event leading to delayed cell death (Mothersill *et al.,* 1998). However studies have also suggested that DNA break without the generalised oxidative stress does not induce the phenotypic expression of persistent instability. Further work is necessary in order to determine the exact mechanism of cell death observed in this study.

One must also consider that, although after treatment the remaining cells were allowed to grow in fresh medium before re-seeding, the trypsinisation of these cells, already under stress from their exposure, may result in their inability to reattach to the culture vessel. Chemical residues may still be present inside the cells after the medium change. The result of this would be a decrease in the plating efficiency of the progeny which would be dose related.

Future study will have to take place to determine what is causing the delayed cell death observed in the results above and why the response differs in type between chemicals. However these results combined with those observed with another aquatic pollutant, cadmium (Mothersill *et al.,* 1998) do suggest that many aquatic pollutants may cause delayed death. These results have serious implications from an ecotoxicological perspective. They demonstrate that the damage caused by nonoxynol, prochloraz and copper is not only acute and immediate but also chronic and persistant. They also demonstrate when considered with the results obtained by Mothersill *et* al., (1998), that

common aquatic pollutants also cause a detrimental cellular reponse previously observed in studies with radiation.

2.5 CONCLUSION

The results of this study demonstrate that all three test substances cause delayed cell death, a phenomenom until recently only observed in studies with radiation. However the mechanism of this delayed death was found to differ between each of the aquatic pollutants tested. Future study will have to take place to determine what is causing the delayed cell death observed in the results above and why the response differs in type between chemicals. There is however a clear link between exposure and the inducible genetic response. These results have serious implications from an ecotoxicological perspective. They demonstrate that the damage caused by the aquatic pollutants studied is not only acute and immediate but also chronic and persistant.

CHAPTER 3

THE TOXICITY OF NONOXYNOL, PROCHLORAZ AND COPPER ON RAINBOW TROUT PRIMARY EPITHELIAL SKIN CELL CULTURES *IN VITRO*

3.1 INTRODUCTION

In a previous study by Mothersill *et al.,* (1995) an *in vitro* technique was developed for the culture of rainbow trout (*Onchorynchus mykiss*) epithelia. In the broad perspective of environmental toxicology and biohazard assessment, epithelial cells are highly significant biological indicators since, all the substances which are received or exerted by the body must transverse an epithelium (Bloom *et al.,* 1970). Epithelial cells form the barrier between an individual and its environment, and a primary target for pollution-related carcinogenises (Langdon, 1983; Iger *et al.*, 1994). Because the characteristics of established cell lines deviate substantially from normal counterparts, fish-derived primary cells in culture may represent an alternative for toxicity studies. If differentiated properties are lost, it is difficult to relate the cultured cells to functional cells in the tissue from which they are derived (Freshney, 1994). Primary cells can be made to express a high number of differentiated characteristics (Mothersill *et al.,* 1995). Rainbow trout (*Onchorynchus mykiss*) skin epithelia contain both epithelial pavement cells and goblet mucus cells (Mothersill *et al.,* 1995). The primary rainbow trout skin cell cultures developed by Mothersill *et al.* (1995) contain both of these cell types (plate 1, p. 59).

The object of this study was to develop the technique for the culture of rainbow trout epithelial skin cell cultures as a workable diagnostic tool in *in vitro* toxicity assessment using the three test substances nonoxynol, prochloraz and copper ie a fast efficient and easy to use method for *in vitro* toxicity assessment. The influence of media composition on the toxicity of the three test substances was also studied by using both serum-containing and serum-free medium. The morphological changes occurring

within the cell cultures post treatment with the pollutants was also noted in order to determine the type of cell death occurring and if the pollutants exerted any alterations in the structure and number of the goblet mucus cells present in the primary cultures.

3.2 MATERIALS & METHODS

3.2.1 Primary Culture:

Healthy Rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial fish farm. Epithelial cell outgrowths were obtained from explants using a modification of the technique developed previously (Mothersill *et al.,* 1995) which is summarised below. A section of skin from the dorsal side was taken; cleaned of muscle tissue and chopped to $2mm^2$ pieces. Individual tissue fragments were placed, scale side up, in $24cm^2$ Nunc flasks containing 2ml of initiating medium containing serum (see Table 1). The cultures were incubated at 19^0C in an atmosphere containing 5% CO_2 in a refrigerated incubator (Leec, U.K.). No medium changes were made as this destroyed the cell growth.

Table 3.1: Composition of Primary culture initiating medium

RPMI-1640 (Sigma)	500ml
Foetal calf serum (Gibco)	60ml
Horse serum (Gibco)	40ml
Hepes buffer (Gibco)	12.5ml
L-Glutamine (Gibco)	20mmol/l
Penstrep (Gibco)	5000IU/ml
Human recombinant insulin (Novo Nordisk A/s)	0.05IU/ml
Hydrocortisone (Sigma)	1µg/ml
Fungizone (Gibco)	1µg/ml

3.2.2 Cytotoxicity Endpoints

Cell growth reduction

The reduction of cell growth in primary cultures by the three test substances, was investigated. Four days post attachment the perimeter of the cultures was outlined on the bottom of the flask with permanent marker pen. Cells that have grown out from the explant form a whitish area that can be observed with the naked eye. The cultures were then treated for 24h, 48h, 72h and 96h with sequential concentrations of the test substances, three cultures per dose point. The experiment was repeated twice. After exposure the cultures were fixed in 10% buffered formalin and counterstained in haematoxylin. The outgrowth area from the explant was estimated using a $1mm^2$ grid by counting the number of squares covered by the explant outgrowth. The area before treatment was also measured. The results are given as a percentage of the control survival rate. The mean values and standard errors were calculated. This method takes into consideration that primary cultures from the same animal do not grow uniformly under the same conditions. The media in a number of the cultures was changed to serum-free Clonetics keratinocyte growth medium (KGM) (Clonetics Corporation USA) prior to the addition of the test substances and the same procedure carried out as described above.

Morphology

The type of cells present in each explant were also counted using a Leica image analyser. Five fields, each $0.55mm^2$, were analysed from each of the explants

described above, two from the inner region, two from the outer region and one from the mid region. The type of cells identified were classified as a) normal epithelia (plate 1),b) goblet (plate 1), c) necrotic (plate 2) and d) apoptotic (plate 3). Goblet cells are recognisable in the epithelium by their basophilic cytoplasm, their basally located nuclei, and the accumulation of mucus secretory granules that fill and distend their apex to give the cells their characteristic goblet shape (Weiss, 1988). Cells were classified as undergoing necrosis if they exhibited the following features; a) swelling of the cytoplasm and organelles, with only slight changes in the nucleus, or b) organelle dissolution and rupture of the plasma membranes resulting in leakage of the cellular contents into the extracellular space (Schwartzman *et al.,* 1993). Cells were classified as undergoing apoptosis if they showed evidence of two or more of the following; a) cell volume shrinkage and picnotic nucleus (chromatin condensation), b) blebbing of the cytoplasm c) nuclear fragmentation and d) development into apoptotic bodies (Kerr *et al.,* 1991; Schwartzman *et al.,* 1993; Martin *et al.,* 1994).

Plate 1: Normal rainbow trout epithelial skin cell culture showing A) normal epithelial cells and B) goblet cells (X20 Magnification)

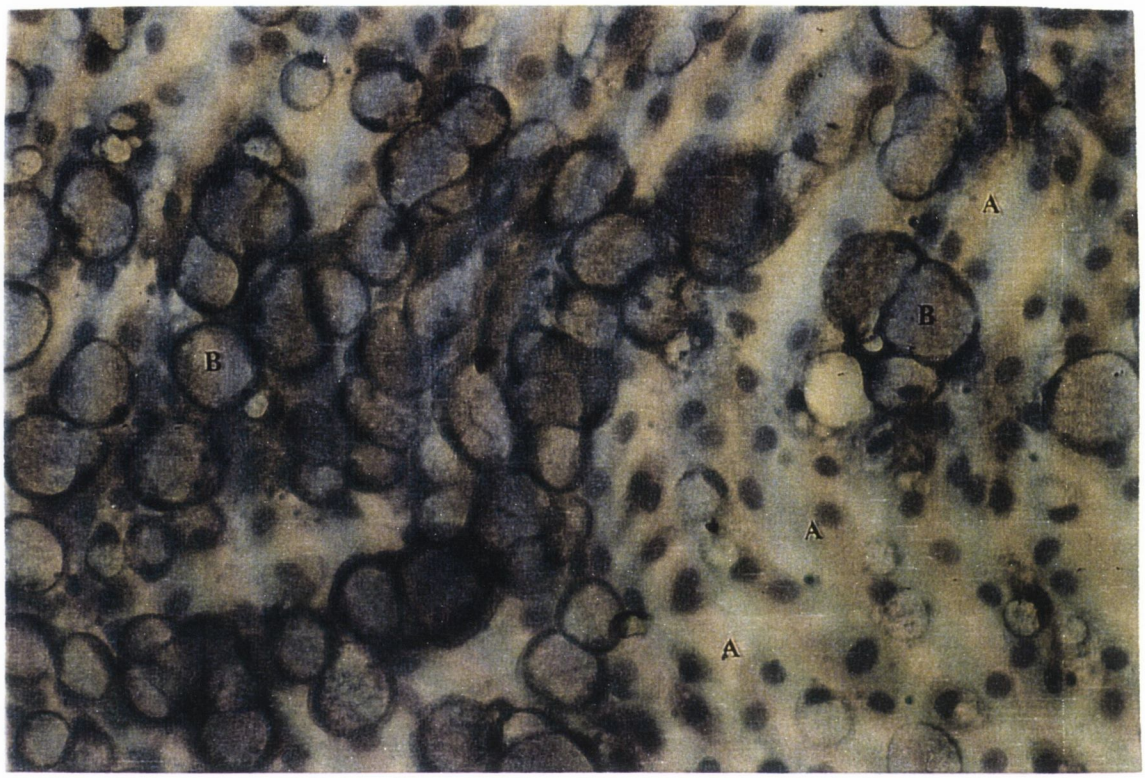


Plate 2: Rainbow trout epithelial skin cell culture showing necrotic cells (N) (X20 Magnification)

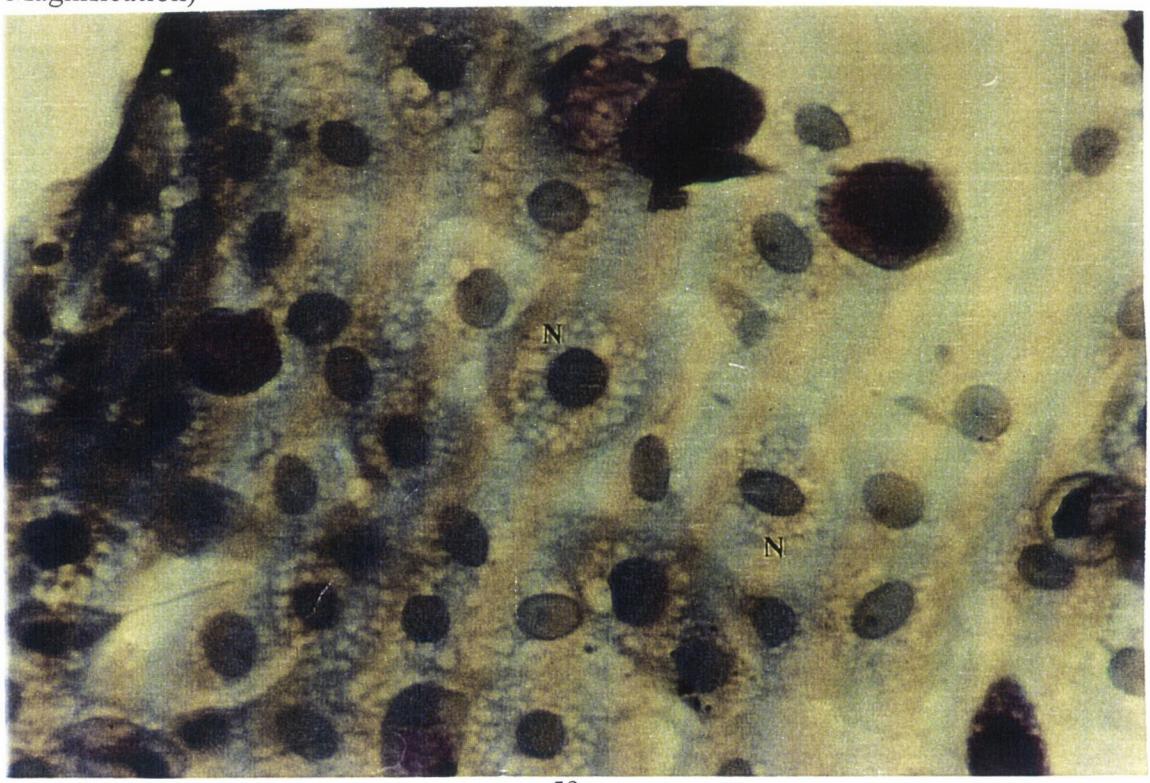
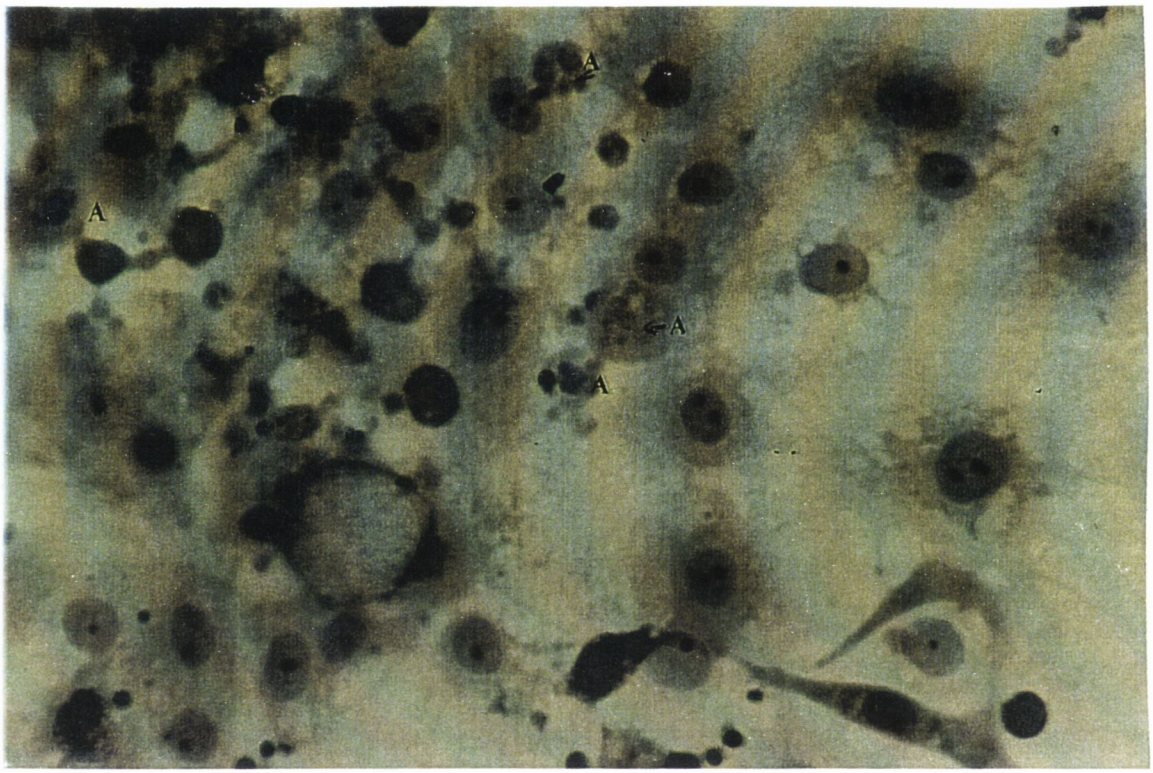


Plate 3: Rainbow trout epithelial skin cell culture showing apoptopic cells (A). (X20 Magnification)



3.3 RESULTS

3.3.1 NONOXYNOL

Table 3.2: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol for 24h, 48h, 72h, 96h in serum-containing medium.

DOSE (µmol/l)	% RESIDUAL OF DAY 3 GROWTH			
	24h	**48h**	**72h**	**96h**
0	100	100	100	100
1.25	91.0 ± 2.7	83.0 ± 2.1	88.9 ± 1.8	95.6 ± 2.2
2.5	83.8 ± 2.1	79.7 ± 3.7	89.7 ± 1.6	99.2 ± 1.8
10	74.8 ± 3.2	75.9 ± 5.1	76.1 ± 2.5	72.8 ± 1.6
12.5	74.0 ± 2.3	75.3 ± 3.2	74.4 ± 3.5	68.0 ± 1.6
25	46.4 ± 1.9	33.9 ± 2.1	30.3 ± 1.4	29.7 ± 0.9
50	15.8 ± 2.3	12.5 ± 2.1	10.0 ± 1.7	10.4 ± 1.2
75	3.3 ± 1.6	2.0 ± 1.3	1.4 ± 0.7	2.1 ± 0.5

Table 3.2 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with nonoxynol in serum-containing medium. The results are expressed as mean ± SEM for n = 6.

Figure 1: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol in serum-containing medium.

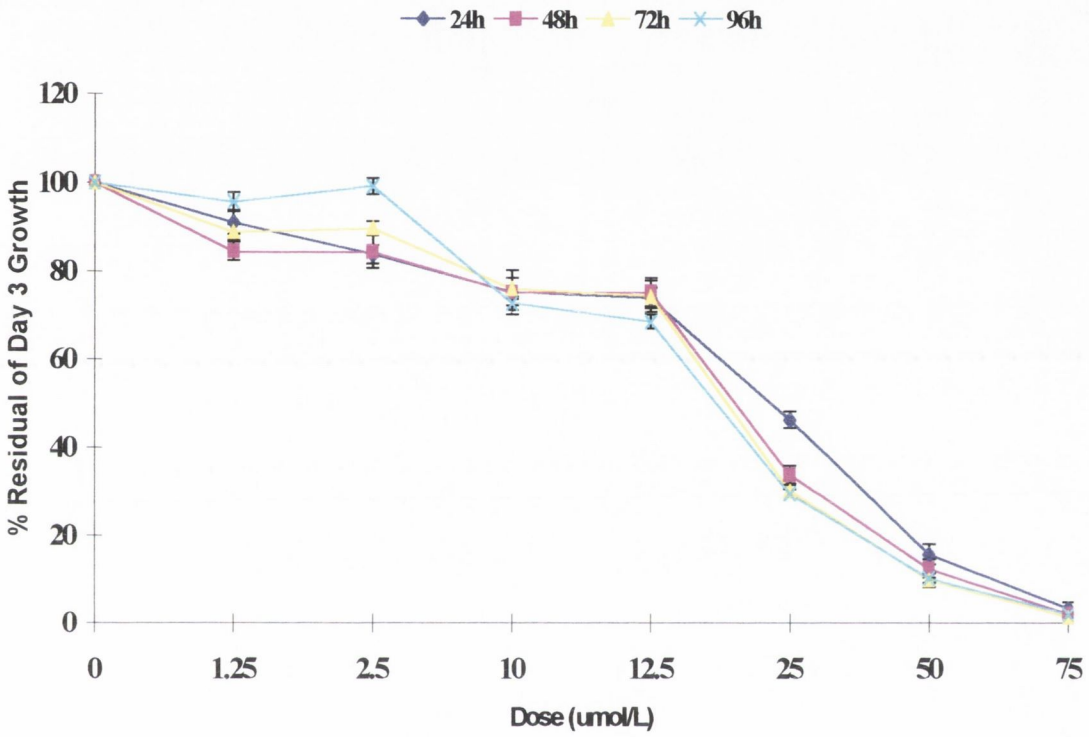


Figure 3.1 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with nonoxynol for 24h, 48h, 72h, 96h in serum - containing medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Table 3.3: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol for 24h and 48h in serum-free medium.

DOSE (µmol/L)	% RESIDUAL OF DAY 3 GROWTH	
	24h	**48h**
0	100	100
1.25	99.5 ± 3.9	99.2 ± 5.7
2.5	91.9 ± 7.0	86.8 ± 1.6
10	53.0 ± 8.4	45.9 ± 3.8
12.5	25.1 ± 2.8	20.3 ± 2.6
25	26.3 ± 4.5	25.8 ± 3.6
50	2.2 ± 1.1	5.0 ± 3.1

Table 3.3 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with nonoxynol in serum-free medium. The results are expressed as mean ± SEM for $n = 6$.

Figure 3.2: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol in serum-free medium.

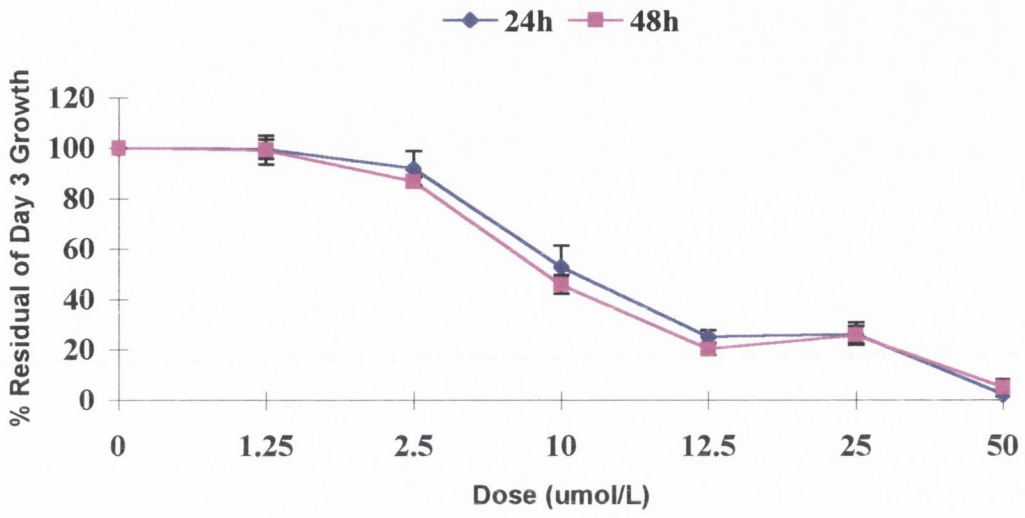
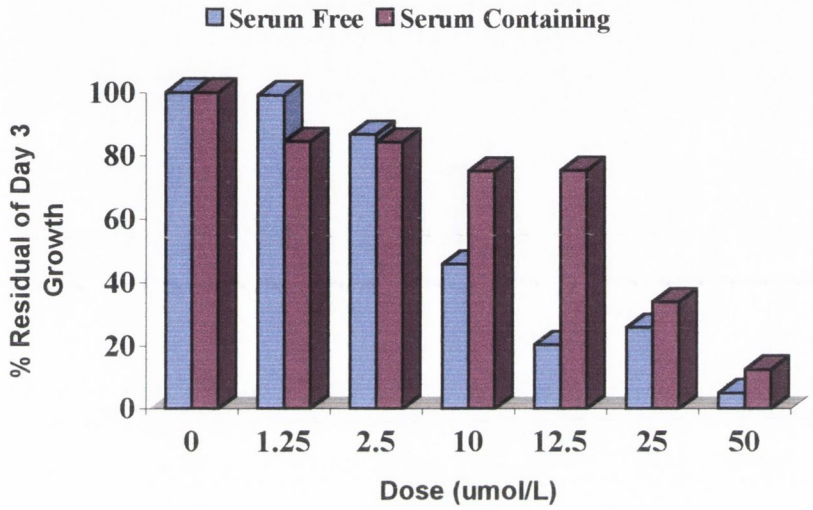


Figure 3.2 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with nonoxynol for 24h and 48h in serum - free medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Figure 3.3: The comparative % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol for 48h in serum-containing and serum-free medium.



Each figure represents the mean for n = 6 .

Table 3.4: Type of cells per epithelial cell culture exposed for 24h with nonoxynol in serum-containing medium.

Dose (μmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No.of Necrotic Epithelial Cells/Field	No. of Apoptotic Epithelial Cells /Field
Control	23.1 ± 1.7	7.7 ± 1.2	0	0
1.25	22.2 ± 1.8	6.9 ± 1.6	0.8 ± 0.5	0.2 ± 0.1
2.5	22.1 ± 1.9	5.5 ± 1.3	2.3 ± 1.9	0.6 ± 0.2
10	13.1 ± 3.6	4.5 ± 1.2	6.9 ± 2.5	0.3 ± 0.3
12.5	12.5 ± 3.6	4.0 ± 1.2	7 ± 2.5	0.4 ± 0.2
25	7.4 ± 4.2	1.9 ± 1.2	14.7 ± 4.6	1.0 ± 0.3
50	2.6 ± 1.8	1.0 ± 0.8	15.2 ± 2.7	0.8 ± 0.4

Each value represents the mean ± SEM for 5 (0.55mm^2) grids counted in n = 6 replicate cultures.

Table 3.5: Type of cells per epithelial cell culture exposed for 48h with nonoxynol in serum-containing medium.

Dose (μmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No.of Necrotic Epithelial Cells/Field	No. of Apoptotic Epithelial Cells /Field
Control	23.2 ± 2.2	8.4 ± 1.7	0.1 ± 0.0	0
1.25	23.1 ± 1.9	6.2 ± 1.1	0.2 ± 0.2	0.2 ± 0.2
2.5	20.9 ± 1.4	6.0 ± 0.7	1.0 ± 0.5	0.3 ± 0.5
10	12.5 ± 2.8	7.7 ± 1.3	4.7 ± 1.5	0.5 ± 0.4
12.5	15.6 ± 2.7	2.3 ± 1.0	7.1 ± 2.1	1.1 ± 0.9
25	6.4 ± 3.2	1.4 ± 0.9	15.2 ± 2.9	2 ± 0.8
50	3.2 ± 2.6	0.8 ± 0.5	13.9 ± 2.3	1.7 ± 0.4

Each value represents the mean ± SEM for 5 ($0.55mm^2$) grids counted in n = 6 replicate cultures.

Figure 3.4: The % cell type contained in rainbow trout primary epithelial cell cultures exposed to nonoxynol for 24h in serum-containing medium.

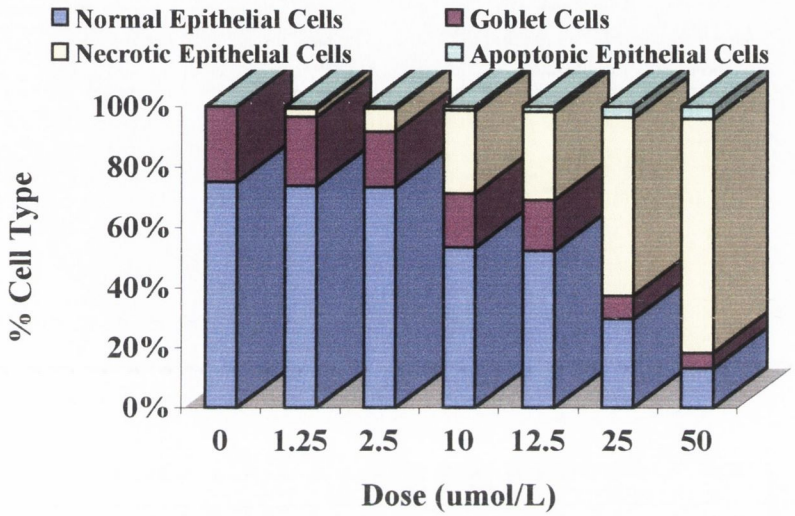


Figure 3.4 shows the % cell type present in rainbow trout primary epithelial cell cultures post exposure to nonoxynol for 24h in serum - containing medium. Each data set represents the % cell type contained in 5 ($0.55mm^2$) grids counted in n = 6 replicate cultures.

Figure 3.5: The % cell type contained in rainbow trout primary epithelial cell cultures exposed to nonoxynol for 48h in serum-containing medium.

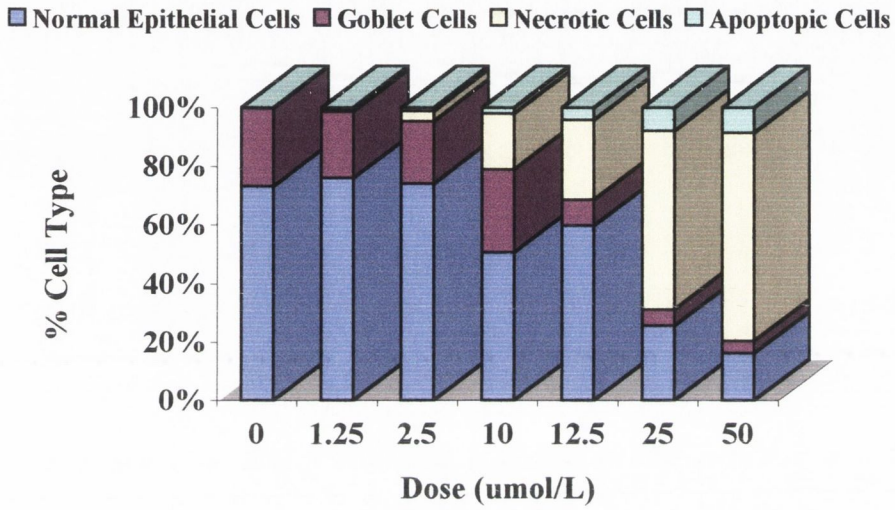


Figure 3.5 shows the % cell type present in rainbow trout primary epithelial cell cultures post exposure to nonoxynol for 48h in serum - containing medium. Each data set represents the % cell type contained in 5 (0.55mm^2) grids counted in n = 6 replicate cultures.

3.3.2 PROCHLORAZ

Table 3.6: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to prochloraz for 24h, 48h, 72h, 96h in serum-containing medium.

DOSE(µmol/l)	% RESIDUAL OF DAY 3 GROWTH			
	24h	**48h**	**72h**	**96h**
0	100	100	100	100
1	98.9 ± 5.2	86.6 ± 4.3	96.5 ± 4.4	71.4 ± 1.5
10	90.5 ± 6.9	91.3 ± 2.8	87.4 ± 4.4	73.3 ± 1.6
25	72.6 ± 2.7	50.5 ± 4.7	63.7 ± 3.9	54.9 ± 1.9
50	31.1 ± 3.7	24.3 ± 2.0	15.3 ± 1.5	13.5 ± 1.8
75	7.9 ± 1.5	8.9 ± 2.2	4.0 ± 1.1	4.5 ± 1.0

Table 3.6 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with prochloraz in serum-containing medium. The results are expressed as mean ± SEM for n = 6.

Figure 3.6: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to prochloraz in serum-containing medium.

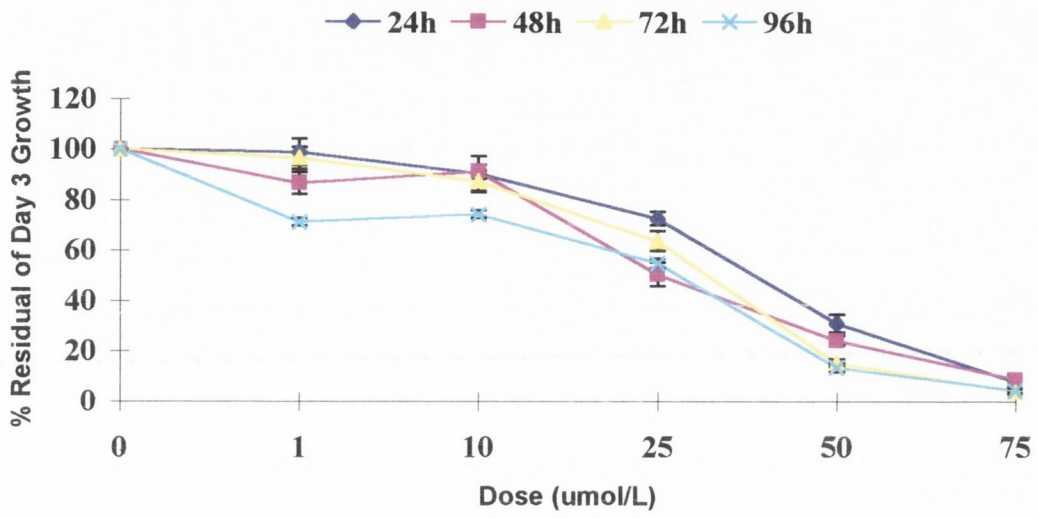


Figure 3.6 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with prochloraz for 24h, 48h, 72h, 96h in serum - containing medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Table 3.7: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to prochloraz for 24h and 48h in serum-free medium.

DOSE(µmol/L)	% RESIDUAL OF DAY 3 GROWTH	
	24h	**48h**
0	100	100
1	96.0 ± 3.7	97.9 ± 2.6
10	93.9 ± 3.5	87.1 ± 1.8
25	44.8 ± 2.1	31.8 ± 3.3
50	10.2 ± 0.7	5.0 ± 1.1
75	0.6 ± 0.5	0.4 ± 0.4

Table 3.7 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with prochloraz in serum-free medium. The results are expressed as mean ± SEM for n = 6.

Figure 3.7: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to prochloraz in serum-free medium.

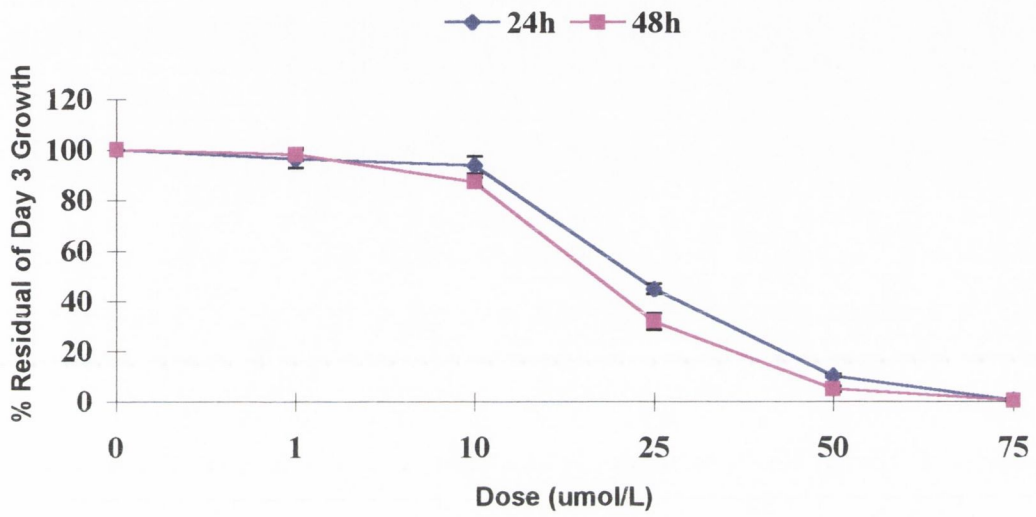
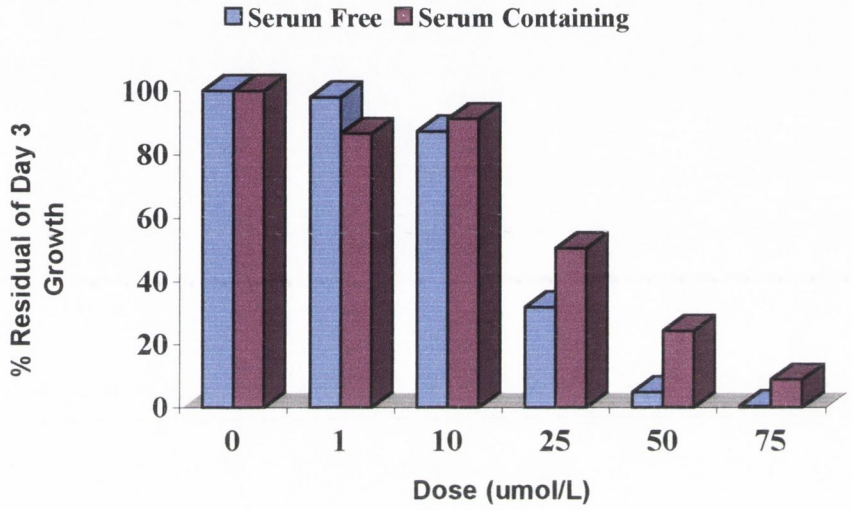


Figure 3.7 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with prochloraz for 24h and 48h in serum - free medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Figure 3.8: The comparative % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to prochloraz for 48h in serum-containing and serum-free medium.



Each figure represents the mean for n = 6 .

Table 3.8: Type of cells per cell culture post 48h exposure to prochloraz

Dose (μmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No.of Necrotic Epithelial Cells/Field	No. of Apoptotic Epithelial Cells /Field
Control	24.5 ± 2.1	7.0 ± 1.7	0.1 ± 0.1	0
1	24.8 ± 1.7	8.2 ± 1.6	0.1 ± 0.1	0
10	21.5 ± 1.6	5.9 ± 0.8	1.4 ± 0.6	0.5 ± 0.3
25	12.5 ± 2.9	5.8 ± 1.1	5.1 ± 1.4	0.6 ± 0.3
50	5.8 ± 2.7	1.3 ± 1.4	11.5 ± 2.4	2.1 ± 0.7
75	3.1 ± 1.3	0.9 ± 0.5	12.7 ± 2.4	1.5 ± 0.3

Each value represents the mean ± SEM for 5 (0.55mm) grids counted in n = 6 replicate cultures.

Figure 3.9: The % cell type contained in rainbow trout primary epithelial cell cultures exposed to prochloraz for 48h in serum-containing media.

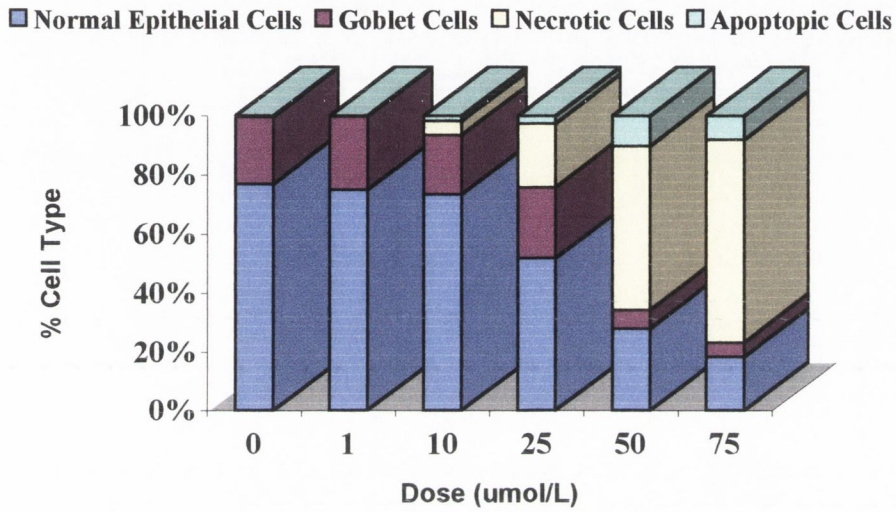


Figure 3.9 shows the % cell type present in rainbow trout primary epithelial cell cultures post exposure to prochloraz for 48h in serum - containing medium. Each data set represents the % cell type contained in 5 ($0.55mm^2$) grids counted in n = 6 replicate cultures.

3.3.3 COPPER

Table 3.9: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to copper for 24h, 48h, 72h, 96h in serum-containing medium.

DOSE(µmol/l)	% RESIDUAL OF DAY 3 GROWTH			
	24h	**48h**	**72h**	**96h**
0	100	100	100	100
1	101.1 ± 2.7	101.4 ± 3.4	101.4 ± 1.5	97.1 ± 1.7
10	102.3 ± 1.8	108.1 ± 2.1	101.1 ± 0.8	99.0 ± 1.7
50	96.8 ± 2.5	101.9 ± 2.8	100.7 ± 0.9	98.5 ± 1.7
100	93.6 ± 3.4	107.7 ± 2.6	99.0 ± 0.6	96.8 ± 1.7
500	35.0 ± 4.1	38.2 ± 5.2	34.0 ± 0.9	33.5 ± 1.5
1000	10.8 ± 1.5	8.0 ± 1.3	8.7 ± 0.2	6.4 ± 0.8

Table 3.9 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with copper in serum-containing media. The results are expressed as mean ± SEM for n = 6.

Figure 3.10: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to copper in serum-containing medium.

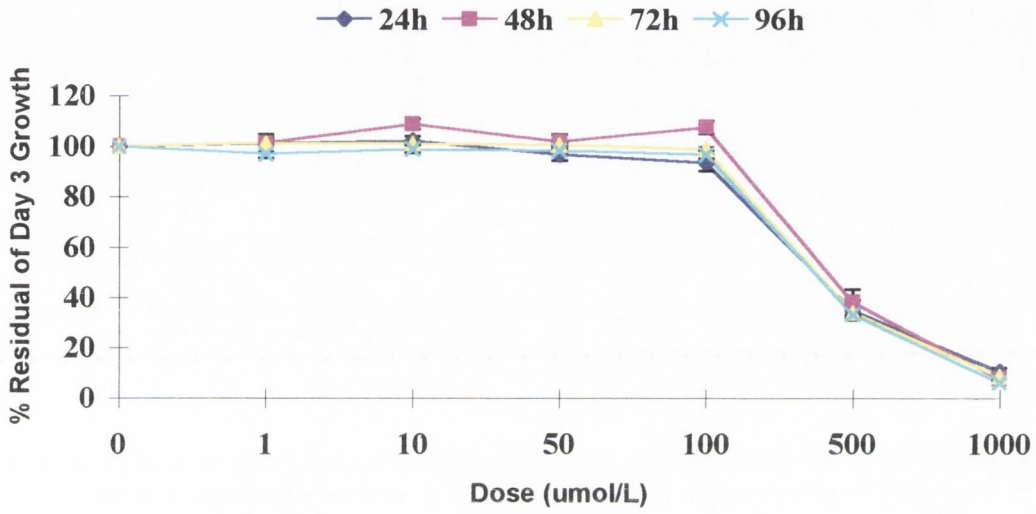


Figure 3.10 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with copper for 24h, 48h, 72h, 96h in serum - containing medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Table 3.10: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to copper for 24h, and 48h in serum-free medium.

DOSE(µmol/L)	% RESIDUAL OF DAY 3 GROWTH	
	24h	**48h**
0	100	100
1	99.4 ± 5.6	101.9 ± 2.9
10	95.9 ± 2.4	98.6 ± 4.6
50	85.5 ± 4.5	92.3 ± 2.8
100	92.4 ± 5.4	85.2 ± 5.6
500	77.6 ± 3.6	67.6 ± 4.5
1000	10.7 ± 1.2	10.3 ± 1.2

Table 3.10 shows the percentage residual of day 3 growth of rainbow trout primary epithelial cell culture systems post treatment with copper in serum-free media. The results are expressed as mean ± SEM for n = 6.

Figure 3.11: The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to copper in serum-free media.

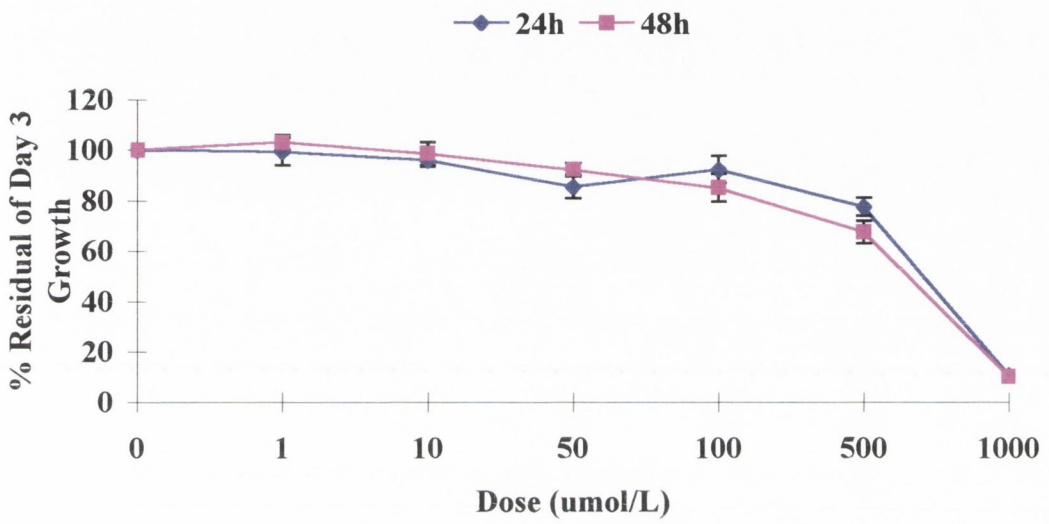
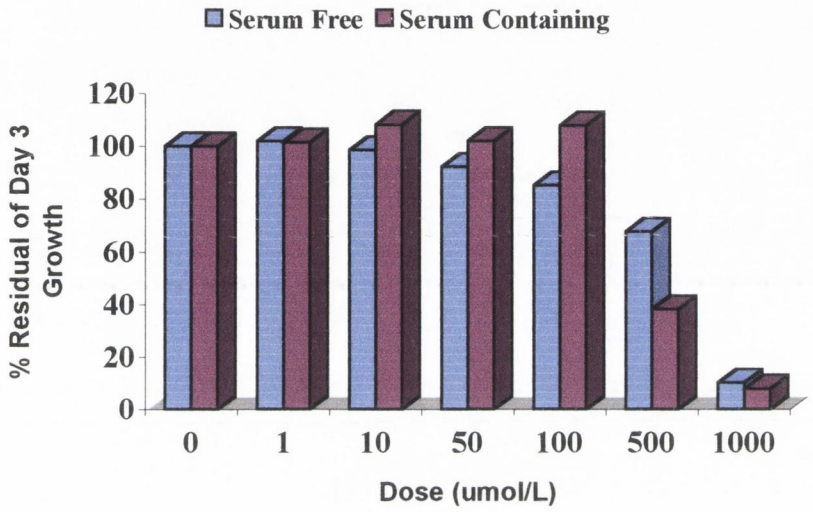


Figure 3.11 shows the % residual of day 3 growth of rainbow trout primary cell culture systems post treatment with copper for 24h and 48h in serum - free medium. Each figure represents the mean ± SEM for n = 6. The values are base on the overall area of the cell culture inclusive of both epithelial cells and goblet cells.

Figure 3.12: The comparative % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to copper for 48h in serum-containing and serum-free media.



Each figure represents the mean for n = 6 .

Table 3.11: Type of cells per culture post 48h exposure to copper

Dose (μmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No.of Necrotic Epithelial Cells/Field	No. of Apoptotic Epithelial Cells /Field
Control	23.2 ± 1.3	7.0 ± 1.7	0.1 ± 0.1	0
10	23.2.± 1.9	7.2 ± 1.2	0.1 ± 0.1	0
50	23.1 ± 1.4	6.2 ± 1.2	0.2 ± 0.1	0
100	20.2 ± 1.1	6.7 ± 1.2	0.2 ± 0.1	0.1 ± 0.1
500	5.9 ± 1.7	2.1 ± 1.4	11.6 ± 2.1	3.2 ± 0.6
1000	1.7 ± 1.4	1.1 ± 0.6	12.6 ± 2.8	5.8 ± 0.4

Each value represents the mean ± SEM for 5 (0.55mm) grids counted in n = 6 replicate cultures.

Figure 3.13: The % cell type contained in rainbow trout primary epithelial cell cultures exposed to copper for 48h in serum containing media.

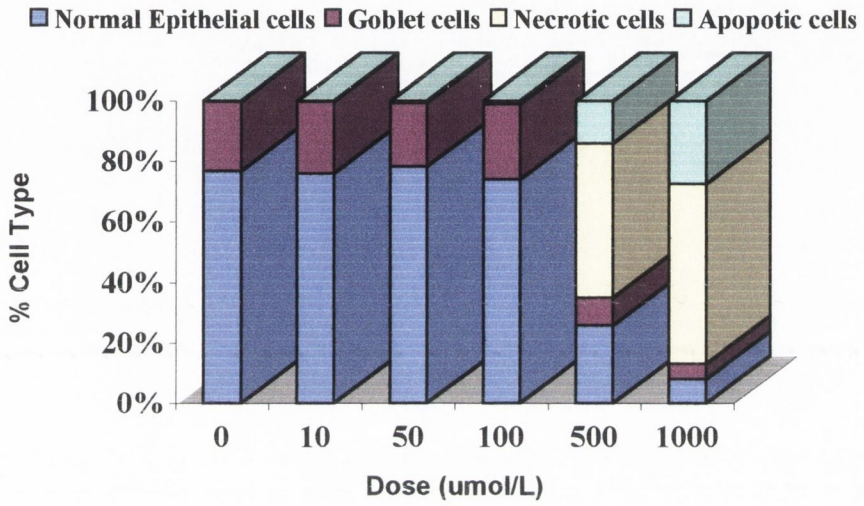


Figure 3.13 shows the % cell type present in rainbow trout primary epithelial cell cultures post exposure to copper for 48h in serum - containing medium. Each data set represents the % cell type contained in 5 (0.55mm^2) grids counted in n = 6 replicate cultures.

3.3.1 Nonoxynol

PRIMARY CULTURE GROWTH DATA

Serum containing medium (Table 3.2 and Fig 3.1)

Nonoxynol was found to kill 50% of the cells at a dose of between 12.5μmol/l and 25μmol/l after 24 hours exposure. Figure 3.1 shows a statistically insignificant ($p<0.05$) increase in cell death was observed over the next 72 hours, the concentration required to kill 50% of the cells was found to be in the same range for 48h, 72h and 96h exposure. However a significant decrease ($P<0.0001$) was observed between the growth of the primary cultures exposed to 25μmol/l for 24h and 96h. The controls were found to exhibit a % residual of day 3 growth of 94.7 ± 1.7, 98.3 ± 1.3, 98.2 ± 1.5 and 97.3 ± 1.5 for 24h, 48h, 72h and 96h exposure periods respectively. Nonoxynol tested on the primary cell system was found to kill over 95% of the cells at 75μmol/l for all exposure times. The threshold level of response, the level of exposure at which a significant decrease in growth begins to occur, is 12.5μmol/l for all exposure times. The residual growth values are based on the overall area of the culture inclusive of both epithelial cells and goblet cells.

Serum-free medium (Table 3.3 and Figure 3.2)

The primary cultures were found to exhibit a significantly different response to Nonoxynol when exposed in serum free media (Fig. 3.3). The dose of the pollutant required to kill 50% of the cells was found to be approximately 10μmol/l for both 24h and 48h exposures. The threshold level of response was found to be 2.5μmol/l for both

exposure periods. The controls were found to exhibit a significantly less % growth ($p<0.0001$) than that of the cultures exposed to the test substance in serum-containing medium. The % residual of day 3 growth of the controls in serum-free media was 76.3 ± 6.3 and 77.5 ± 5.7 for 24h and 48h exposures, respectively.

PRIMARY CULTURE CELL DEATH TYPE (Serum-containing medium)

(Tables 3.4 & 3.5, Figs. 3.4 & 3.5)

The average number of cells per field in the control cultures was 31.3 ± 1.2. The amount of cells per field decreased as the chemical treatment dose increased. The number of cells per field in the cultures treated with 50μmol/l nonoxynol was 16.6 ± 1.8. Tables 3.4 and 3.5 show that an increase in epithelial necrotic cells was observed with increasing nonoxynol exposure in primary cell cultures. Epithelial cells located on the outside edge of the culture underwent necrosis primarily at lower doses. A significant increase in those cells undergoing necrosis was observed at 10μmol/l after 24h (0 to 6.9 ± 2.5 cells/field) and 48h (0.1 to 4.7 ± 1.5 cells/field) exposure to the pollutant. Only a slight increase in apoptopic cell death was observed with increasing nonoxynol exposure (0 to 1.7 ± 0.4 cells/field after 48h exposure to 50μl nonoxynol).

RELATIVE EFFECT ON MUCUS CELLS VERSUS EPITHELIAL CELLS (Serum-containing medium)

(Tables 3.4 & 3.5, Figs. 3.4 & 3.5)

A decrease in the number of goblet cells per field from 7.7 ± 1.2 to 1.0 ± 0.8 (24h), 8.4 ± 1.7 to 0. 8± 0.5 (48h) was also observed as nonoxynol dose increased (Tables 3.4 and 3.5). This decrease was observed uniformly across the explant. The threshold level

of this decrease was found to be 12.5μmol/l for 24h exposure to nonoxynol. The threshold level of this decrease after 48h exposure was slightly lower (10μmol/l). The goblet cells release their secretory contents onto the culture surface. Emptying of the goblet mucous cells results in their loss and consequent decrease in their density. The percentage of epithelial cells, per field, in the cultures was found to increase by 19.9% for 24h exposure and 22.4% for 48h exposure as the dose of pollutant increased.

3.3.2 Prochloraz

PRIMARY CULTURE GROWTH DATA

Serum-containing medium (Table 3.6 and Fig 3.6)

Prochloraz was found to kill 50% of the cells at a dose of between 25μmol/l and 30μmol/l after 24 hours exposure. Figure 3.6 shows a slightly significant increase in cell death was observed over the next 72 hours, the concentration required to kill 50% of the cells was found to be in the same range for 48h, 72h and 96h exposure. However, a significant decrease ($p<0.0001$) was observed between the growth of the primary cultures exposed to 50μmol/l for 24h and 96h. The controls were found to exhibit a % residual of day 3 growth of 73.7 ± 2.4, 79.7 ± 1.3, 82.3 ± 3.2 and 93.7 ± 1.5 for 24h, 48h, 72h and 96h exposure periods, respectively. Prochloraz tested on the primary cell system was found to have an acute lethal dose of 75μmol/l for all exposure times. The threshold level of response, the level of exposure at which a significant decrease in growth begins to occur, is 10μmol/l for 24h, 48h and 72h exposure times and 1μmol/l for 96h exposure. The residual growth values are based on the overall

area of the culture inclusive of both epithelial cells and goblet cells.

Serum-free medium (Table 3.7 and Fig. 3.7)

The primary cultures were found to exhibit a significantly different response to prochloraz when exposed in serum free media (Fig. 3.8). The dose of the pollutant required to kill 50% of the cells was found to be between 10μmol/l and 25μmol/l for both 24h and 48h exposures. The threshold level of response was found to be 10μmol/l for both exposure periods. The controls were found to exhibit a significantly less % growth ($p<0.0005$) than that of the cultures exposed to the test substance in serum-containing medium. The % residual of day 3 growth of the controls in serum-free medium was 68.2 ± 1.0 and 73.8 ± 1.2 for 24h and 48h exposures, respectively.

PRIMARY CULTURE CELL DEATH TYPE (Serum-containing medium)

(Table 3.8 and Fig. 3.9)

The average number of cells per field in the control cultures was 31.6 ± 1.2. The amount of cells per field decreased as the chemical treatment dose increased. The number of cells per field in the cultures treated with 75μmol/l prochloraz was 18.2 ± 0.4. Tables 3.8 shows that an increase in epithelial necrotic cells was observed with increasing prochloraz exposure in primary cell cultures. Epithelial cells located on the outside edge of the culture underwent necrosis primarily at lower doses. A significant increase ($p<0.0001$) in those cells undergoing necrosis was observed at 25μmol/l (0.1 to 5.1 ± 1.4 cells/field) exposure to the pollutant. Only a slight increase in apoptopic

cell death was observed with increasing nonoxynol exposure (0 to 1.5 ± 0.3 cells/field after 48h exposure to 75µl prochloraz).

RELATIVE EFFECT ON MUCUS CELLS VERSUS EPITHELIAL CELLS (Serum-containing medium)

(Table 3.8 and Fig. 3.9)

A decrease in the number of goblet cells per field from 7.0 ± 1.7 to 0.9 ± 0.5) was also observed as prochloraz dose increased (Table 3.8). This decrease was observed uniformly across the explant. The threshold level of this decrease was found to be 25µmol/l. As observed in the cultures exposed to nonoxynol the goblet cells release their secretory contents onto the culture surface. Emptying of the goblet mucous cells results in their loss and consequent decrease in their density. The percentage of epithelial cells, per field, in the cultures was found to increase by 17.21% for 48h exposure as the dose of pollutant increased.

3.3.3 Copper

PRIMARY CULTURE GROWTH DATA

Serum-containing medium (Table 3.9 and Fig 3.10)

Copper was found to kill 50% of the cells at a dose of about 400µmol/l for all exposure duration's. The controls were found to exhibit a % residual of day 3 growth of 101.4 ± 1.4, 101.2 ± 5.2, 98.4 ± 0.6 and 101.5 ± 1.6 for 24h, 48h, 72h and 96h exposure periods, respectively. Copper tested on the primary cell system was found to have an acute

lethal dose of greater than 1000μmol/l for all exposure times. The threshold level of response, the level of exposure at which a significant decrease ($p<0.0001$) in survival begins to occur, is 100μmol/l for all exposure periods. The residual growth values are based on the overall area of the culture inclusive of both epithelial cells and goblet cells.

Serum-free medium (Table 3.10 and Fig 3.11)

The primary cultures were found to exhibit a significantly different response to copper when exposed in serum free media (Fig. 3.12). The dose of the pollutant required to kill 50% of the cells was found to be between 500μmol/l and 1000μmol/l for both 24h and 48h exposures. The threshold level of response was found to be 100μmol/l for both exposure periods. The controls of those cultures exposed for 24h were found to exhibit a slightly significantly less % residual of day 3 growth [$p<0.005$ for 24h, $p=0.9$ (not sig.) for 48h] than that of the cultures exposed to the test substance in serum-containing medium. The % survival of the controls in serum-free medium was 84.6 ± 2.1 and 100.8 ± 2.4 for 24h and 48h exposures respectively.

PRIMARY CULTURE CELL DEATH TYPE (Serum-containing medium)

(Table 3.11 and Fig. 3.13)

The average number of cells per field in the control cultures was 30.3 ± 0.8. The amount of cells per field decreased as the chemical treatment dose increased. The number of cells per field in the cultures treated with 1000μmol/l copper was 21.2 ± 0.4. Tables 3.11 shows that an increase in epithelial necrotic cells and apoptopic cells was observed with increasing copper exposure in primary cell cultures. Epithelial cells located on the outside edge of the culture underwent necrosis primarily at lower doses.

A significant increase ($p<0.0001$) in those cells undergoing necrosis was observed at 500μmol/l (0.1 to 12.6 ± 2.8 cells/field) exposure to the pollutant. A slightly less increase ($p<0.0001$) in apoptopic cell death was observed with increasing copper exposure (0 to 5.8 ± 0.4 cells/field after 48h exposure to 1000μl copper).

RELATIVE EFFECT ON MUCUS CELLS VERSUS EPITHELIAL CELLS (Serum-containing medium)

(Table 3.11 and Fig. 3.13)

A decrease in the number of goblet cells per field from 7.0 ± 1.7 to 1.1 ± 0.6) was also observed as copper dose increased (Table 3.11). This decrease was observed uniformly across the explant. The threshold level of this decrease was found to be 100μmol/l. As observed in the cultures exposed to the other test substances the goblet cells release their secretory contents onto the culture surface. The percentage of epithelial cells, per field, in the cultures was found to increase by 17.41% for 48h exposure as the dose of pollutant increased.

3.4 DISCUSSION

Rainbow trout primary epithelial cell cultures were found to exhibit a decreased sensitivity to all three pollutants tested compared with the cell lines studied in Chapter 2. A similar relationship was observed when primary rainbow trout skin cultures were treated with cadmium and nickel (McSweeney, 1998; Lyons Alacantara *et al.,* 1996). The toxicity of copper to the primary cell culture system was found to be approximately 10 fold less than that of the organic chemicals studied. Nonoxynol was found to be the most toxic chemical studied. Similar results were obtained by Leguen *et al.* (1998) with the three test substances on gill cells *in vitro*. The toxicity curves showed similar slopes to those obtained from the cell line experiments in Chapter 2 but with differing critical concentrations of the test substances. The method of drawing around the outgrowth area of the cell cultures prior to the addition of the chemical described in Section 3.2.2 takes into account that there is a great variability in the initial growth area of the explant culture and as a result the error bars of the toxicity curves are reduced to within acceptable limits. The initial area of the primary cultures was found to range from 15mm^2 to as high as 120mm^2. However by using the technique described it was demonstrated that the % change in area, as a result of exposure to a particular concentration of a test substance, was relatively consistent irrespective of the initial area of the primary cultures.

The influence of medium composition on toxicity testing using the rainbow trout primary skin epithalial cell culture system was studied by exposing the cultures to the test substances in both serum-free and serum-containing medium. An element which is not to be neglected in any bioassay system is the composition of the medium. The

process of changing the media to serum-free medium appeared to kill up to 20% of the control samples. Nonoxynol and prochloraz proved to be more toxic when tested in serum-free medium (Fig. 3.3 and Fig 3.7). A similar result was observed by Bertheussen *et al.* (1997) when testing the toxicity of the pesticides carbofuran, cypermethrin, lindane, glycophosphate and 2,4-D to the IE6 cell line. Lyons Alacantara *et al.* (1996) observed that both human primary urothelium cultures and fish skin primary epithelial cell cultures were more sensitive to cadmium in serum-free medium. Copper, in contrast, was found to be less toxic when added to the test cultures in serum-free medium compared with addition in serum-containing medium (Fig. 3.12). An important parameter of the cellular uptake and subsequent cytotoxicity of metal ions such as Cu^{++} is their binding to extracellular biological components and its resulting influence on their ability to interact with the cell membrane, enter the cell and exert intracellular cytotoxic effects. Whilst it appears that the serum components are preventing nonoxynol from entering the cell, the serum appears to be increasing the uptake of copper into the cell. Webb *et al.* (1972) suggested that nickel ions enter the cell after binding to low molecular weight serum components that actually serve to transport metallic ions into the cell. It appears that something similar may be occurring in the experiments carried out in this study.

As exposure levels to the three test substances increased the quantity of goblet cells decreased. Goblet cells produce mucus and mucus production is a well established defence mechanism of epithelial surfaces against pathogens and pollutants (Shepherd, 1994). The goblet cells release their secretory contents onto the culture surface forming a thick slimy protective coating in an attempt to prevent deleterious effects of the pollutant medium (Roy, 1988). It was observed that the goblet cells were decreasing in

number because they are exocytosing their contents. It was observed by Roy (1988) that an anionic detergent induced similar significant changes in the number and size of goblet mucous cells in the opercular epidermis. This study adds further evidence that mucus production occurs in the presence of an environmental pollutant resulting in a decrease in the quantity of goblet cells present in the skin culture. A decrease in goblet number and density in rainbow trout primary skin culture could be an important marker of toxicity of aquatic pollutants.

An increase in necrotic epithelial cells was observed with increasing dose exposure of nonoxynol and prochloraz. The number of apoptopic epithelial cells remained extremely low and constant with dose exposure. These results suggest that both nonoxynol and prochloraz kill rainbow trout skin epithelial by the process of necrosis. In a previous study a loss of protein mioties was observed from the club cells and epithelial cells of the opercular epidermis as a result of exposure to an anionic detergent (Roy, 1990). Rapid decreases in protein, RNA and DNA levels occur during necrosis (Schwartzman et al., 1993). Other organic and inorganic chemicals have been shown to induce similar responses in fish (Wester *et al.,* 1988; Dietrick *et al.*, 1989). Exposure to waterborne Hg has been demonstrated to induce cellular necrosis and increased mucus secretion by gill cells (Wobster, 1975; Lock *et al.,* 1981).

In contrast copper was found to kill rainbow trout primary skin epithelial by both necrosis and apoptosis in a ratio of approximately 2:1. Pelgrom (1995) observed that epithelial cellular responses of teleosts to Cu^{++} exposure include both increased necrosis and apoptosis of chloride cells and pavement cells. Carp exposed to 1.6μmol/l Cu^{++} for 7 days were observed to show degenerative (apoptopic and necrotic) pavement cells in the skin (Iger *et al.,* 1994). Other *in vivo* studies have shown that *tilapia* exposed to

Cu^{++} have a high incidence of apoptosis in the epidermis (Pilgrom, 1995). As stated in Chapter 1 copper shows a high specific binding to DNA producing DNA strand breaks in several biological systems (Sagripanti *et* al., 1991). Copper has been shown to act as a catalyst in the formation of reactive oxygen species (Chan *et al.,* 1982). Evidence has shown that oxygen radicals may have a role to play in the initiation of apoptosis (Buttke *et al.,* 1994).

The results of this study demonstrate that primary cell cultures are an alternative model for toxicity risk assessment of specific *in vivo* situations to immortalised cell lines. If cultured under the appropriate conditions the cells retain their full, *in vivo*, characteristics for up to 14 days thus allowing sufficient time for investigation of the immediate in vitro effects of toxicants. The cells in the outgrowth area have a high rate of mitotic activity and are actively dividing (Mothersill *et al.,* 1995). The toxicity curves show similar slopes to those obtained from cell lines but with different critical concentrations. However the composition of the media and the binding properties of the substance to be screened should be taken into account when planning toxicity tests using this cell system.

Other work carried out in our group with this system has shown that changes in glycogen deposits, stress proteins and oncogene expression could also be studied using this system (Lyons Alacantara *et al.,* 1998; Lyons Alacantara *et al.*, 1996; McSweeney, 1998). The use of short-term cytotoxicity assays for the initial screening of chemicals not only aids in establishing priorities for the selection of chemicals that should be tested further *in vivo*, but also decreases the time in which potential toxicants can be evaluated (Babich et al., 1987). The primary cell culture system used in this study is an extremely useful tool for such assays for the reasons previously stated. The primary

cell culture system could also be utilised for different areas of study. These include multiple assays/investigations on homologous cultures derived from in vivo exposure which may result a major step in the solving of the problem of comparing in vitro effects with that of in vivo. The cultures can also be infected with viruses and as such provide excellent opportunities for research into fish diseases.

3.5 CONCLUSION

The work stated in this chapter establishes the characteristics of the link between the exposure and inducible response of rainbow trout primary epithelial skin cell cultures exposed to aquatic pollutants. Nonoxynol was found to be the most toxic of the three test substances used, copper the least toxic. It was demonstrated that differences occur in the toxicity of test substances when tested in serum - containing media compared to serum - free media. Both nonoxynol and prochloraz were found to kill the cells by necrosis, copper by a combination of necrosis and apoptosis. Exposure to all three test substances was found to cause a decrease in the number of goblet mucus cells contained in the cell cultures. This reduction was found to be dose related. As a result mucus production may have potential for use as a cellular marker of exposure.

CHAPTER 4

THE DEVELOPMENT OF HEAT SHOCK PROTEIN INDUCTION AND MUCUS SECRETION IN RAINBOW TROUT PRIMARY EPITHELIAL SKIN CULTURES AS AN ACUTE *IN VITRO* TOXICITY TEST.

4.1 INTRODUCTION

4.1.1 Heat Shock Protein

A variety of cellular stresses, especially thermal, but also changes in pH, nutrient deprivation or exposure to toxic chemicals, induce the synthesis of a typical set of proteins, best defined as heat-shock proteins (hsps) (Wolffe *et al.,* 1984). The main groups are categorised by molecular weights and include the hsp70 and hsp60 families. Whereas hsp70s are predominantly cytoplasmic proteins but are also present in many cell compartments, the inducible form of hsp60 is localised in the mitochondrial matrix (Werner *et al.,* 1997). Heat shock proteins are present in all organisms at normal temperatures and play vital roles in maintaining normal cellular homeostasis (Subjeck *et al.,* 1986). Hsps induction is characterised by a rapid reprogramming of the cell's protein manufacturing so the production of the stress proteins is dramatically increased. This is accompanied by an abrupt cessation of much of the cells normal RNA and protein synthesis and a reorganisation of cytoskeletal elements (Wolffe *et al.,* 1984).

Available information indicates that heat shock proteins confer protection from environmentally induced cellular damage (stress response) (Werner *et al.*, 1997; Parsell *et al.,* 1994; Lindquist *et al.*, 1988) and accumulation of these proteins in organisms has been linked to the intensity of the stress. The synthesis of heat shock proteins appears to be part of a general stress response which is universally conserved in organisms from all phylogenic kingdoms (Williams *et al.,* 1996). The gene encoding hsp70 in humans is 73% homologous to the hsp70 gene in *Drosophila* (Lindquist *et al.,* 1988). Some researchers propose that induction and subsequent

accumulation of heat shock proteins, specifically heat shock protein70 (hsp70), may be useful in environmental monitoring and toxicological screening (DiDomonico *et al.*, 1982; Bournias *et al.*, 1983; Hightowe *et al.,* 1985; Mizzen *et al.*, 1988; Sanders *et al.*, 1990; Ryan *et al.*, 1994; Werner *et al.*, 1997) Williams *et al.*, (1996) found that hsp70 levels were significantly increased in the gills of juvenile rainbow trout, *Onchorynchus mykiss*, exposed to metals, in both water and food. In the same study a trend, though not significant , was found toward increased accumulation of hsc/hsp70 in the gills of adult rainbow trout was observed. Werner *et al.* (1997) demonstrated that induction of heat shock protein is both compound and species specific.

Under some conditions in ectotherms such as fish, heat shock proteins are constitutively present in large amounts (Sanders *et al.,* 1992; Yu *et al.,* 1994). The stress protein response has been documented in fish (Ryan *et al.,* 1994). In a study to test whether broad spectrum antibodies could be developed as biomarkers useful in detecting the combined environmental stresses in a wide variety of organisms it was found that the broad spectrum rabbit polyclonal antibody developed against the most conserved epitope of HSP70 reacted with HSP70 or its cognate proteins in fathead minnows and chinook salmon (Dunlap *et al.,* 1997).

The aim of the study presented in this chapter was to investigate the use of anti-rabbit heat shock protein polyclonal antibody on rainbow trout primary skin epithelial cell cultures as a possible acute toxicity test using a common immunocytochemical staining method.

4.1.2 PERIODIC ACID SCHIFFS STAIN FOR MUCUS

As stated in Chapter 3, isolated cells that maintain the essential traits of the *in vivo* state during culture *in vitro* provide an excellent experimental approach to establish diagnostic markers such as early indicators of effect. Rainbow trout primary epithelial cell cultures contain both epithelial cells and goblet mucus cells. Following stress or infection, mucous secretion may be increased from the epithelial layer (Blackstock *et al.,* 1982; Burton *et al.,* 1990; Iger *et al.,* 1988; Benedetti *et al.,* 1989; Perry *et al.,* 1989; Wendelar Bonga *et al.*, 1990; Iger *et al.,* 1992; Iger *et al.,* 1993; Wiipfli *et al.,* 1994). It was discovered in Chapter 3 that the number of goblet cells contained in the primary rainbow trout epithelial cell cultures decreased when the cultures were treated *in vitro* with increasing doses of chemical pollutants. As a result, this study aimed to investigate whether the mucus cells are secreting their contents onto the cell surface in response to chemical exposure using the PAS stain for mucus.

4.2 MATERIALS & METHODS

4.2.1

Rainbow trout primary epithelial cell cultures were set up as previously described (Section 3.2). All skin sections were taken from the same area of the fish, just below the dorsal fin. After 4 days growth the cultures were treated with the following concentrations of the three test substances; control, threshold level, 50% and 80% cell death. These concentrations were determined as described in Chapter 3. After 48h the culture media was removed from the flask. The cultures were then washed in P.B.S. and fixed in 10% formalin. Each experiment was completed in triplicate.

4.2.2 IMMUNOCYTOCHEMICAL ASSAY

Antibody employed for Immunocytochemical Analysis

Heat Shock Protein 70: expression of HSP70 was detected using a rabbit polyclonal antibody (DAKO, Bucks., UK). The antigen used for immunisation was isolated from *E. coli.* The antibody cross-reacts with the two major human heat shock proteins of 72 and 73 kDa. The positive control used for the HSP70 antibody was liver sections which are HSP70 positive.

Method

Immunostaining was performed using the Vectastain ABC kit which is based on the standard indirect immunoperoxidase technique where the cells are blocked with peroxide and a suitable serum before being exposed to the primary antibody. 3,3-Diaminobenzidine (DAB) was used as the chromogen with Harris's haematoxylin employed for counterstaining. The appropriate positive controls (as described above) were included in all experiments and for, negative controls, the cells under investigation were used without any primary antibody. The following procedure was followed:

The culture flasks were cracked open with a pair of pliers in such a fashion as to leave the base of the flask, with the cells attached, intact.

The cultures were washed in P.B.S. for 5 min

To block any endogenous peroxidase activity the cultures were treated with 3% H_2O_2 in P.B.S. for 5 minutes. after which the cultures were washed in buffer for 5 min.

The cultures were then incubated with normal diluted serum (Vectastain ABC kit) for 20 min. Any excess serum was blotted from the cultures.

The relevant primary antibody appropriately diluted in P.B.S. was added to the cultures for 60 min. Please see below for dilution.

The cultures were washed in buffer for 10 min.

The cultures were incubated for 30min with diluted biotinylated antibody solution (Vectastain ABC kit) and then washed for 10 min in buffer.

The cultures were then incubated for 45 min with an avidin DH:biotinylated horseradish peroxidase H complex (Vectastain ABC kit) and washed in buffer for a further 10 min.

The cultures were treated with DAB, the peroxidase substrate until the brown staining becomes apparent (2-7 min).

The cultures were then washed in tap water and counterstained with Harris's haematoxlylin for 1 minute.

The cultures were washed in hot water to blue the haematoxylin and mounted in glycergel.

A positive reaction was indicated by a brown staining.

Heat shock protein is expressed in all cells. It was necessary to carry out a dilution range test of the antibody to determine what dilution could be used that enables the user to determine that any positivity is due to the chemical exposure alone. The recommended dilution for the rabbit polyclonal antibody on mammalian cells is 1:250 (Dako). Untreated cultures were stained with the following dilution's of the primary antibody 1 : 100; 1 : 200; 1 : 250; 1 : 300; 1 : 400; 1 : 500. It was found that all cells stained intensely positive (dark brown) for the first three dilution's. The cells stained light brown for 1: 300 dilution. The cells stained negative for 1:400 dilution and 1:500 dilution. It was decided as a result to use the 1:400 dilution in the experiment.

The PAS (Periodic-Acid Schiff) stain For glycogen deposits

Cultures were fixed in 80% alcohol at 4°C for 30 minutes for glycogen identification. The samples were rinsed repeatedly in distilled water and covered in 1% periodic acid for 5 minutes.

The cultures were then rinsed in distilled water and covered in Schiffs reagent (Sigma) for 10 min.

The cultures were rinsed in several changes of distilled water then placed in running tap water for 10 minutes to aid colour development.

The cell nuclei were counterstained using Mayers haematoxylin for 1 min and washed in hot flowing running tap water for 5 minutes to enhance blue staining.

Coverslips were then attached to stained preparations with Kaiser's glycerol jelly mounting media.

The neutral mucins and glycogen stained magenta pink, the nuclei blue.

Scoring of Immunocytochemical Results

The system employed to score the immunocytochemically stained cultures was an arbitrary one. Cell fields transecting the cultures were analysed using a Leica image analyser. Transects were used to take into account any possible differences in the topographical distribution of the stain. The cells were scored as negative (see Plate 4.1), weakly positive (see Plate 4.2) or intensely positive (see Plate 4.3) depending on the degree of positivity in relation to the negative control. The number of weakly positive cells and intensely positive cells was expressed as a % of the total number of cells.

Scoring of PAS Stain for mucus results

The epithelial cell surfaces were classed as being negative, weakly positive and intensely positive to mucus (see Plate 4.4 and Plate 4.5). Cells were classed as weakly positive and intensely positive to mucus if they were stained light pink and dark pink, respectively. Negative cells stained blue. The mucous cells contained in the cell cultures reacted with intense positivity to PAS staining. The mucous cells were not scored in the results.

Plate 4.1 Rainbow trout primary epithelial cell cultures stained negative for HSP70 (X40 Magnification)

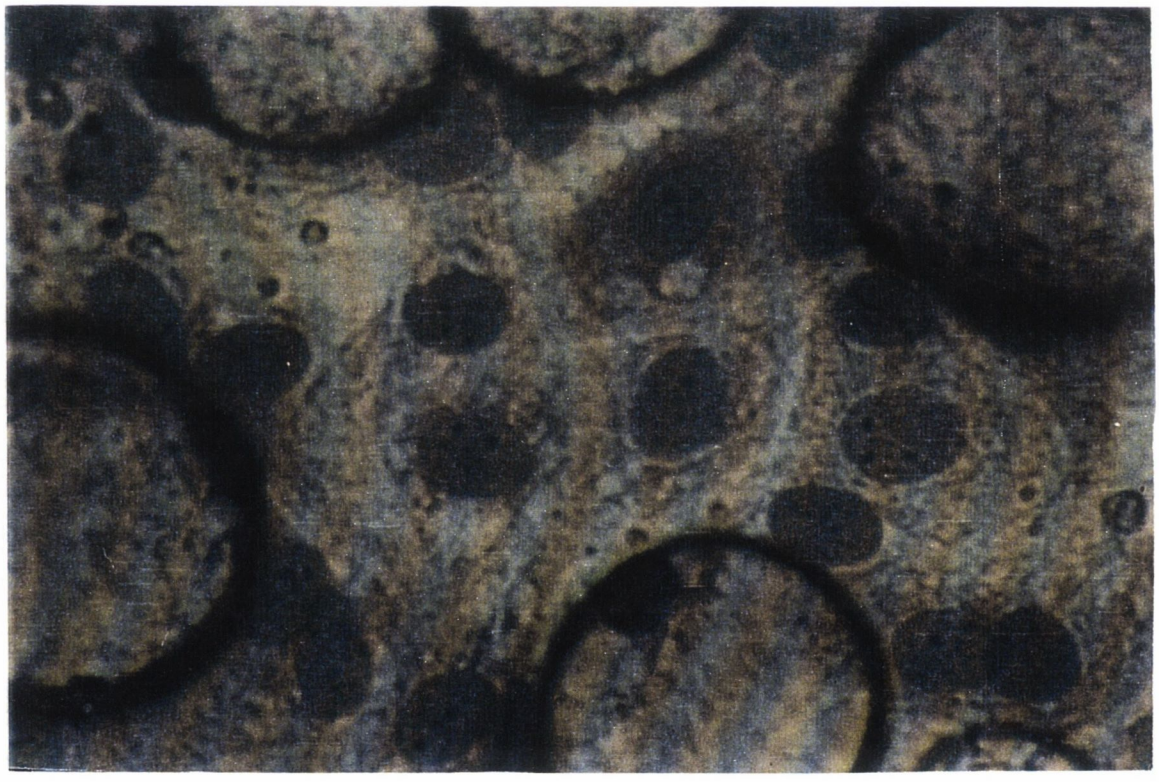


Plate 4.2: Rainbow trout primary epithelial cell cultures stained weakly positive for HSP70 (X10 Magnification).

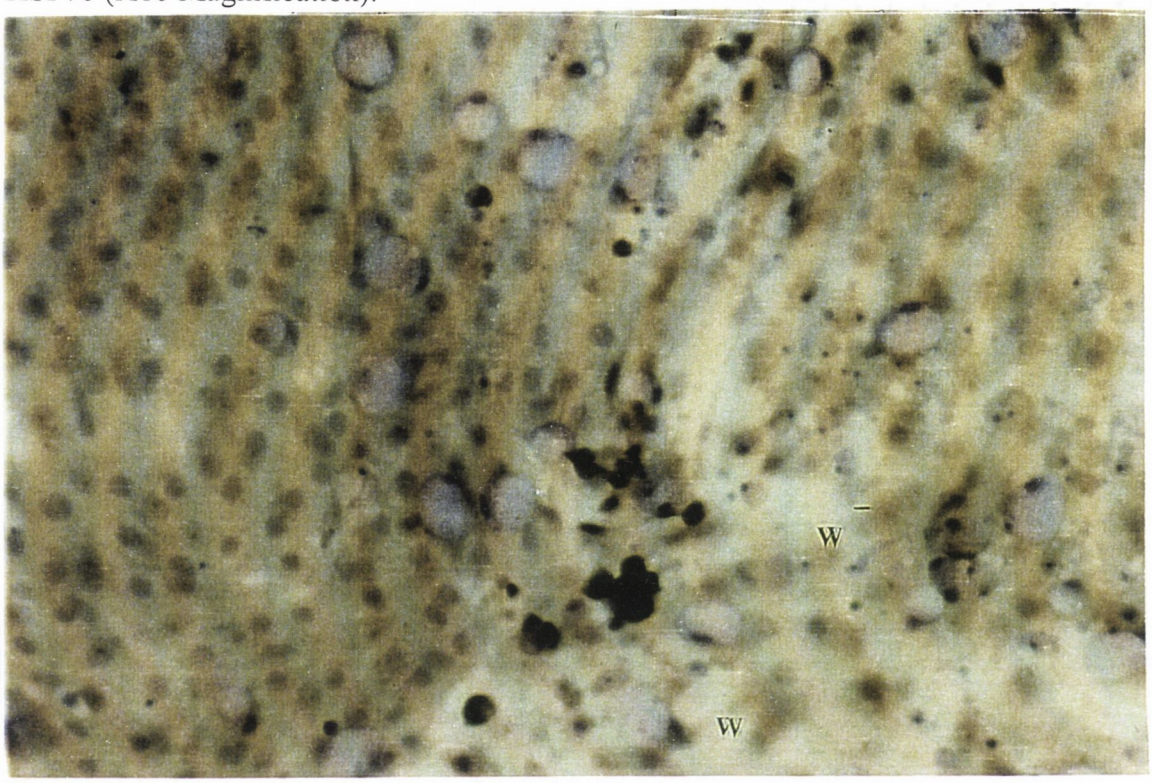


Plate 4.3: Rainbow trout primary epithelial cell cultures stained intensely positive for HSP70 (X20 Magnification).

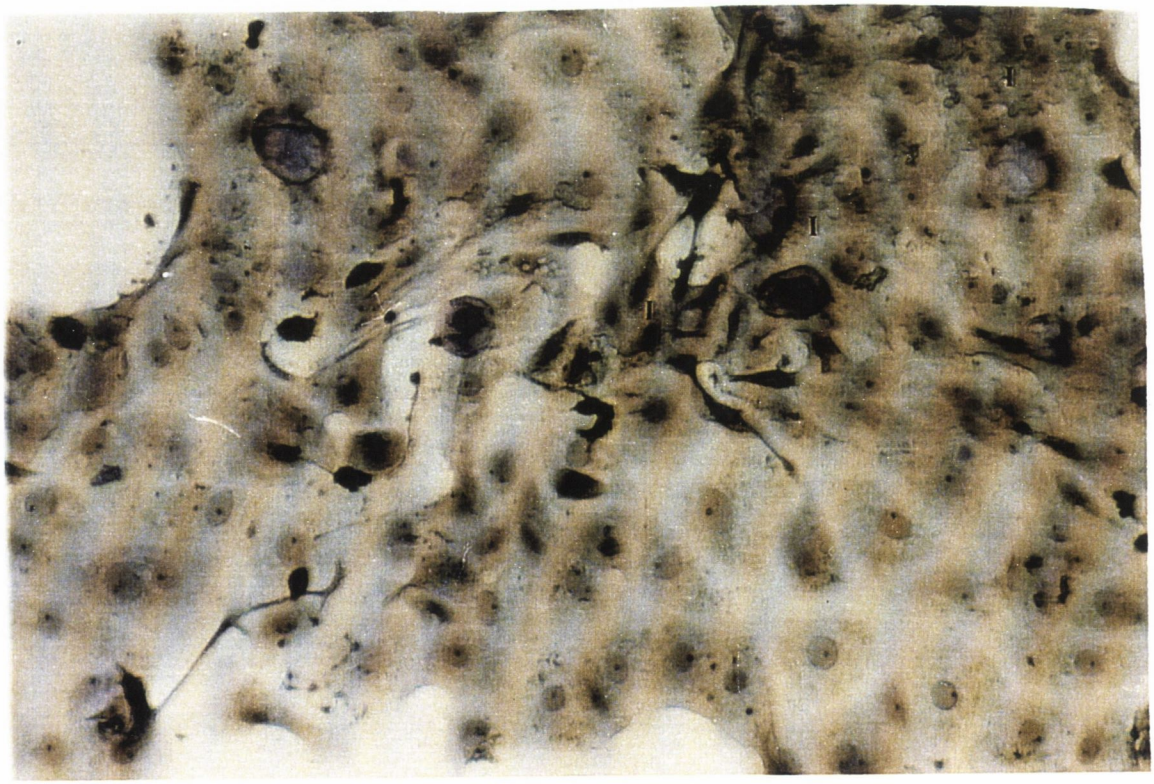


Plate 4.4 Rainbow trout epithelial cells stained negative for mucus. The goblet mucus cells (G) are stained intensely pink. (X20 Magnification)

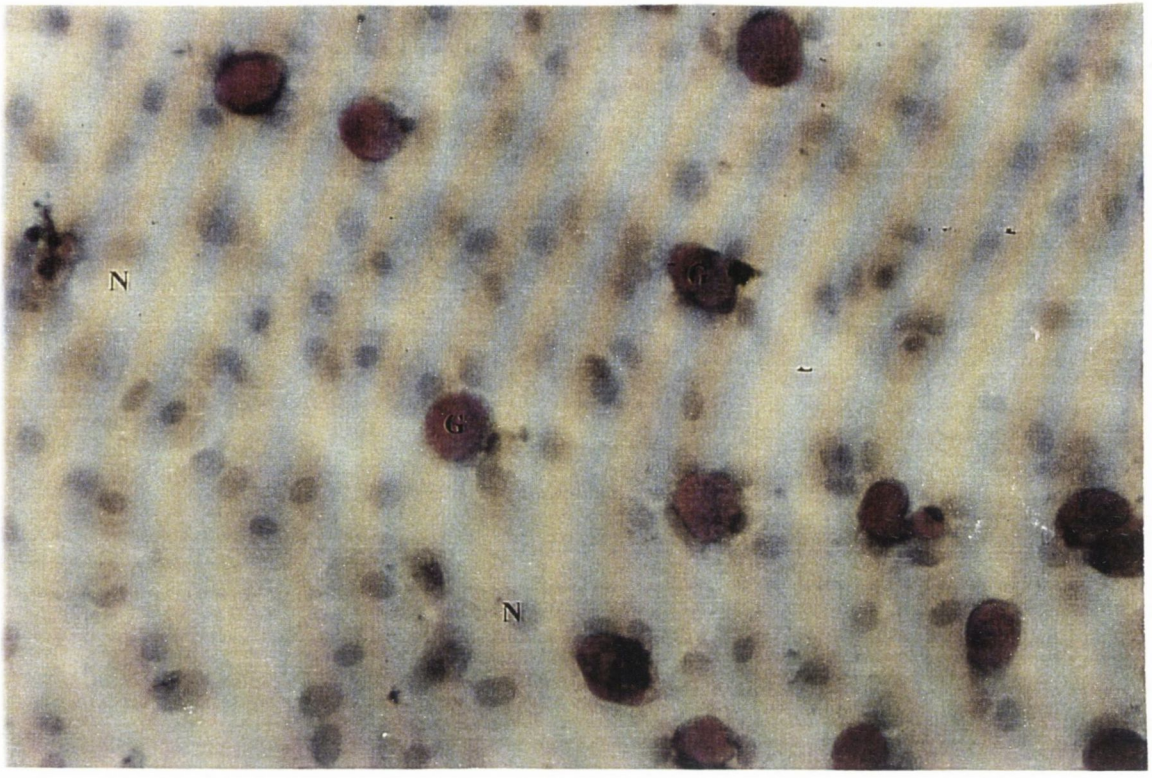
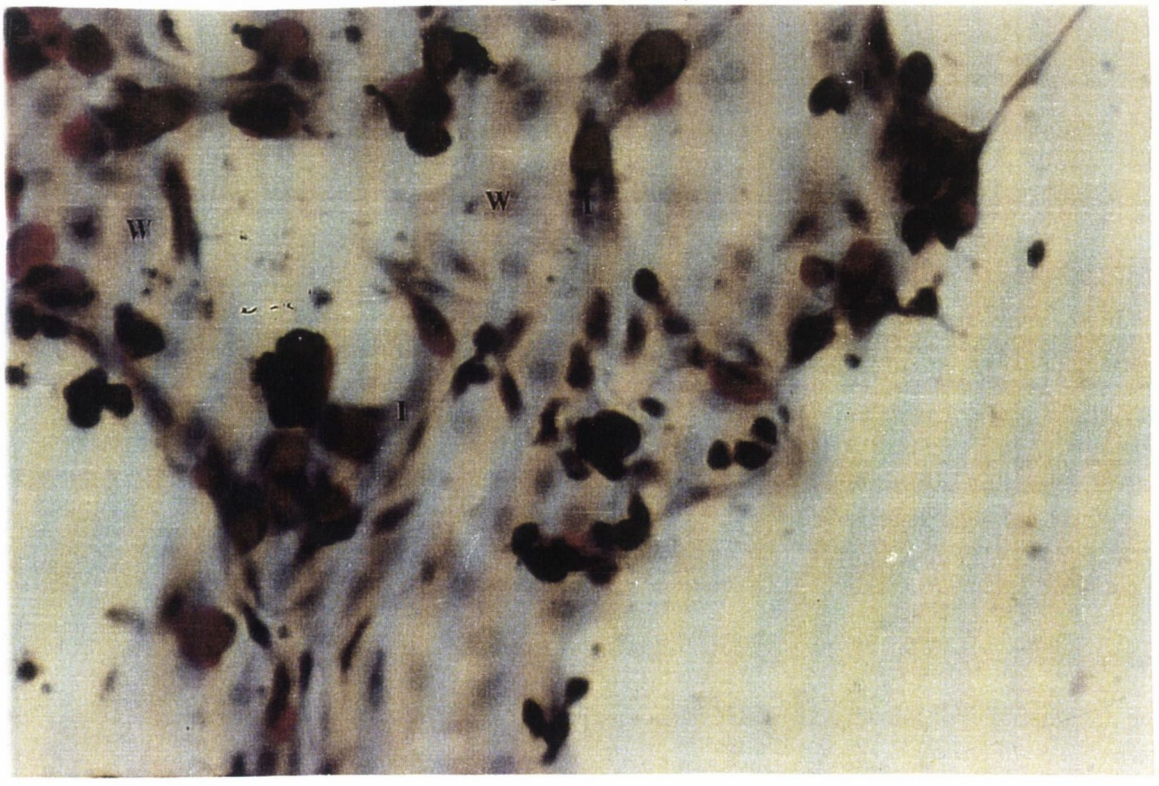


Plate 4.5 Rainbow trout epithelial cell cultures stained weakly positive (W) and intensely positive (I) for mucus. (X20 Magnification).



4.3 RESULTS

4.3.1 HEAT SHOCK PROTEIN

4.3.1.1 NONOXYNOL

Table 4.1: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with nonoxynol in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + DMSO	15.1 ± 2.0	1.7 ± 0.7	16.7 ± 2.5
12.5	47.8 ± 4.4	20.1 ± 5.0	67.8 ± 3.1
25	52.7 ± 5.1	24.6 ± 4.8	77.2 ± 3.8
35	59.9 ± 7.0	31.9 ± 6.9	91.8 ± 3.3

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.1: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with nonoxynol in serum - containing medium:

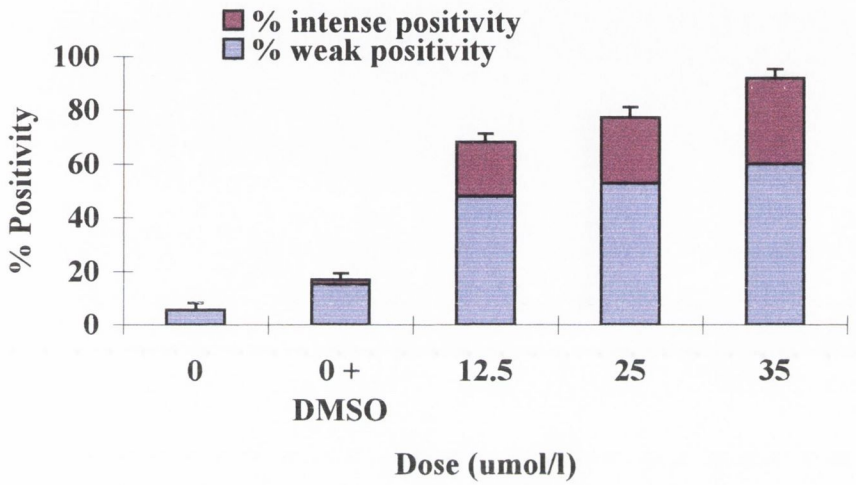


Figure 4.1 shows the % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with nonoxynol in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.1.2 PROCHLORAZ

Table 4.2: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with prochloraz in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + DMSO	32.2 ± 4.7	6.5 ± 5.4	38.7 ± 6.9
10	52.9 ± 5.3	16.9 ± 7.9	69.7 ± 7.2
25	62.3 ± 6.0	17.7 ± 6.6	80.0 ± 4.7
50	63.8 ± 8.4	23.0 ± 8.8	86.8 ± 4.6

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.2: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with prochloraz in serum - containing medium:

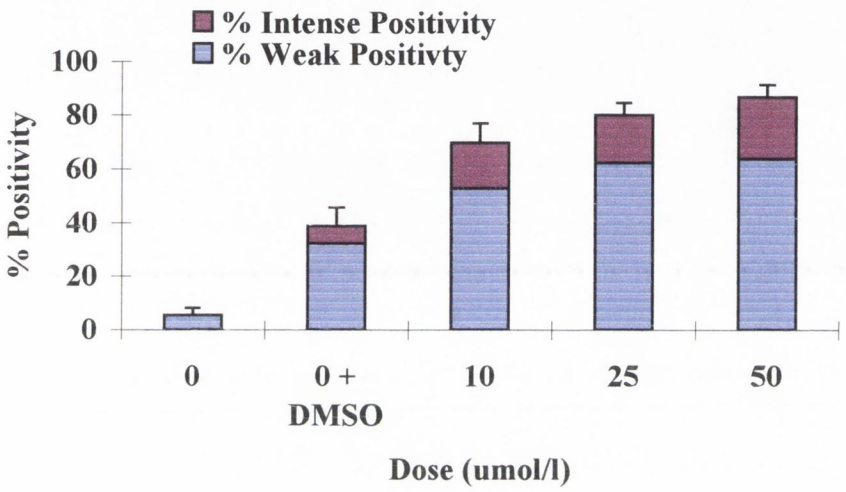


Figure 4.2 shows the % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with prochloraz in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.1.3 COPPER

Table 4.3: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with copper in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + WATER	5.6 ± 2.5	0	5.6 ± 2.5
100	42.9 ± 5.7	35.0 ± 9.4	77.9 ± 8.0
400	60.4 ± 6.8	29.0 ± 5.8	89.4 ± 4.2
700	18.0 ± 5.5	75.3 ± 7.0	93.3 ± 4.0

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.3: The % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with copper in serum - containing medium:

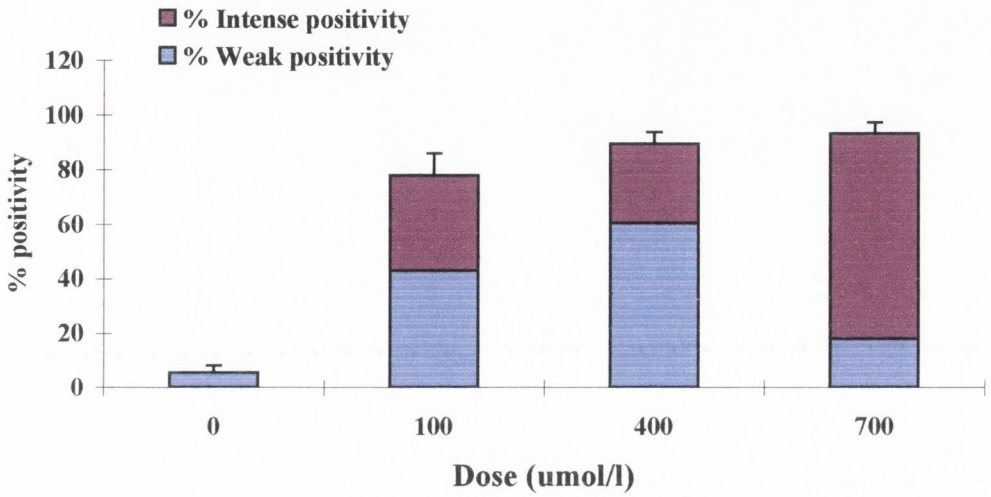


Figure 4.3 shows the % number of rainbow trout primary epithelial cells stained positive for HSP70 post treatment with copper in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.2 PAS STAIN FOR MUCUS

4.3.2.1 NONOXYNOL

Table 4.4: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with nonoxynol in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + DMSO	4.7 ± 0.6	1.7 ± 0.6	6.4 ± 1.0
12.5	9.2 ± 2.0	2.0 ± 0.4	11.2 ± 2.1
25	16.2 ± 1.2	4.3 ± 0.7	20.5 ± 1.5
35	18.7 ± 2.4	5.2 ± 1.6	23.9 ± 1.8

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.4: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with nonoxynol in serum - containing medium:

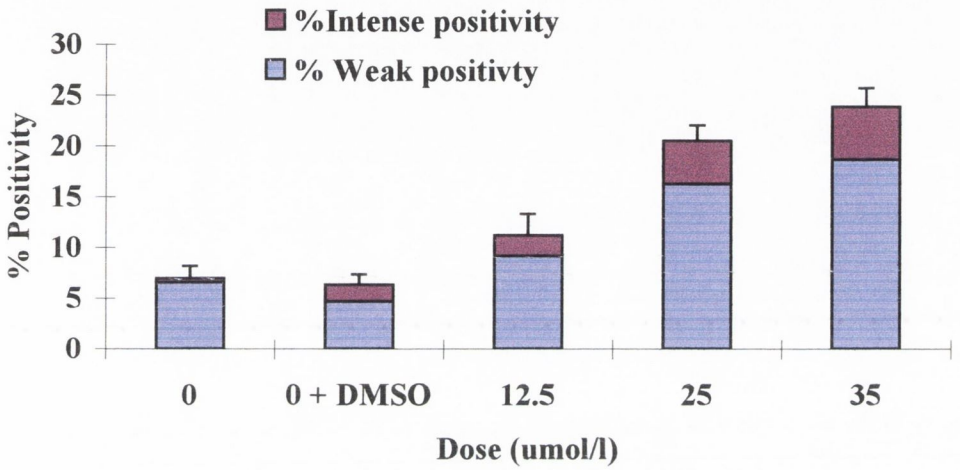


Figure 4.4 shows the % number of rainbow trout primary epithelial cells stained positive for mucus, using the PAS stain, post treatment with nonoxynol in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.2.2 PROCHLORAZ

Table 4.5: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with prochloraz in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + DMSO	7.3 ± 1.3	0.7 ± 0.3	7.9 ± 1.5
10	10.4 ± 2.1	2.3 ± 0.1	12.7 ± 2.6
25	15.6 ± 2.0	3.0 ± 1.0	18.6 ± 2.7
50	15.5 ± 1.6	4.2 ± 1.4	19.6 ± 2.2

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.5: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with prochloraz in serum - containing medium:

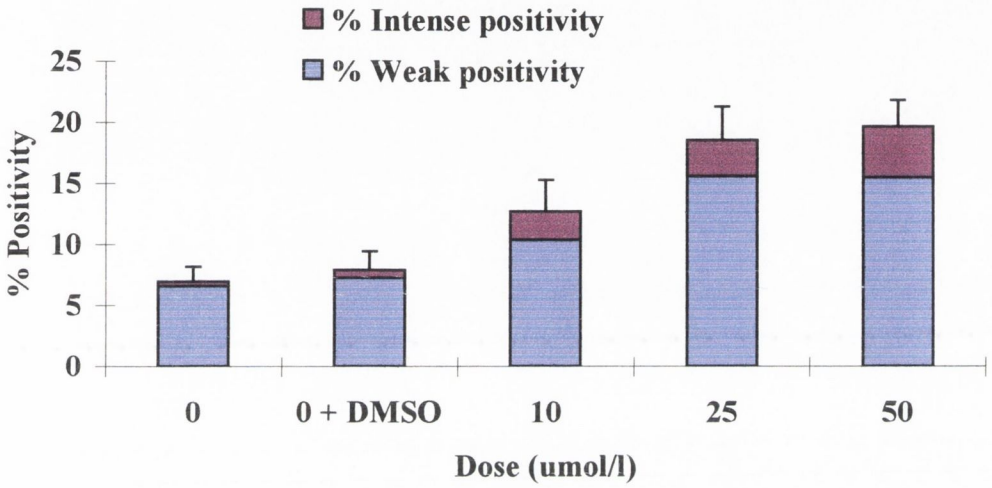


Figure 4.5 shows the % number of rainbow trout primary epithelial cells stained positive for mucus, using the PAS stain, post treatment with prochloraz in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.2.3 COPPER

Table 4.6: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with copper in serum - containing medium:

DOSE (μmol/l)	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVTY
0 + WATER	6.6 ± 1.0	0.4 ± 0.2	7.0 ± 1.2
100	6.9 ± 1.0	5.2 ± 1.9	12.1 ± 1.8
400	10.4 ± 0.8	3.6 ± 1.4	14.0 ± 1.6
700	9.0 ± 1.7	8.8 ± 2.6	17.9 ± 3.0

Each figure represents the mean ± SEM for n = 9 cultures.

Figure 4.6: The % number of rainbow trout primary epithelial cells stained positive for mucus post treatment with copper in serum - containing medium:

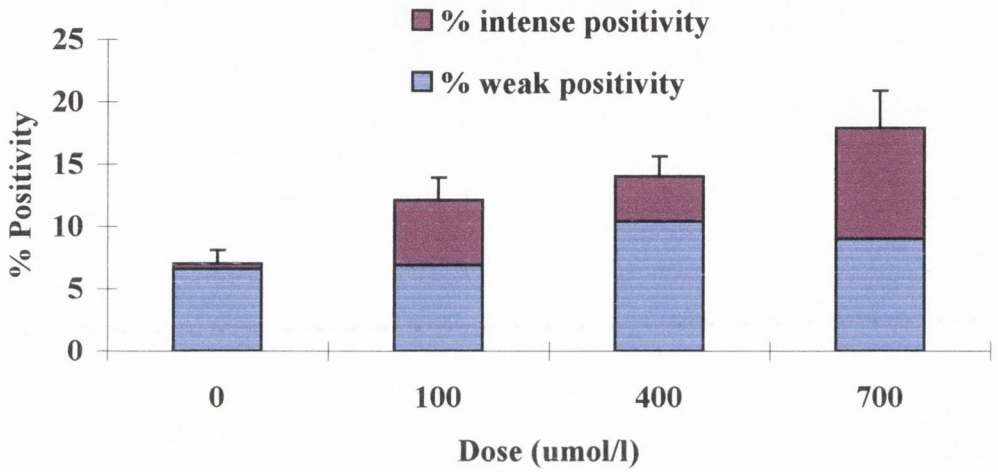


Figure 4.6 shows the % number of rainbow trout primary epithelial cells stained positive for mucus, using the PAS stain, post treatment with copper in serum - containing medium. The cells were scored as been negative, weakly positive and intensely positive. Each figure represents the mean ± SEM for n = 9 cultures.

4.3.1 HEAT SHOCK PROTEIN

The same fish was used for studies with each of the three chemicals. As a result the values for the copper control can be used as a control for each of the three chemicals. The HSP response was equally distributed throughout the explant. However a random distribution of intensely stained cells in the explant was observed. A transect system was used to account for the possibility of differences in the topographical distribution of the stain.

The % of cells expressing heat shock protein was found to increase significantly ($p<0.0001$) with increasing exposure to nonoxynol (Table 4.1; Fig. 4.1). The % of cells expressing positivity in the DMSO controls was 16.7%. The majority of this positivity was weak staining. The number of cells scored per explant in the controls was 527 ± 16. The % of cells expressing HSP70 post exposure with 35 µmol/l nonoxynol was 91.8 ± 3.3. The % of cells stained weakly positive was 59.9 ± 7.0, the % stained with intense positivity was 31.9 ± 6.9 (Table 4.1; Fig. 4.1).

The % of cells expressing heat shock protein was found to increase significantly ($p<0.0001$) with increasing exposure to prochloraz (Table 4.2; Fig. 4.2). The % of cells expressing positivity in the DMSO controls was 38.7%. The majority of this positivity was weak staining. The number of cells scored per explant in the controls was 284 ± 22. The % of cells expressing HSP70 post exposure with 50 µmol/l prochloraz was 86.8 ± 4.6. The % of cells stained weakly positive was 63.8 ± 8.4, the % stained with intense positivity was 23.0 ± 8.8 (Table 4.2; Fig. 4.2).

The % of cells expressing heat shock protein was also found to increase significantly ($p<0.0001$) with increasing exposure to copper (Table 4.3; Fig. 4.3). The % of cells expressing positivity in the water treated controls was 5.55%. All of this positivity

was weak staining. The number of cells scored per explant in the controls was 377 ± 25. The % of cells expressing HSP70 post exposure with 700 μmol/l copper was 93.3 ± 4.0. The % of cells stained weakly positive was 18.0 ± 5.5, the % stained with intense positivity was 75.3 ± 7.0 (Table 4.3; Fig. 4.3). A significant increase ($p<0.0001$) was observed in intense staining in cultures treated with 700 μmol/l copper and those treated with 35 μmol/l nonoxynol and 50 μmol/l prochloraz.

4.3.2 PAS STAIN FOR MUCUS

The % of cells covered with a mucus layer was found to increase significantly ($p<0.0001$) with increasing exposure to nonoxynol (Table 4.4; Fig. 4.4). The % of cells expressing positivity in the DMSO controls was 6.4%. The majority of this positivity was weak staining. The number of cells scored per explant in the controls was 501 ± 70. The % of cells stained positive for mucus post exposure with 35 μmol/l nonoxynol was 23.9 ± 1.8. The % of cells stained weakly positive was 18.7 ± 2.4, the % stained with intense positivity was 5.2 ± 1.6 (Table 4.4; Fig. 4.4).

The % of cells covered with a mucus layer was found to increase significantly ($p<0.0001$) with increasing exposure to prochloraz (Table 4.5; Fig. 4.5). The % of cells expressing positivity in the DMSO controls was 7.9%. The majority of this positivity was weak staining. The number of cells scored per explant in the controls was 408 ± 99. The % of cells stained positive for mucus post exposure with 50 μmol/l prochloraz was 19.6 ± 2.2. The % of cells stained weakly positive was 15.5 ± 1.6, the % stained with intense positivity was 4.2 ± 1.4 (Table 4.5; Fig 4.5).

The % of cells covered with a mucus layer was found to increase significantly ($p<0.0001$) with increasing exposure to copper (Table 4.6; Fig 4.6). The % of cells expressing positivity in the controls treated with water was 7.0%. The majority of this positivity was weak staining. The number of cells scored per explant in the controls was 409 ± 43. The % of cells stained positive for mucus post exposure with 700 µmol/l copper was 17.9 ± 3.0. The % of cells stained weakly positive was 9.0 ± 1.7, the % stained with intense positivity was 8.8 ± 2.6 (Table 4.6; Fig. 4.6).

4.4 DISCUSSION

Heat shock proteins confer protection from environmentally induced cellular damage (stress response) (Werner *et al.*, 1997; Parsell *et al.,* 1994; Lindquist *et al.*, 1988) and accumulation of these proteins in organisms has been linked to the intensity of the stress. Williams *et al.* (1996) suggested that an antibody specific for trout hsp70 would greatly improve the detection of HSP70 in rainbow trout. The results described here show that an available mammalian antibody developed against HSP70 can be used to detect HSP70 induction in response to chemical exposure. All three chemicals tested were found to induce HSP70 production. However copper was found to cause an significant difference ($p<0.001$) in intense positive staining for the stress protein compared with the two organic pollutants tested at the dose level required to cause 80% cell death (fig. 4.3). The decrease observed in chapter 3 in the % residual of day 3 growth in response to the chemicals tested was found to correspond to a significant increase in HSP70 induction. For example primary skin cultures exposed to nonoxynol for 48h in serum - containing medium were found to exhibit a % residual of day 3 growth of 33.9% (table 3.2). The corresponding % of cells expressing positivity for HSP70 at this level of exposure was found to have increased from 5.6 ± 2.5 to 77.2 ± 3.8 in relation to the control (table 4.1, fig 4.1). Similar relationships were observed in the studies with prochloraz (table 3.6, table 4.2 & fig. 4.2) and copper (table 3.9, table 4.3 & fig. 4.3). Other environmental contaminants have been found to elicit HSP70 expression in rainbow trout primary hepatocyte cultures. HSP70 was found to increase in response to β-naphthoflavone (BNF) and cadmium chloride exposure (Iwama *et al.,* 1998).

The use of DMSO was found to induce a slight production of the stress protein. However a significant increase ($p<0.0001$) was observed in HSP70 induction in relation to increasing chemical exposure (Figs. 4.1, 4.2, 4.3). Even at concentrations as low as that required to provide the threshold level of response a significant increase was observed in the production of the stress protein (table 4.1, 4.2, 4.3). The level of HSP70 expressed in the cells at the threshold dose of exposure was found to be the more or less the same for all three chemicals at around 70%. A similar response was observed by Bauman *et al.* (1993). Heat shock proteins were found to be synthesised at low metal concentrations that do not cause cellular toxicity. Low concentrations of both cadmium and arsenite were found to trigger the synthesis of heat shock proteins without causing a loss of intracellular potassium. Low levels of exposure to other metals such as gallium were also found to induce the heat shock proteins without causing gross evidence of cytotoxicity (Aoki *et al.,* 1990). In a previous study an increase in HSP70 in response to SDS and BKME exposure *in vivo* occurred at concentrations that were several-fold lower than the LC_{50} values (Vijayan *et al.,* 1997).

The fact that an increase in HSP70 induction can be detected in both the DMSO controls and the cultures treated with the threshold doses shows that the antibody used may be a very sensitive marker for HSP70 induction.

It would be most interesting to test the antibody on cells cultured from fish treated with *in vivo* concentrations of pollutants. This study shows that it is possible to use mammalian antibodies on fish. Other work, which could be carried out in the future, would be to use existing mammalian antibodies to stain for other stress proteins, proliferation proteins and oncogenes. If the antibodies are verified to exist in fish

their study could dramatically aid in the study of cellular responses to specific classes of chemical pollutants.

The level of mucus on the surface of the epithelial cultures was found to increase with exposure to increasing doses of the chemicals tested figs. 4.4, 4.5, 4.6). As observed in chapter 3 the number of mucus cells was found to decrease in the cell cultures in response to increasing dose exposure. As the number of goblet mucus cells per culture decreases the % of pavement cells expressing positivity for mucus was found to increase. The number of goblet cells per field exposed to 25μmol/l nonoxynol in serum-containing media was found to decrease from 7.7 ± 1.2 to 1.9 ± 1.2 in relation to the control cultures (table 3.4). The percentage of cells expressing positivity per culture exposed to 25μmol/l nonoxynol for mucus was found to increase from 6.4 ± 1.0 to 20.5 ± 1.5 in relation to the controls (fig. 4.4). A similar relationship was observed between number of goblet mucus cells per skin culture and the % number of cells expressing positivity for mucus per skin culture in the studies with prochloraz (table 3.8, fig 4.5) and copper (table 3.11, fig 4.6). It appears that the mucus cells are secreting their contents onto the surface of the cells. The mucus content of the culture media was not analysed and hence the amount of mucus secreted may actually be higher than that observed in this study. The results of this study demonstrate that *in vitro* rainbow trout primary epithelial cell cultures maintain the essential traits of the *in vivo* response of the skin to chemical stresses. The goblet cells contained in the epithelial cell cultures are retaining their *in vivo* functions *in vitro*.

4.5 CONCLUSION

This chapter demonstrated that an available mammalian antibody developed against HSP70 can be used to detect HSP70 induction in response to chemical exposure. The % of cells per primary culture expressing positivity for HSP70 was found to increase as chemical exposure increased. The nature of the response demonstrates the possible use of HSP70 induction using the a rabbit polyclonal antibody described as a sensitive marker of chemical stress. However it shall be necessary to carry out *in vivo* studies to verify this.

The % number of cells expressing positivity for mucus was found to increase as chemical exposure increased. This increase was found to correspond to the decrease in mucus cell number, observed in chapter 3, in relation to increased chemical exposure. The results of this study demonstrate that *in vitro* rainbow trout primary epithelial cell cultures maintain the essential traits of the *in vivo* response of the skin to chemical stresses. The goblet cells contained in the epithelial cell cultures are retaining their *in vivo* functions *in vitro*.

CHAPTER 5

THE GROWTH AND MUCUS SECRETION OF PRIMARY EPITHELIAL CELL CULTURES GROWN FROM RAINBOW TROUT EXPOSED *IN VIVO* TO PROCHLORAZ AND A PROCHLORAZ/NONOXYNOL MIXTURE.

5.1 INTRODUCTION

Many different agricultural and industrial chemicals enter aquatic environments and pose a potential threat to the indigenous biota. Changes in the quality of these environments are a major problem associated with the deposition of chemical pollutants into freshwater and marine environments (Babich *et al.*, 1986). The emphasis in the development of ecotoxicology assessment methods has moved from the measurement of acute, lethal effects to the early detection and evaluation of chronic sublethal stresses. Over the years there has been increasing interest in the use of biomarkers for environmental assessment. Biomarkers are defined as a biological response to a chemical or chemicals that give a measure of exposure and sometimes also, of toxic effect (Peakall, 1994).

The body surface is in intimate contact with the water and therefore is the first tissue exposed to water pollutants. The skin of fish is a metabolically very active tissue, that quickly responds to stressors (Whitear, 1986; Iger *et al.,* 1992). The epidermal mucus layer constitutes the primary biological interface between fish and the aqueous environment (Ottesen *et al.,* 1997). A number of functions have been ascribed to the mucus layer, such as protection against mechanical injury (Pickering *et al.,* 1980) and friction reducing properties (Pickering *et al.,* 1974). Following stress or infection, mucous secretion may be increased (Blackstock *et al.,* 1982; Iger *et al.,* 1988). Increased mucus secretion has been reported in fish exposed to various pollutants, acidified water or parasitic infection (Burton *et al.,* 1984; Benedetti *et al.,* 1989; Perry *et al.,* 1989, Wendelar Bonga *et al.,* 1990; Iger 1992; Iger *et al.,* 1993; Wiipfli *et al.,* 1994). Iger *et al.* (1994) demonstrated that within 3h after the start of

temperature elevation to rainbow trout, abundant mucous cells extruded their contents onto the epidermal surface. Exposure to water-borne mercury promoted increased mucus production in goldfish (*Carassius auratus*) (McKone, 1971) and rainbow trout (Varanasi *et al.*, 1975). Exposure to 150ppb of either water-borne lead or cadmium resulted in a substantial increase in the production of mucus in coho salmon (Varanasi *et al.*, 1977).

Isolated cells that maintain the essential traits of the *in vivo* state during culture in vitro provide an excellent experimental approach to establish diagnostic markers such as early indicators of effect. The rainbow trout (*Onchorynchus mykiss*) epithelial cell cultures contain both epithelial cells and goblet mucus cells. *In vitro* the number of goblet cells contained in these culture were found to decrease with exposure to increasing levels of the pesticide prochloraz . It was found that the mucus cells excreted their contents onto the surface of the cell cultures (Chapter 3 & 4).

As stated in Chapter 1 in order to understand the ecotoxicological implications of the cellular indicator response, *in vitro* studies on isolated cells have to integrated with *in vivo* studies on the intact animal. Also substantial research and development is needed to establish early indicators of exposure as a tool for the timely threat to biological systems.

The purpose of this study was to see if rainbow trout primary skin epithelial cell cultures cultured *in vitro* from fish exposed to the pesticide prochloraz and a mixture of prochloraz and nonoxynol *in vivo* demonstrated a mucus response in relation to the pollutant similar to that observed in Chapter 4 with cells exposed *in vitro* to different chemicals. It was also investigated whether changes in the growth of primary skin epithelial cell cultures cultured *in vitro* from fish exposed to pollutants *in vivo* occur as a result of this exposure.

5.2 MATERIALS & METHODS

5.2.1) Work done on the test site

Twenty four bath tubs (enamelled iron; volume: 200 liters; colour: clear sand) were installed on two superimposed levels (12 tubs per level). Water was renewed continuously at the rate of 100% (ie 200 liters per tub per day) during the experiment. The water used was dechlorinated tap water. Discarded water flowed out at the top of one extremity of every tub and was filtered on a charcoal filter before flowing into the sewer. Oxygen was bubbling continuously from the bottom at the opposite extremity of the tubs. The test substance was mixed with the incoming water, just before it flowed into the tub in the area of oxygen bubbling (to allow rapid mixing). The chemicals were pumped from stock bottles to the tubs using a 24 channel-BVK pump and Tygon catheters (id 2.06mm), the shortest ones being 4 meters long and the longest ones 9 meters long. The variation coefficient for the flow rate between the 24 catheters was equal to ±4.95%. The mean temperature of water was between 11 and 13^0C. Organic matter (faeces and unused pellets) in suspension in the water was not removed, but a part was permanently eliminated with the water flowing out of the tubs.

Forty rainbow trout were placed in each of the twenty four tanks. Six of the tanks were exposed to a dose of 100ppb Prochloraz, Six to a dose of 10ppb Prochloraz, Six to 60:40 Mixture of Prochloraz/Nonoxynol and Six tanks were used as controls. The Prochloraz was dissolved in 2.7% Ethanol. The control tanks were treated with 2.7% Ethanol. Ethanol was use as the solvent control. The fish were exposed for twelve days to the test chemicals . The Tanks were divided in distance from the pump. The

pump was placed so that supply tube length from the pump to some of the tanks was shorter than the length to the remaining tanks.

Eight fish were taken from eight different tanks before any chemical entered the tanks to be used as controls for the experiment. The fish were killed by a swift blow to the back of the head, weighed and measured. A piece of skin tissue was then immediately removed from the upper dorsal side of the fish and primary epithelial cultures plated in $24cm^2$ flasks. Six of these cultures were plated to study survival, three for the PAS stain. After three days growth at eighteen degrees all the cultures except those for the survival study and protein synthesis studies were then fixed as follows. The culture media was removed from the flask. The cultures were then washed in P.B.S. and fixed in 10% formalin.

The growth cultures were taken out of the incubator after three days and the cell outgrowth outlined with a permanent marker as previously described in the *in vitro* toxicity testing. These were then fixed as described above 5 days post culture, 7 days post culture, 9 days post culture, 11 days post culture and 14 days post culture. One fish were taken and killed as previously described 18 hours post exposure to the chemical from one tank for each of the exposure doses and the above carried out.

One fish was taken and killed from three separate tanks for each exposure level after five days exposure to the test substances and the above carried out. After 12 days exposure to the test substances one fish was taken from the same tanks as that of 5 days exposure and the same procedure carried out.

During the exposure period water samples were taken at regular intervals to determine the actual concentration of chemical in the water. Water samples were also taken to determine the pH, dissolved oxygen concentration, nitrate concentration and nitrite concentration, contained in the water over the exposure period.

5.2.2) WORK CARRIED OUT IN THE LABORATORY

GROWTH OF PRIMARY CULTURES

The cultures were stained with Harris's Haemotoxylin. The outgrowth areas were determined in the same manner as that previously described (Section 3.2) by overlaying the culture flask with a clear Xerox transparency onto which a sheet of $1mm^2$ grid paper had been photocopied. The number of $1mm^2$ grids covered the stained explant were counted. The area outlined after three days growth was also counted using the same grid. The % residual survival of the day 3 growth area for each culture was then calculated using the following formula

$$\frac{\text{Area of stained Cells}}{\text{Area of outlined area}} * 100$$

PERIODIC ACID SCHIFFS (PAS) TEST FOR MUCUS.

The PAS stain for mucus was also carried out. The procedure involved is described in Section 4.2.2

5.3 RESULTS

Measurements were taken from a number of sample tanks to see what concentration of chemical was actually in the water and to detect the effect of differences in the length of the supply tubing.

Table 5.1: The actual amount chemical contained in the tanks during the experiment.

Values = ppb								
			Dates of sampling					
Tank No.	Length of Tube	Chemical	20/3	24/3	26/3	28/3	36/3	1/4
9	S	P10		6	8	9	11	9
10	L	P10		5	7	7	7	6
13	S	P100	45	54	62	75	63	78
18	L	P100	39	10	22	15	19	18
21	S	M	45	53	73	78	71	80
22	L	M	31	14	16	13	18	23

S = short length tube, L = long length tube, P10 = 10ppb prochloraz added, P100 = 100ppb added, M = mixture of nonoxynol/prochloraz added.

The following water quality measurements were taken and analysed.

Table 5.2: The water parameters in one tub without chemicals taken during the experiment.

The Parameters of water in one tub without chemicals						
Date	**Temp. (^{O}CR)**	**pH**	**Oxygen (mg/l)**	**N-NH_{3-4} (mg/l)**	**N-NO_3 (ppm)**	**N-NO_2 (ppm)**
17-3-98	12.1	7.44	11.5	0.05	0	1.5
18-3-98	12.2	7.17	7.5	1.0	0	2.0
25-3-98	11.5	7.48	9.5	1.5	0.25	3.0
1-4-98	12.7	7.36	8.1	0.2	0.08	4.0
3-4-98				.155	0.025	4.0

N-NH3-4 = Dissolved Ammonia, N-NO3 = Dissolved Nitrites

N-NO2 = Dissolved Nitrates

Table 5.3: The mean value of four water parameters determined at different times during the exposure period.

The mean value of 4 parameters determined at different times during the exposure period						
	pH	**Oxygen (mg/l)**	**Oxygen (mg/l)**	**Oxygen (mg/l)**	**N-NH_{3-4} (ppm)**	**N-NO_3 (ppm)**
	1-4-98	19-3-98[a]	23-3-98[b]	30-3-98	3-4-98	3-4-98
+0	7.35±0.04	8.52±0.70	9.53±0.50	8.52±0.93	0.19±0.03	0.05±0.05
+10μg/l	7.32±0.04	7.78±1.14	8.42±0.65	7.00±0.70	0.18±0.02	0.13±0.07
+100μg/l	7.27±0.02	8.75±0.73	9.65±0.52	6.43±2.92	0.17±0.04	0.06±0.03
Mix	7.28±0.02	7.37±0.81	8.32±1.07	6.38±0.45	0.15±0.00	0.05±0.02
Oxyg Sat.		12.05	11.7	11.1		

a=4 hours before feeding b=3 hours before feeding

Due to these findings it has been decided to group the data on the bases of a dose range of prochloraz in the tanks.

Dose Range ppb	Tank	Fish No. day 5+12
45+	14,13	9,8
1-45	16,9,8,12	7*,6,5,4
0	2,1,4	3,2,1
M high	21,20	12,11
M low	10	10

* fish no.7 was exposed to a level of prochloraz of between 10-20 ppb.

All water parameter readings were taken by the technical staff of the INRA laboratory in Rennes, France, where the study took place.

5.3.1 GROWTH OF PRIMARY EPITHELIAL CULTURES

Tables 5.4 to 5.8 show the average % residual of day 3 growth in relation to the amount of chemical in the water. The relative water quality measurements are also included.

Table 5.4: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to 2.7% Ethanol.

Days Exp.	pH	°cr	O_2 mg/l	'NH_3 mg/l	'NO_3 ppm	'NO_2 ppm	% Residual of Initial Day3 Growth					
							D. 3	D. 5	D. 7	D. 9	D.11	D.14
0	7.17	12.2	8.11	1	0	2	100	110± 9.2	119± 4.8	159± 19.9	116± 18.5	133± 15.6
5	7.48	11.5	9.51	1.5	.25	3	100	105± 4.2	105± 4.2	81± 23.4	76± 24.9	400
12	7.36	12.7	8.31	.2	0.8	4	100	100± 3.5	88± 12.6	76± 10.0	77± 4.2	-

Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 3 cultures

Figure 5.1: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to 2.7% Ethanol

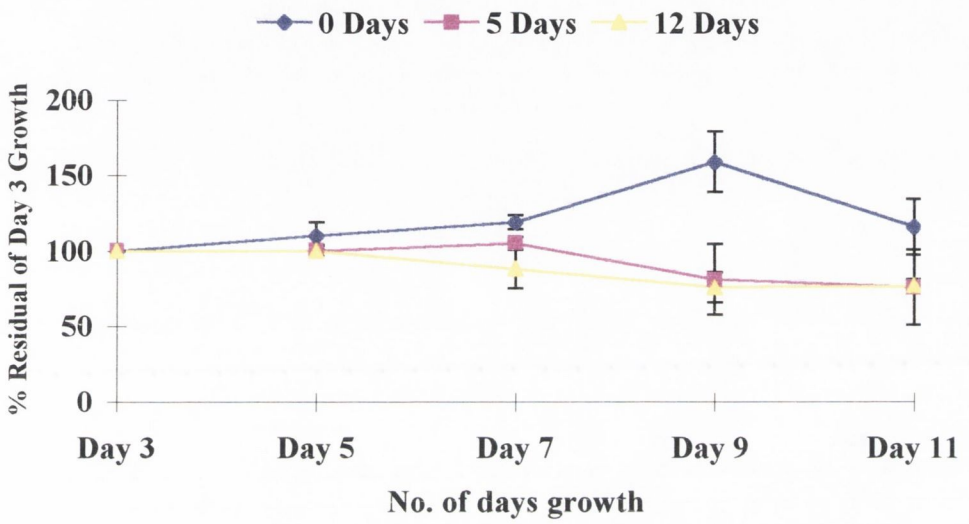


Figure 5.1 shows the % residual of day 3 growth of primary epithelial cell cultures cultured from rainbow trout exposed to 2.7% ethanol for 0 days, 5 days and 12 days. Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 3 cultures.

Table 5.5: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to 1-45ppb Prochloraz

Days Exp.	pH	^{O}cr	O_2 mg/l	'NH$_3$ mg/l	'NO$_3$ ppm	'NO$_2$ ppm	% Residual of Initial Day3 Growth					
							D. 3	D. 5	D. 7	D. 9	D.11	D.14	D.21
0	7.17	12.2	8.11	1	0	2	100	110± 9.2	119± 4.8	159± 19.9	116± 18.5	133± 15.6	-
18h							100	139	191	216	168	234	-
5	-	-	8.42	-	-	-	100	110± 5.2	138± 7.7	145± 35.1	161± 12.4	246± 23.8	-
12	7.32	-	7.00	..18	0.13	-	100	89± 15.4	83± 5.8	49± 9.4	67± 17.3	-	20± 1.2

Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 4 cultures.

Figure 5.2: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to 1-45ppb Prochloraz.

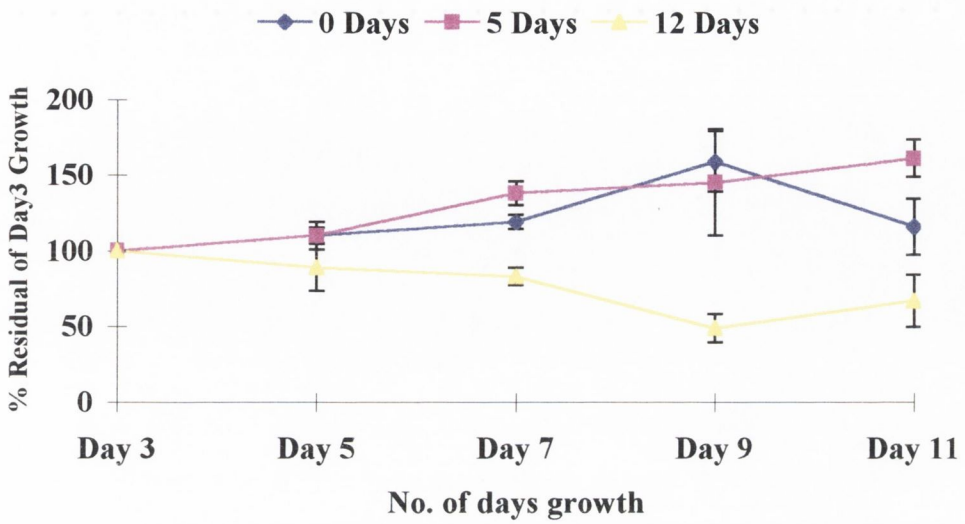


Figure 5.2 shows the % residual of day 3 growth of primary epithelial cell cultures cultured from rainbow trout exposed to 1 - 45ppb prochloraz for 0 days, 5 days and 12 days. Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 4 cultures.

Table 5.6: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to >45ppb Prochloraz

Days Exp.	pH	°cr	O_2 mg/l	'NH_3 mg/l	'NO_3 ppm	'NO_2 ppm	% Residual of Initial Day3 Growth					
							D. 3	D. 5	D. 7	D. 9	D.11	D.14	D.21
0	7.17	12.2	8.11	1	0	2	100	110± 9.2	119± 4.8	159± 19.9	116± 18.5	133± 15.6	-
18h							100	115	65	64	154	174	-
5	-	-	9.65	-	-	-	100	120± 30	57± 9.6	118± 18.5	63± 0	102± 29.5	-
12	7.32	-	6.43	.17	0.06	-	100	107± 7.8	91± 8.9	58± 11.4	23± 5.0	-	23± 4.9

Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 2 cultures.

Figure 5.3: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to >45ppb Prochloraz.

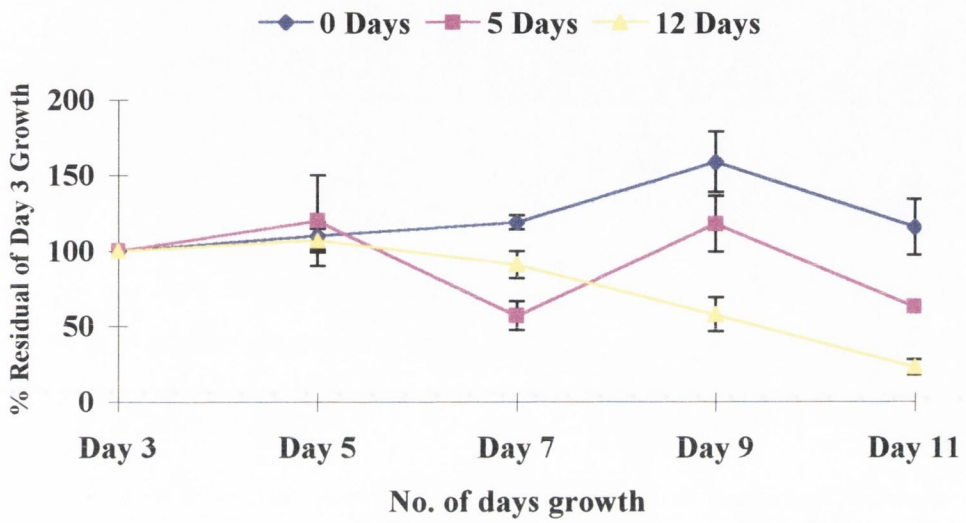


Figure 5.3 shows the % residual of day 3 growth of primary epithelial cell cultures cultured from rainbow trout exposed to >45ppb prochloraz for 0 days, 5 days and 12 days. Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 2 cultures.

Table 5.7: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to the high concentration of the mixture.

Days Exp.	pH	°cr	O_2 mg/l	'NH_3 mg/l	'NO_3 ppm	'NO_2 ppm	% Residual of Initial Day3 Growth					
							D. 3	D. 5	D. 7	D. 9	D.11	D.14	D.21
0	7.17	12.2	8.11	1	0	2	100	110± 9.2	119± 4.8	159± 19.9	116± 18.5	133± 15.6	-
18H							100	137	240	330	281	221	-
5	-	-	8.32	-	-	-	100	107± 5.0	84± 13.6	124± 42.5	154± 0.9	105± 48.7	-
12	7.28	-	6.38	.15	0.05	-	100	94± 7.8	81± 10.1	70± 6.2	92± 0.0	-	31± 1.5

Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 2 cultures.

Figure 5.4: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to high concentration of the mixture.

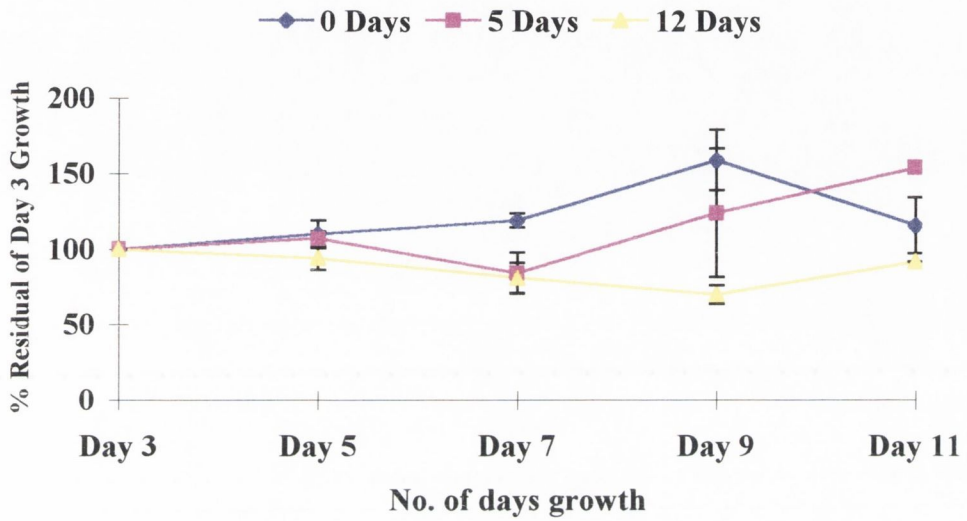


Figure 5.4 shows the % residual of day 3 growth of primary epithelial cell cultures cultured from rainbow trout exposed to the high concentration of the mixture of prochloraz/nonoxynol for 0 days, 5 days and 12 days. Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean ± SEM for n = 2 cultures.

Table 5.8: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to the low concentration of the mixture.

Days Exp.	pH	°cr	O_2 mg/l	'NH_3 mg/l	'NO_3 ppm	'NO_2 ppm	% Residual of Initial Day3 Growth						
							D. 3	D. 5	D. 7	D. 9	D.11	D.14	D.21
0	7.17	12.2	8.11	1	0	2	100	110± 9.2	119± 4.8	159± 19.9	116± 18.5	133± 15.6	-
5	-	-	-	-	-	-	100	192	300	319	191	150	-
12	-	-	-	-	-	-	100	94	86	105	83	-	22.2

Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean for n =1 cultures.

Figure 5.5: The % residual of day 3 growth of primary epithelial explants cultured from rainbow trout exposed to a low concentration of the mixture.

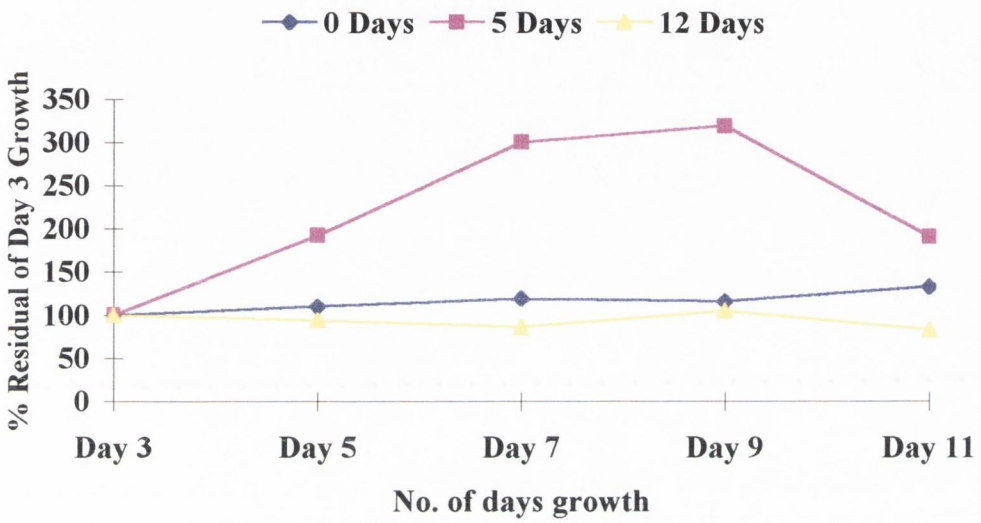


Figure 5.5 shows the % residual of day 3 growth of primary epithelial cell cultures cultured from rainbow trout exposed to the low concentration of the mixture of prochloraz/nonoxynol for 0 days, 5 days and 12 days. Day 0 figures represent the mean ± SEM for n = 7 cultures. Day 5 and Day 12 figures represent the mean for n =1 cultures.

Table 5.9 & 5.10 show the % residual of day 3 growth of each explant in relation to the number of days each explant grew compared to the chemical dose each fish was exposed to.

Table 5.9: 5 Days Exposure to the test Substances, 2.7% ethanol, 1-45ppb prochloraz, >45ppb prochloraz, high and low dose of the mixture.

	Day 3	Day 5	Day 7	Day 9	Day 11
CONTROL	100	109.71 ± 9.3	119.17 ± 4.9	159.11 ± 20.0	115.97 ± 18.6
0	100	105.13 ± 4.2	105.13 ± 4.2	81.41 ± 23.4	76.23 ± 25.0
Mix -Low	100	192.31	300	318.18	190.91
Pro. 1-45ppb	100	110.36 ± 5.2	137.58 ± 7.8	145.4 ± 35.2	161.09 ± 12.4
Mix - High	100	107.23 ± 5.0	84.23 ± 13.6	124.46 ± 42.6	153.61 ± 0.9
Pro. >45ppb	100	120.14 ± 30.0	57.04 ± 9.6	118.28 ± 18.6	62.5

The control values represent % residual of day 3 cultures from fish exposed to the test substances for 0 days. Control figures represent the mean ± SEM for n = 7 cultures. 1-45ppb prochloraz figures represent the mean ± SEM for n = 4 cultures. >45ppb prochloraz figures and the high mixture figures represent the mean ± SEM for n = 2 cultures. The low mixture figure represents the mean for n = 1 cultures.

Figure 5.6: The % residual of day 3 growth of primary epithelial cultures from rainbow trout exposed to the test substances for 5 days.

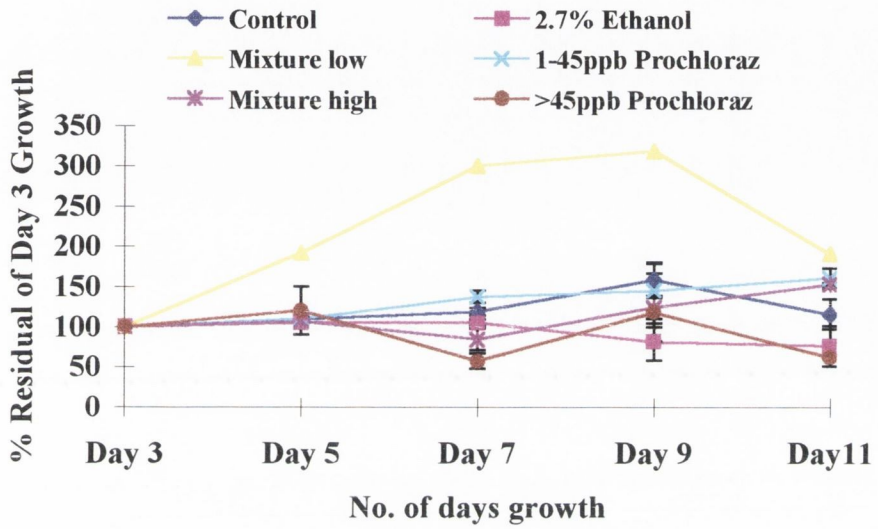


Figure 5.6 shows the % residual of day 3 growth of primary epithelial cell cultures for rainbow trout exposed to 2.7 % ethanol, 1-45ppb prochloraz, >45ppb prochloraz, the high concentration of the mixture of prochloraz/nonoxynol and the low concentration of the mixture of prochloraz/nonoxynol for 5 days. The control values represent % residual of day 3 cultures from fish exposed to the test substances for 0 days. Control figures represent the mean ± SEM for n = 7 cultures. 1-45ppb prochloraz figures represent the mean ± SEM for n = 4 cultures. >45ppb prochloraz figures and the high mixture figures represent the mean ± SEM for n = 2 cultures. The low mixture figure represents the mean for n = 1 cultures.

Table 5.10: 12 Days Exposure to the Test Substances, 2.7% ethanol, 1-45ppb prochloraz, >45ppb prochloraz, high and low dose of the mixture.

	Day 3	Day 5	Day 7	Day 9	Day 11
CONTROL	100	109.71 ± 9.3	119.17 ± 4.9	159.11 ± 20.0	115.97 ± 18.6
0	100	100.18 ± 3.5	87.80 ± 12.6	75.83 ± 10.1	77.38 ± 4.3
Mix -Low	100	94.12	85.71	105	83.33
Pro. 1-45ppb	100	88.89 ± 15.4	82.52 ± 5.8	49.13 ± 9.5	66.5 ± 17.3
Mix - High	100	94.30 ± 7.8	80.95 ± 10.1	70.12 ± 6.3	92.31
Pro. >45ppb	100	107.17 ± 7.8	91.21 ± 9.0	57.74 ± 11.4	21.92 ± 5.1

The control values represent % residual of day 3 growth of cultures from fish exposed to the test substances for 0 days. Control figures represent the mean ± SEM for n = 7 cultures. 1-45ppb prochloraz figures represent the mean ± SEM for n = 4 cultures. >45ppb prochloraz figures and the high mixture figures represent the mean ± SEM for n = 2 cultures. The low mixture figure represents the mean for n = 1 cultures.

Figure 5.7: The % residual of day 3 growth of primary epithelial cultures from rainbow trout exposed to the test substances for 12 days.

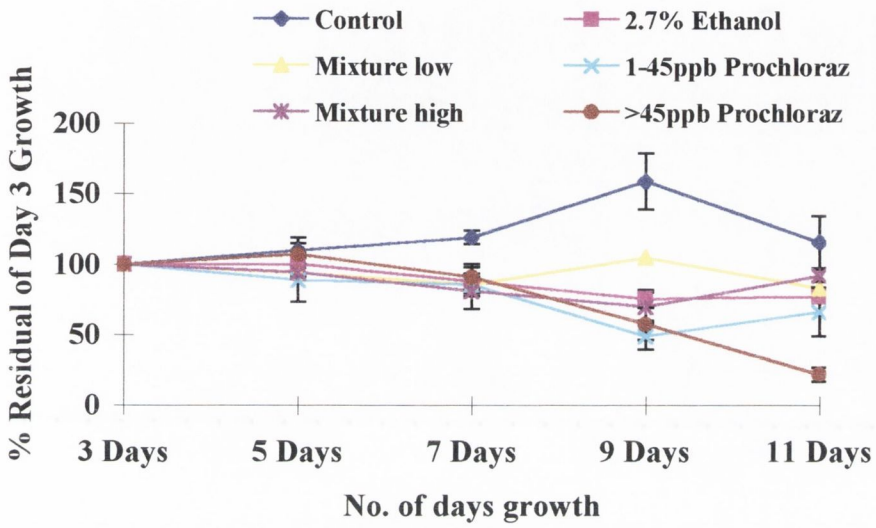


Figure 5.7 shows the % residual of day 3 growth of primary epithelial cell cultures for rainbow trout exposed to 2.7 % ethanol, 1-45ppb prochloraz, >45ppb prochloraz, the high concentration of the mixture of prochloraz/nonoxynol and the low concentration of the mixture of prochloraz/nonoxynol for 12 days. The control values represent % residual of day 3 growth of cultures from fish exposed to the test substances for 0 days. Control figures represent the mean ± SEM for n = 7 cultures. 1-45ppb prochloraz figures represent the mean ± SEM for n = 4 cultures. >45ppb prochloraz figures and the high mixture figures represent the mean ± SEM for n = 2 cultures. The low mixture figure represents the mean for n = 1 cultures.

5.3.2 PAS STAIN (% Cells Staining Positive for Mucus)

Table 5.11: The % Epithelial cells stained positive for mucus from cultures derived from fish exposed to Prochloraz and Nonoxynol/Prochloraz mixture for 5 and 12 days.

DOSE	DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
0	0	9.0 ± 1.0	1.7 ± 0.6	10.7 ± 1.3
	+18h	5.8 ± 0.9	0.2 ± 0.1	6.1 ± 0.9
2.7% Ethanol	5	7.0 ± 0.8	0.5 ± 0.2	7.5 ± 0.9
1-45ppb Pro.	5	11.2 ± 1.2	0.8 ± 0.3	12.0 ± 1.2
>45ppb Pro.	5	16.8 ± 2.9	3.2 ± 1.6	20.0 ± 3.6
Mixture - Low	5	8.3 ± 1.1	0.3 ± 0.6	8.6 ± 1.2
Mixture - High	5	13.6 ± 2.8	1.0 ± 0.5	14.6 ± 2.9
2.7% Ethanol	12	8.2 ± 1.3	0.2 ± 0.1	8.3 ± 1.2
1-45ppb Pro.	12	11.4 ± 1.7	0.6 ± 0.2	12.0 ± 1.7
>45 ppb Pro.	12	14.8 ± 2.4	1.7 ± 0.6	16.4 ± 2.7
Mixture - Low	12	11.1 ± 1.6	0.4 ± 0.2	11.5 ± 1.8
Mixture - High	12	8.4 ± 1.0	0.7 ± 0.3	9.1 ± 1.2

The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol values for 5 days and 12 days represents the mean ± SEM for n = 9 cultures. 1-45ppb prochloraz value for 5 days represents the mean ± SEM for n = 12 cultures. > 45ppb value for 5 days represents the mean ± SEM for n = 6 cultures. High dose mixture value for 5 days represents the mean ± SEM for n = 6 cultures. Low dose mixture value for 5 days represents the mean ± SEM for n = 3 cultures. 1-45ppb prochloraz value for 12 days represents the mean ± SEM for n = 11 cultures. > 45ppb value for 12 days represents the mean ± SEM for n = 6 cultures. High dose mixture value for 12 days represents the mean ± SEM for n = 4 cultures. Low dose mixture value for 12 days represents the mean ± SEM for n = 3 cultures.

Figure 5.8: % Epithelial cells stained positive for mucus in primary cultures derived form fish exposed to Prochloraz for 5 days.

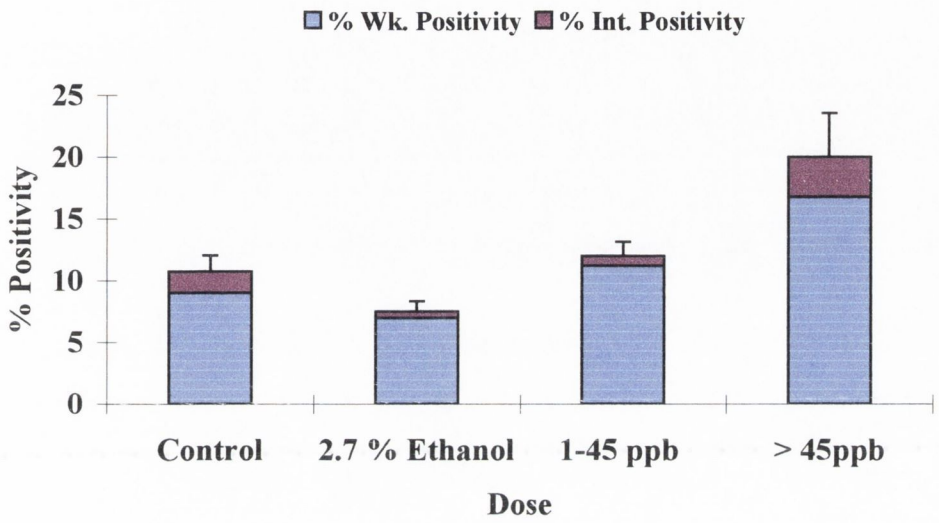


Figure 5.8 shows the % epithelial cells stained positive for mucus, using the PAS stain, in primary cultures from fish exposed to prochloraz for 5 days. The control cultures were taken from fish exposed for 0 days. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol value represents the mean ± SEM for n = 9 cultures. 1-45ppb prochloraz value represents the mean ± SEM for n = 12 cultures. > 45ppb value represents the mean ± SEM for n = 6 cultures.

Figure 5.9: % Epithelial cells stained positive for mucus in primary cultures derived form fish exposed to Nonoxynol/Prochloraz Mixture for 5 days.

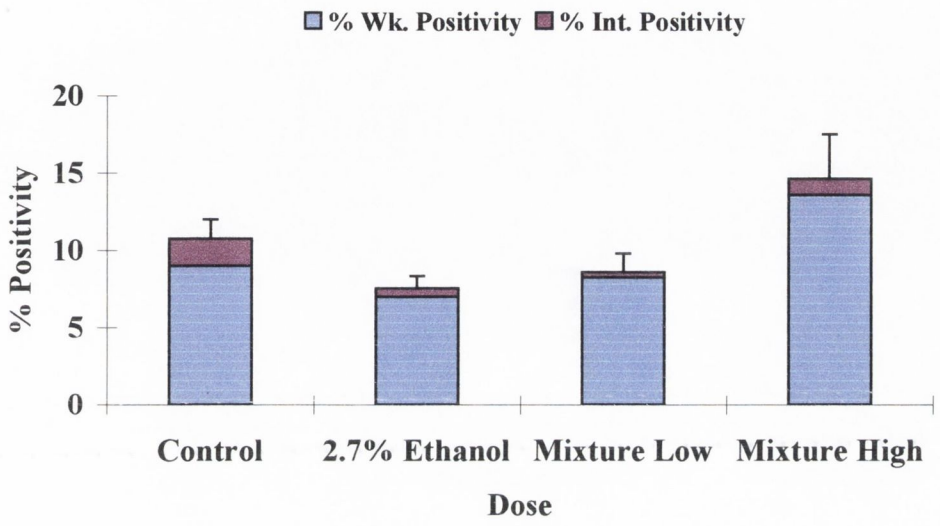


Figure 5.9 shows the % epithelial cells stained positive for mucus, using the PAS stain, in primary cultures from fish exposed to nonoxynol/ prochloraz mixture for 5 days. The control cultures were taken from fish exposed for 0 days. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol value represents the mean ± SEM for n = 9 cultures. High dose mixture value represents the mean ± SEM for n = 6 cultures. Low dose mixture value represents the mean ± SEM for n = 3 cultures.

Figure 5.10: % Epithelial cells stained positive for mucus in primary cultures derived form fish exposed to Prochloraz for 12 days.

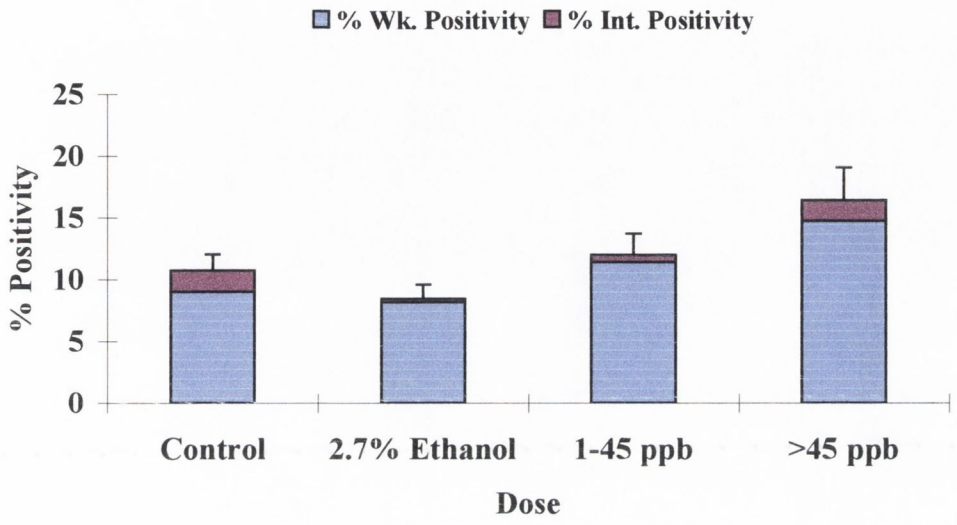


Figure 5.10 shows the % epithelial cells stained positive for mucus, using the PAS stain, in primary cultures from fish exposed to prochloraz for 12 days. The control cultures were taken from fish exposed for 0 days. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol value represents the mean ± SEM for n = 9 cultures. 1-45ppb prochloraz value represents the mean ± SEM for n = 11 cultures. > 45ppb value represents the mean ± SEM for n = 6 cultures.

Figure 5.11: % Epithelial cells stained positive for mucus in primary cultures derived form fish exposed to Nonoxynol/Prochloraz for 12 days.

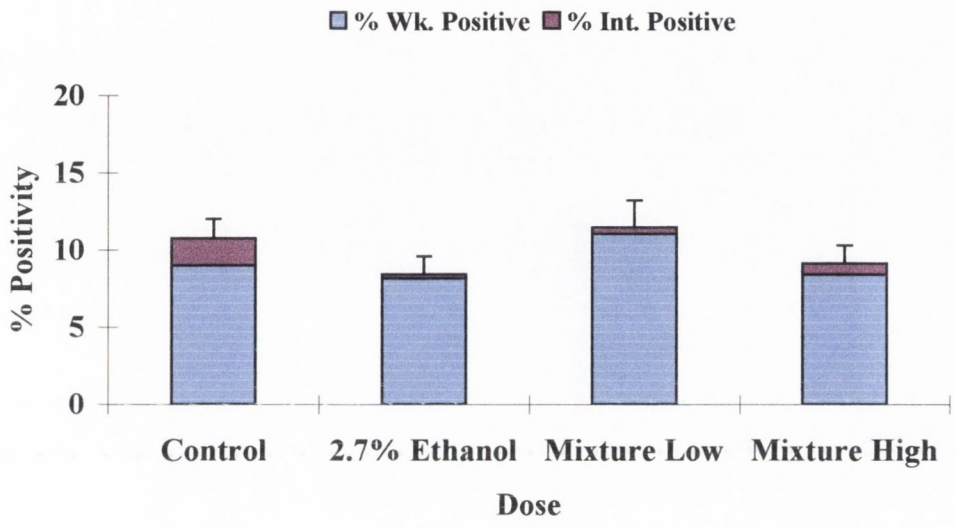


Figure 5.11 shows the % epithelial cells stained positive for mucus, using the PAS stain, in primary cultures from fish exposed to nonoxynol/ prochloraz mixture for 12 days. The control cultures were taken from fish exposed for 0 days. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol value represents the mean ± SEM for n = 9 cultures. High dose mixture value represents the mean ± SEM for n = 4 cultures. Low dose mixture value represents the mean ± SEM for n = 3 cultures.

Table 5.12: % Epithelial cells stained positive for mucus from cell cultures derived from fish exposed to 2.7% Ethanol for 5 and 12 days.

NO. OF DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
CONTROL	9.0 ± 1.0	1.7 ± 0.6	10.7 ± 1.3
5	7.0 ± 0.8	0.5 ± 0.2	7.5 ± 0.9
12	8.2 ± 1.2	0.2 ± 0.1	8.3 ± 1.2

The control value represents the mean ± SEM for n = 21 cultures. 2.7% ethanol values for 5 days and 12 days represents the mean ± SEM for n = 9 cultures.

Table 5.13: % Epithelial cells stained positive for mucus from cell cultures derived from fish exposed to 1-45 ppb Prochloraz for 5 and 12 days.

NO. OF DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
CONTROL	9.0 ± 1.0	1.7 ± 0.6	10.7 ± 1.3
5	11.2 ± 1.2	0.8 ± 0.3	12.0 ± 1.2
12	11.4 ± 1.7	0.6 ± 0.2	12.0 ± 1.7

The control value represents the mean ± SEM for n = 21 cultures. 1-45ppb prochloraz value for 5 days represents the mean ± SEM for n = 12 cultures. 1-45ppb prochloraz value for 12 days represents the mean ± SEM for n = 11 cultures.

Table 5.14: % Epithelial cells stained positive for mucus from cell cultures derived from fish exposed to >45 ppb Prochloraz for 5 and 12 days.

NO. OF DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
CONTROL	9.0 ± 0.10	1.7 ± 0.6	10.7 ± 1.3
5	16.8 ± 2.9	3.2 ± 1.6	20.0 ± 3.6
12	14.8 ± 2.4	1.7 ± 0.6	16.4 ± 2.7

The control value represents the mean ± SEM for n = 21 cultures. > 45ppb values for 5 and 12 days represents the mean ± SEM for n = 6 cultures.

Table 5.15: % Epithelial cells stained positive for mucus from cell cultures derived from fish exposed to a low level of Nonoxynol/Prochloraz Mixture for 5 and 12 days.

NO. OF DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
CONTROL	9.0 ± 1.0	1.7 ± 0.6	10.7 ± 1.3
5	8.3 ± 1.1	0.3 ± 0.6	8.6 ± 1.2
12	11.1 ± 1.6	0.4 ± 0.2	11.5 ± 1.8

The control value represents the mean ± SEM for n = 21 cultures. High dose mixture value for 5 days represents the mean ± SEM for n = 6 cultures. High dose mixture value for 12 days represents the mean ± SEM for n = 4 cultures.

Table 5.16: % Epithelial cells stained positive for mucus from cell cultures derived from fish exposed to a high level of Nonoxynol/Prochloraz Mixture for 5 and 12 days.

NO. OF DAYS EXPOSED	% WEAK POSITIVE	% INTENSE POSITIVE	TOTAL POSITIVITY
CONTROL	9.0 ± 1.0	1.7 ± 0.6	10.7 ± 1.3
5	13.6 ± 2.8	1.0 ± 0.5	14.6 ± 2.9
12	8.4 ± 1.0	0.7 ± 0.3	9.1 ± 1.2

The control value represents the mean ± SEM for n = 21 cultures. Low dose mixture values for 5 and 12 days represents the mean ± SEM for n = 3 cultures.

5.3.1 GROWTH OF PRIMARY EPITHELIAL CULTURES

The experiment explored whether or not a response would be observed in how long primary epithelial cultures which were cultured from fish exposed over a period of time to varying concentrations of an aquatic pollutant, continued to grow.

It was decided to take a random sample of fish from the tanks prior to the experiment starting. The % residual of day 3 growth of cultures taken from these fish was used as a control point for the entire experiment. The cultures were found to expand until day 9 and then decrease slightly until day 14 (fig. 5.1). However these samples were taken just after the fish had been added to the tanks and they may have been under some stress. The handling of these fish may also have slightly affected the growth of these explants. Three more fish were taken from three tanks, one for each dose eighteen hours post the start of the experiment. These cultures were still expanding after 14 days.

After 5 days exposure to the test substances cultures grown from fish exposed to the low dose of prochloraz (1-45ppb) were found to be still expanding after 14 days (Fig. 5.2). The % residual after fourteen days was found to be 246.15%± 23.8. Cultures from fish exposed to the high dose of prochloraz (>45ppb) for 5 days were found to on average expand for 9 days and then die back to the level of growth achieved after 3 days (fig. 5.3). The % residual of Day 3 growth after 14 days growth was 101.74± 29.6. Cultures grown from fish exposed to the high dose of the mixture of prochloraz and nonoxynol were found to expand until Day 11 and then begin to decrease in % residual of day 3 growth (fig. 5.4). The % residual of day 3 growth after 11 days growth was 153.61± 0.9, the % residual of day 3 growth after 14 days was 105.57± 48.7. A similar response was found with cultures taken from the fish exposed to the

low dose of the mixture for 14 days (fig. 5.5). The explants form this fish were found to expand quite rapidly and then begin to decrease in survival from 11 days growth. However the % residual of day 3 growth after 14 days was 150%, higher than that of the cultures grown from fish exposed to the high mixture dose and the high dose of the prochloraz.

After 12 days exposure to the test substances a different picture was observed however. All the cultures taken from all the fish, including those taken from the fish only exposed to 2.7% ethanol (fig. 5.1) were found to have a lower residual of day 3 growth for all days growth, from 5 days up to 11 days. The lowest residual of day 3 growth cultures after eleven days growth was found to be cultures taken from fish exposed to the highest dose of prochloraz (21.92%± 5.1) (fig. 5.3). The highest residual of day 3 growth values was found to be from fish exposed to the mixture.

5.3.2 PAS STAIN FOR MUCUS

The epithelial cell surfaces were classed as being negative, weakly positive and intensely positive to mucus (see Plate 4.4 and Plate 4.5). Cells were classed as weakly positive and intensely positive to mucus if they were stained light pink and dark pink respectively. Negative cells stained blue. The mucous cells contained in the cell cultures reacted with intense positivety to PAS staining. The mucous cells were not scored in the results.

The total positivity for mucus expression of the cultures derived from fish taken before exposure commenced was 10.7 ± 1.3 (Table 5.11). The average number of cells scored per explant was 874 ± 187. The % of cells expressing weak positivity for

mucus was 9.0 ± 1.0. The % of cells expressing intense positivity for mucus was 1.7 ± 0.1.

The number of cells scored per explant from fish exposed to only 2.7% ethanol for five days was 670 ± 118. The % of cells expressing weak positivity for mucus in these cultures was 7.0 ± 0.8. The % of cells expressing intense positivity for mucus was 0.5 ± 0.2. The total positivity for mucus expression was 7.5 ± 0.9 (Table 5.11). This value was not significantly different from that of the control (p=0.12). The % of cells expressing mucus was found to increase with the exposure level of the chemical pollutant added (figs. 5.8, 5.9). The average number of cells scored per explant from fish exposed to 1-45ppb prochloraz was 1027 ± 197. The % of cells in these cultures expressing weak positivity for mucus was 11.2 ± 1.2. The % number of cells expressing intense positivity for mucus was 0.8 ± 0.3. The total positivity for mucus expression was 12.0 ± 1.2 (Table 5.11). This value was not significantly different (p=0.48) to that of the control value but significantly different (p=.009) from that of the tanks containing 2.7% ethanol. The level of positivity was found to increase significantly in the cultures derived from fish exposed to >45ppb Prochloraz (fig. 5.8). This increase in positivity however, although very significant (p=.0011) in relation to the control, was only slightly significantly different (p=0.015) to the level of positivity of cells from fish exposed to 1-45ppb prochloraz. The average number of cells scored per explant derived from fish exposed to >45ppb prochloraz was 1008 ± 172. The % of cells expressing weak positivity for mucus was 16.8 ± 2.9. The % of cells expressing intense positivity for mucus was 3.2 ± 1.6. The total positivity for mucus expression was 20.0 ± 3.6 (Table 5.11).

A similar pattern of increased positivity was observed in the cultures derived from fish from fish exposed to the test pollutant for twelve days. A significant increase in positivity ($p=0.008$) was observed between cultures derived from fish exposed only 2.7% ethanol and those exposed to >45 ppb prochloraz (Fig 5.10). However no significant difference was observed between cultures from fish exposed to the test substances for five days and those exposed for twelve days ($p>0.4$ for all dose levels)(Table 5.11). The average number of cells scored per explant derived from fish exposed to 2.7% Ethanol was 740 ± 131. The % of the cells expressing weak positivity for mucus was 8.2 ± 1.2. The % of cells expressing intense positivity for mucus was 0.2 ± 0.1. The total positivity for mucus expression was 8.3 ± 1.2. The average number of cells scored per explant from fish exposed to 1-45ppb prochloraz was 1362 ± 278. The % of these cells expressing weak positivity for mucus was 11.4 ± 1.7. The % of cells expressing intense positivity for mucus was 0.6 ± 0.2. The total positivity for mucus expression was 12.0 ± 1.7. The average number of cells scored per explant derived from fish expose to >45ppb for 12 days was 1243 ± 167. The % of these cells expressing weak positivity for mucus was 14.8 ± 2.4. The % of cells expressing intense positivity for mucus was 1.7 ± 0.6. The total positivity for mucus expression was 16.4 ± 2.7 (Table 5.11).

5.4 DISCUSSION

Rainbow trout primary epithelial skin cell cultures cultured from fish exposed to the test substances were found to exhibit a markedly lower residual survival than that of the controls (fig. 5.7). Cultures grown after 5 days exposure to the higher dose of prochloraz (>45ppb) were found to exhibit a lower residual survival than the samples used as control points and the samples taken from fish exposed to the lower dose of prochloraz (fig 5.6). After 12 days exposure to this concentration of prochloraz the samples taken from fish in these tanks were found to exhibit a significantly lower % residual of day 3 growth than the controls (fig 5.7). This response suggests that the growth and survival of rainbow trout primary epithelial cell cultures is impeded after exposure of the host fish to greater than 45ppb prochloraz..

Cultures grown from exposed to the low dose of the prochloraz/nonoxynol mixture for 5 days were found to exhibit a % residual of day 3 growth very similar to that of fish exposed to the low dose of prochloraz for the same period of time (fig. 5.6). However samples taken from fish after 12 days exposure to the low dose of the mixture all exhibited a lower % residual of day 3 growth than that of the controls (Fig 5.7). Cultures grown from fish exposed to the high dose, for five days, of the mixture exhibited a % residual of day 3 growth similar to that of the control cultures (fig 5.6). However this % residual was found to decrease in samples grown for 9 days from fish exposed to the dose for twelve days (fig 5.7).

The % residual survival of the primary cultures from fish contained in the tanks exposed only to solvent control ,2.7% ethanol, demonstrate that there is not a significant difference between cultures grown from fish exposed for 5 days (fig. 5.6) and fish exposed for 12 days (fig. 5.7). The figures for both these days however are

below that of the controls (fig. 5.6 & fig 5.7). These results suggest that the changes in water quality over the experiment is affecting the survival of the samples. Due to problems in the experimental design of the apparatus these changes in water quality occurred.

These results demonstrate that there is an effect on the residual survival of primary epithelial cell cultures grown from fish exposed to a pollutant over a period of time. The primary skin cell cultures appear to demonstrate a kind of "memory effect". The growth and proliferation of the cells appear to be affected by the conditions that fish were exposed to. However this experiment however will have to be repeated because of problems encountered due to the experimental design of the apparatus and the small number of fish sampled. Because of the pilot nature of the study many uncertainties in the experimental conditions occurred. Some data points had to be regrouped according to what concentration of pollutant actually was entering the water. The result of this was that both the mixtures points and the high dose of prochloraz for the growth parameter were not of a statistically viable number. Only one explant was taken for each day of growth per each fish for the survival experiments resulting occasionally in large errors due to a variety of possible factors (individual variability, a damaged piece of tissue etc.). This was due to the lack of incubator space available . It was only discovered into the experiment that the length of the tubing from the pumping apparatus affected the amount of chemical entering the water. Some data points had to be regrouped according to what concentration of pollutant actually was entering the water. However these results in their crude form suggest that there is a use for the survival of primary epithelial cell cultures as a diagnostic tool in determining whether or not a fish is under stress, be it from a pollutant or other stress agents.

The % of cells expressing positivity for mucus in rainbow trout primary epithelial cultures cultured from control fish was found not to be significantly different form that for the cultures cultures from fish exposed to both 2.7% ethanol and the low dose of prochloraz. However a significant increase in positivity for mucus was observed between these cultures and the cultures cultured from fish exposed to the high dose of the pollutant (table 5.11). A certain quantity of the mucus contained on the control cultures was probably due to the handling and killing method of the fish. Pickering *et al.* (1977) found that stress, as represented by a single incidence of handling, increased mucus cell frequency in charr, *Salvelinus alpinus*.

No significant difference was found between cultures cultured from fish exposed for five days to the test substances and those exposed for twelve days (table 5.13 - table 5.15). These results suggest that the mucus response is dose related but not time related. Under conditions of stress fish epithelial after the secretion of the contents of the mucus cells are capable of differentiating into new mucus cells after 4 days. The appearance of newly-differentiated mucous cells at least partially compensates for losses of mucus cells caused by the intense secretion that occurs after initial exposure to the stress agent.(Iger *et al.,* 1994). Iger *et al.* (1988) found that fish maintained in organically fertilised water show a progressive reduction in the number of mucous cells in the epidermis during the first three days. A new wave of mucus cells had appeared by day 8. Our results demonstrate that the mucus cells in the epithelial cell cultures secrete a sufficient level of mucus onto the surface of the cells to protect against the particular dose level the fish has been exposed too. This quantity of mucus remains constant over the period of exposure at constant dose rates (table 5.13).

As stated in the introduction isolated cells that maintain the essential traits of the *in vivo* state during their culture *in vitro* provide an excellent experimental approach to establish diagnostic markers. This study demonstrates that *in vitro* primary skin epithelial cell cultures retain the characteristics of the *in vivo* condition of that of the fish they were cultured from. Cell cultures cultured from fish that were exposed to the highest dose of prochloraz were found to contain more mucus on their surface than those from fish exposed to low doses or controls. A similar result was obtained when rainbow trout primary epithelial cell cultures were exposed *in* vitro to prochloraz. The level of mucus on the surface of the epithelial cultures was found to increase *in vitro* with exposure to increasing doses of prochloraz (fig 4.5). The number of mucus cells contained per skin cell culture was also found to decrease in relation to increasing exposure of prochloraz *in vitro* (3.8). Hence primary skin epithelial cell cultures and their mucus content may have a valuable use as a biomarker of pollution and a stress indicator.

A new "*ex vivo*" approach was used in this study. As stated previously some uncertainties arose in the carrying out of this experiment due to the pilot and inniovative nature of the approach used. It is clear however that significant *ex vivo* results in the parameters tested were obtained which can be causatively traced back to the *in vivo* situation. As a result this approach merits further investigation as a suitable method for evaluating *in vivo* conditions. The parameters tested also merit further investigation as possible biological indicators of chemical exposure and stress indicators.

5.5 CONCLUSION

A new "*ex vivo*" approach was used in this study. Primary epithelial skin cell cultures were cultured from rainbow trout that were exposed *in* vivo to prochloraz and a mixture of prochloraz and nonoxynol in order to investigate whether a similar response observed in cultures exposed *in vitro* to prochloraz could be observed in cultures cultured from fish exposed *in vivo* to the test substances Significant *ex vivo* results in the parameters (growth and mucus production) tested were obtained which can be causatively traced back to the *in vivo* situation. These results indicate that the biological response detected in the isolated cell in an *in vitro* environment could be detected again in an *ex vivo* situation. As a result the approach used in this chapter evaluating *in vivo* conditions merits further investigation. The parameters tested also merit further investigation as possible biological indicators of chemical effect, exposure and stress indicators.

CHAPTER 6

THE % RESIDUAL GROWTH AND POSITIVITY FOR MUCUS OF RAINBOW TROUT PRIMARY SKIN CELL CULTURES CULTURED FROM FISH EXPOSED TO NONOXYNOL, *IN VIVO*, IN DECHLORINATED TAP WATER AND RIVER WATER

6.1 INTRODUCTION

As a result of the experiment described in Chapter 5 it was decided to carry out another experiment to clarify and investigate the ability of primary epithelial cell cultures cultured *in vitro* to demonstrate the condition of the water from which the fish that they are cultured from were kept in. It was decided to use the pollutant nonoxynol for the experiment in order to investigate if the effects observed in Chapter 5 are similar to those observed when the fish are exposed to differing pollutants. It was also decided to expose the fish to the test substance in river water as well as dechlorinated tap water. This was done in order to attempt to take the exposure set up one step nearer the conditions observed in the wild.

It was also decided to add the test substances to the water manually so that the dose levels remained constant during the experiment. This was due to the problems observed in the use of varying lengths of tubing in the experiment outlined in chapter 5.

The two endpoints investigated were the growth of the primary cultures and the level of mucus contained on the surface of the primary cultures. It was also investigated whether a relationship existed between the level of mucus contained on the surface of the cultures and the survival of these cultures.

6.2 MATERIALS AND METHODS

6.2.1 Experimental set up

Fifteen bath tubs (enamelled iron; volume: 200 liters; colour: clear sand) were installed on two superimposed levels. Water was renewed once daily at the rate of 50% (i.e. 100 liters per tub per day) during the experiment. Nine of the tanks contained dechlorinated water. The other six contained river water from the Couesnon river in Rennes, France. Oxygen was bubbling continuously from the bottom of the tubs. The chemicals were added manually each day directly after the water change. Organic matter (faeces and unused pellets) in suspension in the water was not removed, but a part was permanently eliminated with the water removed out of the tubs.

Rainbow trout were placed in each of the fifteen tanks and allowed to acclimatise to the conditions for 7 days. Three of the tanks containing dechlorinated tap water were exposed to 100µg/l nonoxynol, three to 400µg/l nonoxynol and three were exposed to 2.7% Ethanol (the solvent control). Three of the tanks containing river water were exposed to 400µg/l nonoxynol. The remaining three tanks containing river water to which 2.7% ethanol was added. The fish were exposed to the pollutants for nine days. During the exposure period water samples were taken at regular intervals to determine the actual concentration of chemical in the water. Water samples were also taken to determine the quality of the water over the exposure period.

6.2.2 Sampling

Six fish were taken from three different tanks before any chemical entered the tanks to be used as controls for the experiment prior to the commencement of the exposure to the pollutant. The fish were killed by a swift blow to the back of the head, weighed and

measured. One fish was taken and killed from each of the three separate tanks for each exposure level after nine days exposure to the test substances and the above carried out.

6.2.3 Primary culture Technique

Primary epithelial cell cultures were set up as described in Chapter 3 Section 2.1.

6.2.4 Growth of the primary cell cultures

Three days post attachment the perimeter of the cell cultures was outlined on the bottom of the flask with permanent marker. Three cultures from each fish sampled were fixed after 3 days, 5 days, 7 days and 9 days growth in 10% buffered Formalin and counterstained in haematoxylin. The outgrowth area from the explant was estimated using a $1mm^2$ grid by counting the number of squares covered by the explant outgrowth. The area before treatment was also measured. The results are given as a percentage of the control survival rate. The mean values and standard errors were calculated.

6.2.5 Staining

After three days growth half the primary epithelial cultures were fixed as follows. The culture media was removed from the flask. The cultures were then washed in P.B.S. and fixed in 10% formalin. The primary epithelial cell cultures were stained with periodic acid Schiff (PAS) for mucus as described in Chapter 4 Section 4.2.2.

6.3 RESULTS

The following water physiochemical parameters were taken during the experiment.

a = after adding the chemical;

b = before adding the chemical

TC = Tap Water Control; T100 + 100μg/l nonoxynol; T400 = Tap Water + 400μg/l nonoxynol; RC = River Water Control; R400 = River Water + 400μg/l nonoxynol;

Table 6.1: Temperature °C

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	-	10.9	11.25	11.5	12.6	11.1
R400 T13	9	10.5	11.1	11.4	12.6	11.1
TC T19	9	11.1	11.5	11.6	12.4	11.1
T100 T20	-	10.8	11.2	11.8	12.2	11.1
T400 T14	-	10.8	11.1	11.3	12.2	11.1

Table 6.2: pH

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	-	7.12	9.05	7.2	-	7.56
R400 T13	-	7.14	7.11	7.44	-	7.55
TC T19	-	7.61	7.47	7.50	-	7.59
T100 T20	-	7.54	7.45	7.36	-	7.65
T400 T14	-	7.45	7.37	7.86	-	7.77

Table 6.3: Oxygen

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	-	-	3	4.9	8.7	10.6
R400 T13	-	-	6.2	6.8	8.8	10.5
TC T19	-	-	8.6	9.7	9.3	10.1
T100 T20	-	-	8.3	8.5	9.6	10.2
T400 T14	-	-	9	10.1	10.4	10.8

Table 6.4: $N\text{-}NO_3$ (mg/l)

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	6	6.8	7.6	8.0	6.8	7.8
R400 T13	6	6.3	6.8	8.0	6.6	6.9
TC T19	2.5	2.8	2.4	2.8	2.9	3.2
T100 T20	2.5	2.3	2.8	2.7	2.6	3.4
T400 T14	2.5	2.5	2.6	2.9	2.6	3.9

Table 6.5: $N\text{-}NH_{3\text{-}4}$ (mg/l)

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	0.87	0.99	0.31	0.52	0.38	0.36
R400 T13	0.65	0.49	0.32	0.56	0.38	0.30
TC T19	0.46	0.61	0.60	0.45	0.48	0.16
T100 T20	0.61	0.38	0.84	0.65	0.43	0.17
T400 T14	0.48	0.54	0.32	0.31	0.23	0.13

Table 6.6: $N\text{-}NO_2$ (mg/l)

Tank	23/11/98a	23/11/98b	27/11/98b	27/11/98a	30/11/98a	02/12/98b
RC T21	0.039	0.050	0.180	0.125	0.083	0.037
R400 T13	0.079	0.105	0.320	0.205	0.078	0.056
TC T19	0.008	0.008	0.018	0.012	0.123	0.141
T100 T20	0.009	0.002	0.013	0.022	0.063	0.158
T400 T14	0.003	0.003	0.038	0.029	0.043	0.131

Table 6.7: Apparent food intake

Tank	Treatment	24/11	25/11	26/11	27/11	30/11	1/12	Aver.
6	TC	0.5	0.5	0.5	0.5	0.5	0.2	0.45
10	T100	0.3	0.3	0.5	0.5	0.5	0.2	0.38
11	T400	0.1	0.2	0.1	0.1	0.1	0.1	0.12
14	T400	0.1	0.2	0.1	0.1	0.1	0.1	0.12
17	T100	0.3	0.4	0.1	0.1	0.1	0.1	0.18
19	TC	0.1	0.1	0.1	0.1	0.1	0.1	0.12
20	T100	0.3	0.3	0.3	0.2	0.3	0.1	0.25
22	T400	0.1	0.1	0.3	0.1	0.1	0.1	0.13
23	TC	0.1	0.1	0.1	0.1	0.1	0.1	0.12

Table 6.8: Actual Nonylphenol diethoxylate concentration in water (ppb)

Tank	Treatment	Conc. of Chem. (ppb)
17	T100	50
22	T400	215
24	R400	84

All water parameter readings were taken by the technical staff of INRA, Rennes France.

6.3.1 SURVIVAL OF EPITHELIAL PRIMARY CULTURES

Table 6.9 The % residual of day 3 growth of rainbow trout primary epithelial cell cultures exposed to nonoxynol in dechlorinated tap water and river water.

Tank + Dose	No. of Days Exposed	% Residual of Initial Day 3 Growth			
		Day 3	Day 5	Day 7	Day 9
Control	0	100	114.24 ± 8.1	153.02 ± 12.8	204.32 ± 36.2
Tap Control	9	100	122.52 ± 11.7	108.48 ± 10.9	106.17 ± 13.1
Tap +100ppb nonoxynol	9	100	115.56 ± 11.6	145.94 ± 16.8	125.68 ± 25.5
Tap +400ppb nonoxynol	9	100	109.10 ± 3.1	97.87 ± 17.5	81.31 ± 9.1
River Control	9	100	101.60± 20.9	165.60 ± 31.7	151.60± 28.2
River +400ppb nonoxynol	9	100	150.30 ± 14.3	138.43 ± 19.2	165.59 ± 30.4

The control - 0 days exposure represents the mean ± SEM for n = 18. The tap + 100ppb values, tap + 400ppb values, tap control (tap water + 2.7% ethanol) values, river + 400ppb values and river control (river + 2.7% ethanol) values represent the mean ± SEM for n = 9.

Figure 6.1: The % residual of day 3 growth of epithelial primary cell cultures cultured from fish exposed to nonylphenol diethoxyate for 9 days in dechlorinated tap water and river water.

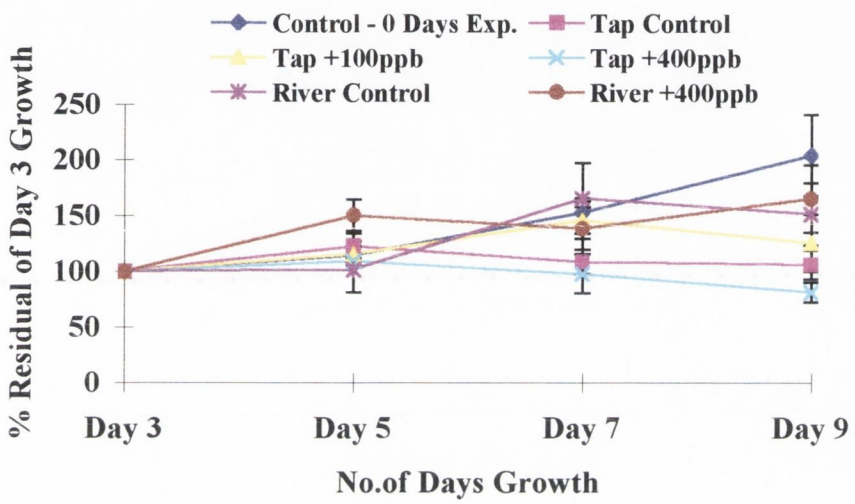


Figure 6.1 shows the % residual of day 3 growth of rainbow trout primary epithelial cell cultures from fish exposed to 100ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in river water, 2.7% ethanol in both dechlorinated tap water and river water. The control values for 0 days exposure represent the % residual of day 3 growth of rainbow trout primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The control - 0 days exposure represents the mean ± SEM for n = 18. The tap + 100ppb values, tap + 400ppb values, tap control (tap water + 2.7% ethanol) values, river + 400ppb values and river control (river + 2.7% ethanol) values represent the mean ± SEM for n = 9.

Figure 6.2: The % residual of day 3 growth of epithelial primary cell cultures cultured from fish exposed to nonylphenol diethoxyate for 9 days in dechlorinated tap water.

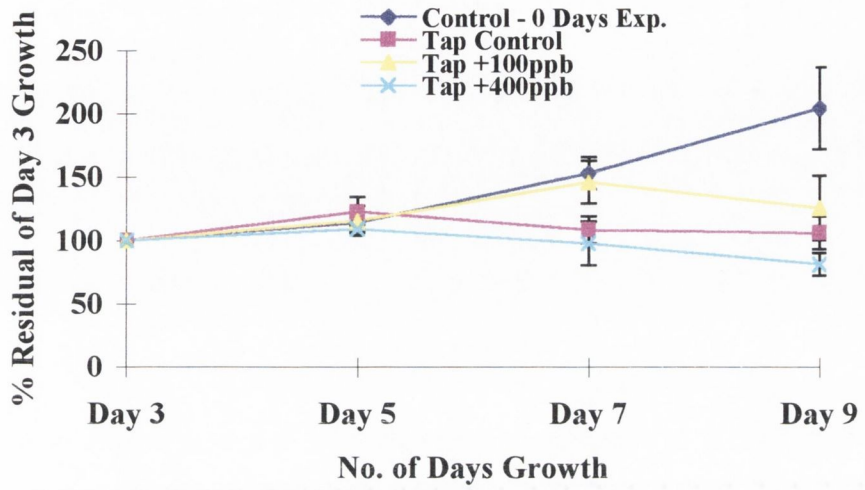


Figure 6.2 shows the % residual of day 3 growth of rainbow trout primary epithelial cell cultures from fish exposed to 100ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in dechlorinated tap water and 2.7% ethanol in both dechlorinated tap water. The control values for 0 days exposure represent the % residual of day 3 growth of rainbow trout primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The control - 0 days exposure represents the mean ± SEM for n = 18. The tap + 100ppb values, tap + 400ppb values and tap control (tap water + 2.7% ethanol) values represent the mean ± SEM for n = 9.

Figure 6.3: The % residual of day 3 growth of epithelial primary cell cultures cultured from fish exposed to nonylphenol diethoxyate for 9 days in river water.

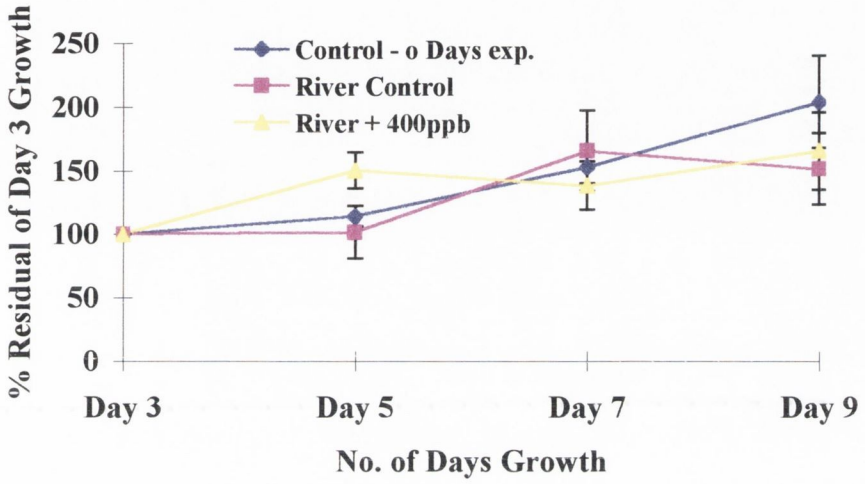


Figure 6.3 shows the % residual of day 3 growth of rainbow trout primary epithelial cell cultures from fish exposed to 400ppb nonoxynol in river water and 2.7% ethanol in both dechlorinated river water. The control values for 0 days exposure represent the % residual of day 3 growth of rainbow trout primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The control - 0 days exposure represents the mean ± SEM for n = 18. The river + 400ppb values and river control (river + 2.7% ethanol) values represent the mean ± SEM for n = 9.

Table 6.10: The % residual of day 3 growth of cultures grown for 9 Days versus the conc. of nonoxynoldiethoxylate actually contained in the water.

Tank	Days Exposure	Chemical Conc. (ppb)	Day 9 % Survival
Control	0	0	204.32 ± 36.2
Tap Control	9	0	106.17 ± 13.1
T17	9	50	117.77 ± 5.2
T22	9	215	91.88 ± 5.0
River Control	9	0	151.60 ± 28.2
R24	9	84	258.57 ± 70.0

6.3.2 PAS STAIN FOR MUCUS

Table 6.11: The % Epithelial cells stained positive for mucus from derived from fish exposed to Nonoxynoldiethoxylate in tap water and river water for 9 days.

DOSE	DAYS EXPOSURE	% WEAK POSITIVITY	% INTENSE POSITIVITY	TOTAL POSITIVITY
0	0	6.9 ± 1.2	2.3 ± 0.7	9.2 ± 1.7
Tap Water + 0	9	19.3 ± 1.7	2.3 ± 0.8	21.6 ± 1.7
Tap Water + 100mg/l nonoxynol	9	15.3 ± 2.4	3.8 ± 1.2	19.1 ± 3.2
Tap Water + 400µg/l nonoxynol	9	20.6 ± 1.6	8.4 ± 2.3	29.0 ± 2.3
River Water + 0	9	13.2 ± 1.7	5.0 ± 1.3	18.2 ± 2.1
River Water + 400µg/l nonoxynol	9	14.3 ± 1.1	3.8 ± 1.3	18.1 ± 1.5

The control value represents the mean ± SEM for n = 17. The tap + 100ppb value represents the mean ± SEM for n = 7. The tap + 400ppb value represents the mean ± SEM for n =9. The tap control (tap water + 2.7% ethanol) value represents the mean ± SEM for n = 9. The river + 400ppb value represents the mean ± SEM for n =8. The river control (river + 2.7% ethanol) value represent the mean ± SEM for n = 7.

Figure 6.4: The % Epithelial cells stained positive for mucus from primary cultures derived from fish exposed to Nonoxynoldiethoxylate in tap water and river water for 9 days.

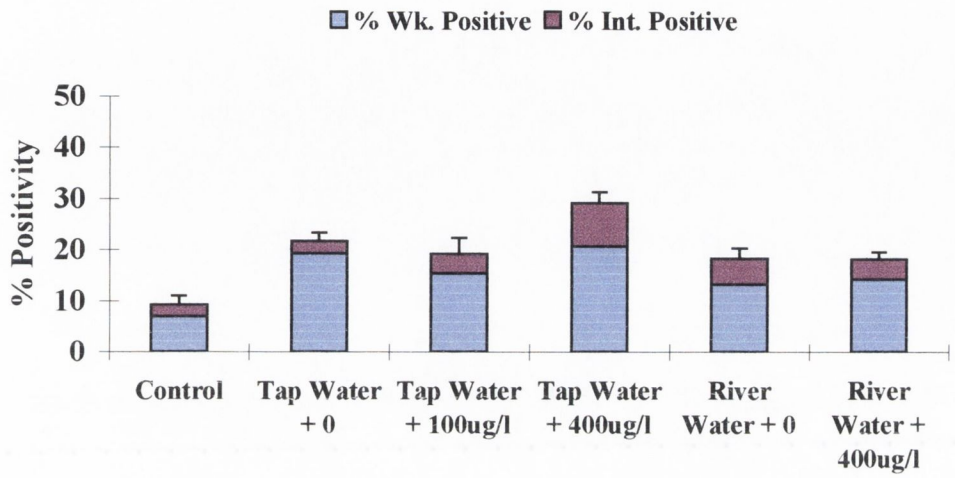


Figure 6.4 shows the % epithelial cells stained positive for mucus in primary epithelial skin cell cultures from fish exposed to 100ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in river water, 2.7% ethanol in both dechlorinated tap water and river water for 9 days, using the PAS stain. The control values for 0 days exposure represent the % epithelial cells stained positive for mucus in primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 17. The tap + 100ppb value represents the mean ± SEM for n = 7. The tap + 400ppb value represents the mean ± SEM for n =9. The tap control (tap water + 2.7% ethanol) value represents the mean ± SEM for n = 9. The river + 400ppb value represents the mean ± SEM for n =8. The river control (river + 2.7% ethanol) value represent the mean ± SEM for n = 7.

Figure 6.5: The % Epithelial cells stained positive for mucus from primary cultures derived from fish exposed to Nonoxynoldiethoxylate in tap water for 9 days.

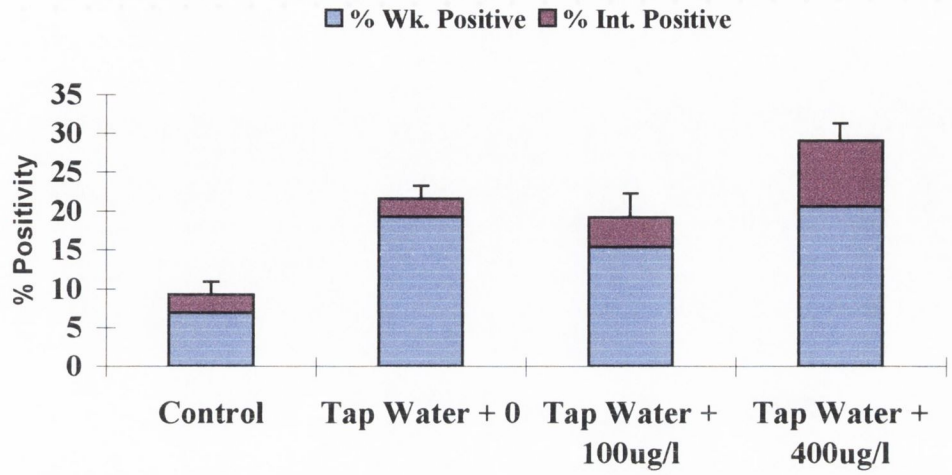


Figure 6.5 shows the % epithelial cells stained positive for mucus in primary epithelial skin cell cultures from fish exposed to 100ppb nonoxynol in dechlorinated tap water, 400ppb nonoxynol in dechlorinated tap water and 2.7% ethanol in both dechlorinated tap water, using the PAS stain. The control values for 0 days exposure represent the % epithelial cells stained positive for mucus in primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 17. The tap + 100ppb value represents the mean ± SEM for n = 7. The tap + 400ppb value represents the mean ± SEM for n =9. The tap control (tap water + 2.7% ethanol) value represents the mean ± SEM for n = 9.

Figure 6.6: The % Epithelial cells stained positive for mucus from primary cultures derived from fish exposed to Nonoxynoldiethoxylate in tap water for 9 days.

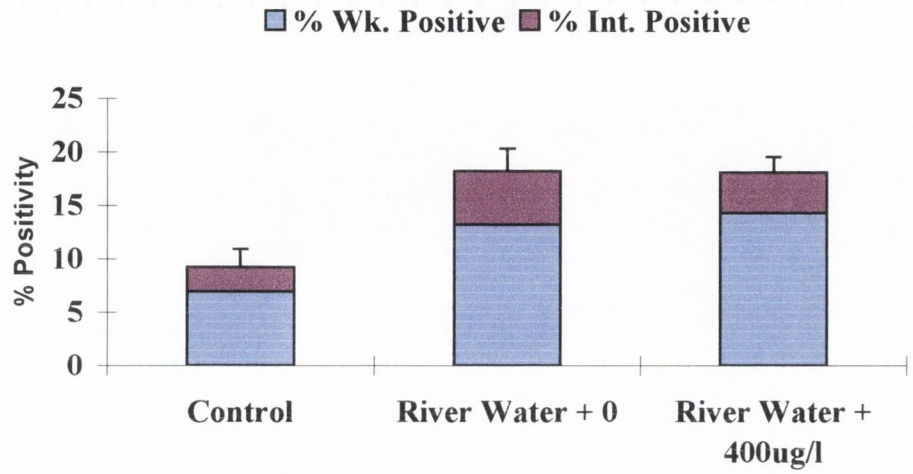


Figure 6.6 shows the % epithelial cells stained positive for mucus in primary epithelial skin cell cultures from fish exposed 400ppb nonoxynol in river water and 2.7% ethanol in river water for 9 days, using the PAS stain. The control values for 0 days exposure represent the % epithelial cells stained positive for mucus in primary epithelial cultures from fish which were allowed to acclimatise in dechlorinated tap water for 7 days in the experimental set up prior to the addition of the test substances. The cells were scored as been negative, weakly positive and intensely positive. The control value represents the mean ± SEM for n = 17. The river + 400ppb value represents the mean ± SEM for n =8. The river control (river + 2.7% ethanol) value represent the mean ± SEM for n = 7.

Table 6.12: The % Residual of day 3 growth of cultures grown for 9 days from fish exposed to Nonoxynoldiethoxylate in tap water and river water versus the % number of cells expressing positiviy for mucus from cultures grown from the same fish.

DOSE	NO. OF DAYS EXPOSED	% RESIDUAL SURVIVAL OF DAY 3 GROWTH	% MUCUS POSITIVITY
0	0	204.32 ± 36.2	9.2 ± 1.7
Tap Water + 0	9	106.17 ± 13.1	21.6 ± 1.7
Tap Water + 100µg/l nonoxynol	9	125.68 ± 25.5	19.1 ± 3.2
Tap Water + 400µg/l nonoxynol	9	81.31 ± 9.1	29.0 ± 2.3
River Water + 0	9	151.60 ± 28.2	18.2 ± 2.1
River Water + 400µg/l nonoxynol	9	165.59± 30.4	18.1 ± 1.5

Table 6.12: The % residual of day 3 growth of cultures grown for 9 Days and mucus positivity compared to water physiochemical parameters readings taken the day of sampling.

Tank + Dose	Days Exp.	% growth.	% Muc. Pos.	Food Int. (%)	^0C	Ph	O_2	N-NO_3 mg/l	N-NH_{3-4} mg/l	N-NO_3 mg/l
T19 0ppb	0	238.5 ± 93	12.5 ±4.4		9			2.5	0.46	0.008
T19 0ppb	9	81.55 ± 11	24.5 ±3.0	0.12	11.1	7.59	10.1	3.2	0.16	0.141
T20 100 ppb	9	175.9 ± 48	21.7 ±5.1	0.25	11.1	7.65	10.2	3.4	0.17	0.158
T14 400 ppb	9	57.77 ± 11	30.8 ±3.2	0.12	11.1	7.77	10.8	3.9	0.13	0.131
R21 0ppb	9	170.4 ± 57	14.0 ±3.2		11.1	7.56	10.6	7.8	0.36	0.037
R13 400 ppb	9	134.2 ± 28	19.4 ±3.1		11.1	7.55	10.5	6.9	0.30	0.056

6.3.1 The quantity of chemical pollutant contained in the water.

The actual quantity of chemical pollutant in the water was as follows. The tanks exposed to 100ppb nonoxynol in dechlorinated tap water contained 50 ppb of pollutant. The tanks exposed to 400ppb nonoxynol in dechlorinated tap water contained 215 ppb of pollutant. The tanks exposed to 400ppb nonoxynol in river water were found to contain 84 ppb of the pollutant. The decrease in the concentration of nonoxynol actually contained in the water could have been as a result of the following factors 1) absorption onto the surface of the fish 2) absorption onto the surface of the tanks and 3) binding to faeces and sediment contained in the water.

6.3.2 Explant Survival (see table 6.9).

The explants cultured on day 0 were found to continue to grow for up to 9 days. The % residual of initial day 3 growth area was 204.32 ± 36.2 (Table 6.9, fig 6.1). The response of the cultures derived from fish exposed to in dechlorinated tap water for 9 days differed extremely significantly from that of the control ($p<.0001$). The % residual of initial day 3 growth was found to expand until day 5 to 122.52 ± 11.7 and then decreased slightly until day 9 (Table 6.9). The % residual of initial day 3 growth after 9 days was 106.17 ± 13.1. The % residual of cultures from fish exposed to 100ppb nonoxynol in dechlorinated tap water was found to be significantly different from that of the control values ($P<0.0001$) but not significantly different ($p=0.06$) from the cultures derived from fish contained for 9 days in dechlorinated tap water (Table 6.9, fig 6.2). The explants were found to expand for 7 days and then decreased slightly in area. The % residual of initial day 3 growth after 9 days was found to be 125.68 ± 25.5. The % residual of day 3 growth of the cultures grown from fish treated with 400ppb nonoxynol for 9 days in dechlorinated water was found to be significantly different

from that of the control values ($p<.0001$), from the cultures grown from fish exposed in for 9 days ($p<.0001$)and from fish exposed to 100ppb nonoxynol for 9 days ($p<.0001$). The cultures grown from fish exposed to the high dose of the pollutant for 9 days in dechlorinated tap water was found to increase slightly in area until day 5 before dying off (Table 6.9, fig 6.2).. The % residual of day 3 growth after 9 days was 81.31 ± 9.1. The % residual survival of cultures grown from fish exposed in river water, although exhibiting a significantly lower residual growth than that of the control cultures ($p<.0001$), had a significantly higher residual of day 3 growth than that of the cultures derived from fish exposed in dechlorinated tap water. The % residual of day 3 growth of the primary cultures cultures from fish exposed to in river water after 9 days was 151.60 ± 28.2 (Table 6.9, fig 6.3). The % residual of day 3 growth of cultures derived from fish exposed to 400ppb nonoxynol after 9 days was 165.59 ± 30.4. These values were not found to be significantly different from each other ($p=0.3$) (Table 6.9, fig 6.3).

3.3 % Positive staining for mucus (see table 6.3).

The epithelial cell surfaces were classed as being negative, weakly positive and intensely positive to mucus (see plate 4.1 and plate 4.2). Cells were classed as weakly positive and intensely positive to mucus if they were stained light pink and dark pink respectively. The mucous cells contained in the cell cultures reacted with intense positivity to PAS staining. The mucous cells were not scored in the results.

The % of cells expressing positive staining for mucus in cultures derived from fish taken before exposure to the test substances was 9.2 ± 1.7. The % of cells expressing weak positivity for mucus was 6.9 ± 1.3, the % of cells expressing intense positivity was 2.3 ± 0.7. The average number of cells counted per culture was 427 ± 65 (Table 6.10).

The % of cells which expressed positivity for mucus in the cultures derived from the fish taken as controls for the experiment was found to differ significantly for that of the cultures derived from the fish exposed for 9 days to the test substances ($p<0.0001$). The % of cells expressing % staining for mucus derived from fish exposed to 2.7% ethanol in dechlorinated tap water for 9 days was 21.6 ± 1.7, the % of cells expressing weak positivity for mucus was 19.3 ± 1.7, the % of cells expressing intense positivity was 2.34 ± 0.8 (Table 6.10). The average number of cells scored per culture was 414 ± 75. The % of cells expressing positivity for mucus in cultures derived from fish exposed to 100ppb nonoxynol for 9 days did not differ significantly ($p=.06$) (Fig. 6.5). The % positivity for mucus was 19.1 ± 3.2. The % of cells expressing weak positivity was 15.3 ± 2.4, the % of cells expressing intense positivity for mucus was 3.8 ± 1.2 (Table 6.10). The average number of cells scored per explant was 424 ± 66. The % of cells expressing positivity for mucus was found to increase significantly ($p<.0001$) in the cultures grown from fish exposed to 400ppb nonoxynol for 9 days in dechlorinated tap water (Fig. 6.5). The % of these cells expressing positiviy for mucus was 29.0 ± 2.3. The % positivity of cells expressing weak positivity was 20.6 ± 1.6, the % of cells expressing intense positivity was 8.4 ± 2.3 (Table 6.10). The average number of cells scored in these cultures was 498 ± 87.

The cultures derived from fish exposed to in river water for 9 days were found to exhibit a significantly lower % positivity for mucus than the cultures grown from fish exposed in dechlorinated tap water for 9 days ($p=.003$) (Fig. 6.4). The % of cells expressing positivity for mucus was 18.2 ± 2.1. This figure was made up of 13.2 ± 1.7 % weak positivity and 5.0 ± 1.3 intense positiviy (Table 6.10). The average number of cells counted per culture was 468 ± 59. The level of positiviy was not found to

significantly change in the cultures derived from fish exposed to 400ppb nonoxynol for 9 days (p=0.9) (Fig. 6.4). The % of cells expressing weak positivity to mucus was 14.3 ± 1.1, the % number of cells expressing intense positivity for mucus was 3.8 ± 1.3. The total % of cells expressing positivity for mucus was 18.1 ± 1.5 (Table 6.10). The average total of cells scored per explant was 655 ± 111.

6.4 DISCUSSION

The results show that the explants cultured from fish under stress do not continue to grow at the same rate as primary cultures cultured from control fish. These results suggest that below a certain level nonoxynol was not found to be harmful to the cells. The difference between the growth values obtained from fish exposed to the solvent control and the low level of nonoxynol for 9 days and those values obtained from fish sampled before the experiment started, may be due to stress associated with the experimental set up and not the chemical dose. A similar pattern emerges if one looks at the level of mucus contained on the primary cultures from these fish. The amount of mucus on the control fish was found to be very similar to that contained on the controls in chapter 5. However the level of mucus was found to be quite high in fish contained in the experimental set up for the exposure period (Fig. 6.5). Again no significant difference was found in the mucus level of the primary skin cultures from fish taken from tanks containing less than 100 ppb nonoxynol and the primary cultures from fish taken from tanks containing the same water type as the treated fish (i.e. dechlorinated tap water and river water). The % of cells containing a mucus coat was highest in cultures from fish exposed to 400µg/l nonoxynol in dechlorinated tap water. The actual concentration of nonoxynol contained in the culture was 215µg/l. A high percentage of this mucus was found to stain intensely.

The residual growth of the skin cultures was found to be directly related to the amount of mucus contained on their surface (R=0.95). Iger *et al.* (1994) found that the pavement skin cells of rainbow trout exposed to Rhine water contained significantly more secretory vessels than control fish and that an increase in necrosis was apparent after 24h exposure, an increase in apoptosis was apparent after 4 days exposure. The

authors also observed mucus secretion was intense and high differentiation of mucus cells was stimulated in exposed fish compared with control fish and that some of these cells synthesised mucus of high electron density. Iger *et al.* (1988) observed that carp maintained in water fertilised with organic manure developed a thick mucus coat on the outside of the epidermis compared with controls. The mucus level contained on the primary skin cultures cultured from fish contained in river water was found to be significantly higher than control fish in my study. This is very similar to that observed in the experiments carried out by Iger *et al.* (1994, 1988). The continuously forming glycocalyx probably acts as a defence against bacterial penetration into the skin, reduces the friction between the body surface and suspended particles and acts as a defence against any pollutants contained in the water.

One obvious question exists when one looks in detail at the results obtained from this experiment. If, as it appears, that both primary skin culture growth and survival and mucus secretion are directly related to the amount of chemical the fish are exposed to why are untreated fish kept in dechlorinated tap water for the exposure period less healthy that the control fish and fish contained in river water? If one looks at the feeding rates of these fish they appear to be quite low. This suggests that the fish were under stress caused by the experimental set up or the dechlorinated tap water. However difficulties arose in calculating the feeding rates of the fish contained in the river water. All feeding rate data was done by the naked eye. It was impossible to differentiate between suspended organic matter and suspended feed. Reduced feeding rates is a well documented response of fish to a stress situation (Collvin, 1984; Waiwood *et al.*, 1978). It is also possible that the colour of the clear dechlorinated water can cause stress to the fish causing and an increase in the amount of mucus secreted onto the surface of the fish. Due to the clear water the fish are exposed to more light than normal which would

result in stress. This may help to explain why fish contained in the darker river water are more healthy than those contained in clear water. It appears that only a portion of the mucus secretion is due to the effect of the pollutant, the remaining mucus production resulting from the stress caused by the experimental conditions.

Other problems existed in this study. It was assumed that the level of pollutant and other water quality parameters in the water were the same in each tank per dose level. Hence only one tank was sampled per dose to assess the amount of chemical contained in the water and the water chemistry of the tanks. As a result it is impossible to state if the effects observed are definitely due to chemical pollution or the experimental conditions. In future each tank will have to be monitored separately for all parameters to build up a fuller picture of the actual conditions which exist in the experiment for each population of fish contained per tank. Also in future three to six fish should be taken from each taken so that differences between populations can be taken into accounts. However the modified design of the experiment resulted in an improved set of results compared with the first study. The growth data obtained is vastly improved from that obtained in the study with prohloraz and gave a clearer picture that the *ex vivo* growth and survival of rainbow trout primary cultures is dependent on the conditions that the host fish were exposed to. Also confirmation of the mucus response *ex vivo* to the *in vivo* conditions observed in the study with prochloraz was obtained in this study with nonoxynol. These results, combined with those obtained in the previous chapter demonstrate that primary rainbow trout skin cell cultures exhibit potential as a tool in the monitoring of the aquatic environment.

6.5 CONCLUSION

The results obtained in this study add support to those described in chapter 5. Mucus secretion and cell growth are affected by stress. However it is too difficult to say from these studies whether the response is a specific chemical response or just as a result of general stress situations. It is however possible to state that primary rainbow trout skin cultures show potential uses in environmental monitoring. Significant *ex vivo* results in the parameters (growth and mucus production) tested were obtained which can be causatively traced back to the *in vivo* situation. These results indicate that the biological response detected in the isolated cell in an *in vitro* environment could be detected again in an *ex vivo* situation. Hence primary skin epithelial cell cultures, their growth and proliferation and mucus content may have a valuable use as a biomarker of pollution and a stress indicator. Primary skin cultures may provide a cost effective rapid technique to determine if a problem exists in a particular aquatic environment.

CHAPTER 7

GENERAL DISCUSSION

As stated in the introduction, the object of this study was to develop fish epithelial cellular markers as diagnostic tools for effect assessment in ecotocicology. The project comprised of three major tasks. The first task consisted of research on cultured cells in order to establish the basic characteristics of the link between exposure and inducible response. The second task centred on the development of cellular markers of exposure and stress. The third task centred on research on intact fish in the laboratory in order to clarify a) whether the biological response detected in the isolated cell culture in an *in vitro* environment can be detected again in the intact *in vivo* environment and b) if the biological response has the potential for use as a suitable biomarker for effect or exposure.

The first piece of work carried out in this study involved the use of immortalized cell lines. The major advantage of their use was that it is possible to study the effects of a chemical in future generations. This property was used to investigate whether or not the chemicals caused delayed cell death. As was demonstrated in Chapter 2 all three chemicals caused this phenomenon. However, with the exception of been enable to carry out research into the effect of chemicals into future generations cell lines do not present as adequate a diagnostic tool as rainbow trout primary skin epithelial cell cultures. Isolated cells that maintain the essential traits of the *in vivo* state during culture *in vitro* provide an excellent experimental approach to establish diagnostic markers Because the characteristics of established cell lines deviate substantially from normal counterparts, fish-derived primary cells in culture may represent an alternative for toxicity studies. Primary cells can be made to express a high number of differentiated characteristics (Mothersill *et al.,* 1995) If differentiated properties are lost, it is difficult to relate the

cultured cells to functional cells in the tissue from which they are derived (Freshney, 1994). The primary cell culture used in this study contains both pavement cells and goblet mucus cells. It was possible to study the changes in both of these cell types when exposed to chemical stresses.

The toxicity of each of the three chemicals nonoxynol, prochloraz and copper was tested using the primary cell cultures. The first parameter used was survival. A system was developed where it was possible to screen rapidly the toxicity of chemicals by measuring the area of the cell culture before the chemical was added and comparing this area to the area of the cell culture after the exposure. However one major point became evident when testing the chemicals. One must consider the properties of the chemical to be tested when one is deciding whether or not to test the chemical on the cell culture in media containing serum or serum-free medium. One most have prior knowledge of the composition of the serum if one is to adequately assess different chemicals in relation to each other. Components of serum (e.g. albumin) are capable of binding to certain chemicals resulting in either an increase or decrease in their uptake by the cell.

The next step was to look at the cultures under a microscope to determine the changes occurring within the cell system during exposure to the cell system. It was discovered that as the dose of the chemical pollutant increased the number of goblet cells present in the cell culture decreased (Chapter 2). It was also discovered that the goblet cells appear to be secreting their contents onto the culture surface in response to increasing doses of the chemicals. Evidence was gained to support this by using the PAS stain for mucus on cultures treated with increasing doses of the three test substances. The effect was found to be exposure dependent and uniform between chemicals. As stated earlier in this thesis,

increased mucus secretion has been reported in fish exposed to various pollutants and stresses *in vivo*. The primary rainbow trout epithelial cell cultures were able to respond to the chemical stresses in a similar manner to that seen in the skin of fish in an *in vivo* situation.

Some researchers had proposed that induction and subsequent accumulation of heat - shock proteins may be useful in environmental monitoring and toxicological screening (Williams *et al.,* 1996). This study investigated whether or not the available rabbit polyclonal antibody for Hsp70 could be used as an acute toxicity test for fish epithelia. It was found that it was possible to obtain an increase in heat shock protein expression in response to increasing levels of chemical exposure. The induction appeared to be quite sensitive. It was possible to show that DMSO induced heat shock protein to a small degree and that at the threshold level of response obtained in the survival assays, all three chemicals were found to produce an induction in 70% of the cells contained in the cell culture. Copper was found to result in more intense positivity for the stress protein than the two organic pollutants studied. However more work would have to be carried out to determine if it is possible to distinguish between differing chemicals or if the induction of the stress protein is just a general response to chemical or oxidative stress.

To understand the ecotoxicological implications of the cellular indicator response, *in vitro* studies on isolated cells have to be integrated with *in vivo* studies on the intact animal. As a result of the findings of Chapter 3 and Chapter 4 it was decided to see if primary cultures grown from fish exposed *iv vivo* to one of the chemical pollutants prochloraz would exhibit the same mucus response as that observed *in vitro*. It was found that the level of mucus contained on the surface of the cultures increased in

relation to the concentration of the pollutant the parent fish were exposed to. The mucus secretion was found to be dose dependent but not time dependent. The levels of mucus positivity seen were very similar to that obtained *in vitro*. However due to cost and the time available it was not possible to expose the fish for longer than 12 days to the test substances. It was also investigated whether the growth of the primary cell cultures would be affected by the *in vivo* conditions the fish were exposed in. It was found that cell cultures cultured from fish exposed to the highest dose of the chemical for the full exposure period did grow at the same rate as cultures grown from control fish. These results however, due to problems with the test system, were not of the statistical number required to be definitive.

As a result of the *in vivo* experiment with prochloraz it was decided to repeat this experiment using nonoxynol and also to treat the fish in river water as well as dechlorinated tap water in an attempt to get nearer real *in vivo* conditions. It was discovered that a similar response was obtained in cultures taken from fish exposed to the high dose of the chemical. It was also discovered that the growth rate of the cultures was directly related to the amount of mucus contained on the surface of their cells. However it became apparent in this study that only a portion of the response may be in relation to the chemical exposure, the rest due to general stress conditions. It appears that fish kept in river water were under less stress than those contained in dechlorinated tap water. As with all *in vivo* studies many factors can alter the study such as population behavior, dominance etc. However, the controls for this experiment were similar to those in the first experiment. Another interesting fact also became apparent. This was that cultures grown from fish exposed to the high dose of the chemical did not continue to proliferate as long

as the cultures cultured from control fish. There are two possible reasons for this. Firstly the level of mucus contained on the cell culture surface is actually secreted *in vivo* and hence prevents the epithelial cells from adequately dividing *in vitro*. The second possibility is that a similar event is taking place as demonstrated when studying the effect of the test substances on the CHSE cell line. All the cultures appeared to grow and attach equally for the first few days. However it is their division and multiplication that appeared to be affected. It may be possible that a delayed death is occurring in the cultures. This possibility would certainly merit further investigation.

The first task consisted of research on cultured cells in order to establish the basic characteristics of the link between exposure and inducible response. Chapter 2 demonstrated that all three test substances cause delayed cell death, a phenomenon until recently only observed in studies with radiation. A clear link was found between exposure and the inducible genetic response. However the mechanism of this delayed death was found to differ between each of the aquatic pollutants tested. Chapter 3 established the characteristics of the link between the exposure and inducible response of rainbow trout primary epithelial skin cell cultures exposed to aquatic pollutants. It was demonstrated that differences occur in the toxicity of test substances when tested in serum-containing media compared to serum-free media. Both nonoxynol and prochloraz were found to kill the cells by necrosis, copper by a combination of necrosis and apoptosis. Exposure to all three test substances was found to cause a decrease in the number of goblet mucus cells contained in the cell cultures. This reduction was found to be dose related.

The second task centred on the development of cellular markers of exposure and stress. It was demonstrated in chapter 4 that an available mammalian antibody developed against HSP70 can be used to detect HSP70 induction in response to chemical exposure. The % of cells per primary culture expressing positivity for HSP70 was found to increase as chemical exposure increased. The nature of the response demonstrates the possible use of HSP70 induction using the rabbit polyclonal antibody described as a sensitive marker of chemical stress. The % number of cells expressing positivity for mucus was also found to increase as chemical exposure increased. This increase was found to correspond to the decrease in mucus cell number, observed in chapter 3, in relation to increased chemical exposure. The results of this study demonstrate that *in vitro* rainbow trout primary epithelial cell cultures maintain the essential traits of the *in vivo* response of the skin to chemical stresses. The goblet cells contained in the epithelial cell cultures are retaining their *in vivo* functions *in vitro*. As a result mucus production may have potential for use as a cellular marker of exposure.

The third task of this project centered on research on intact fish in the laboratory in order to clarify a) whether the biological response detected in the isolated cell culture in an *in vitro* environment can be detected again in the intact *in vivo* environment and b) if the biological response has the potential for use as a suitable biomarker for effect or exposure. Primary epithelial skin cell cultures were cultured from rainbow trout that were exposed *in* vivo to prochloraz and nonoxynol in order to investigate whether a similar response observed in cultures exposed *in vitro* to prochloraz could be observed in cultures cultured from fish exposed *in vivo* to the test substances Significant *ex vivo* results in the parameters (growth and mucus production) tested were obtained which can

be causatively traced back to the *in vivo* situation. These results indicate that the biological response detected in the isolated cell in an *in vitro* environment could be detected again in an *ex vivo* situation.

Although it appears that the use of mucus production in rainbow trout primary epithelial cell cultures and the growth of these cell cultures has potential for use as a suitable biomarker of stress further work is needed into the nature of the response. Is this response specific or is it a general stress response. I would venture to suggest that the response observed in this study demonstrates that mucus production and cell survival is a general response to stress be it chemical or other. Further studies are also needed to determine whether this response is a) part of an adaptation response or of a pathological response and b) translates into other levels of biological organisation. However the results do show that rainbow trout primary skin epithelial cell culture is an extremely useful tool for ecotoxicity testing both *in vitro* and *in vivo.*

Much work is needed in order to develop the use of rainbow trout primary epithelial skin cell cultures as a suitable tool in studying the toxicity of chemical stressors. Different proliferation markers such as PCNA and KI67 should be developed to investigate if the reduction in residual growth, as observed in this study, is as a result of reduced cell survival or reduced cell proliferation. Different antibodies such as c-Myc, Bax and Bcl2 should also be developed to help investigate the nature of any resulting cell death and reduced cell proliferation. It may then be possible to give conclusive evidence on the different modes of action between chemicals on fish epithelia.

It would also be very interesting to investigate whether primary cell cultures grown from fish exposed *in vivo* to chemical pollutants show an increase in Hsp70 induction as

compared to untreated fish using the immunocytochemical technique using the rabbit polyclonal antibody as described in chapter 4. If the results of such a study were favorable it may be possible to develop Hsp70 induction in rainbow trout primary epithelial cell cultures as a suitable biomarker. Other stress proteins could also be developed to aid in the study of the cellular response to an exposure situation.

Future work that is essential in order to develop the potential biological markers (cell growth and mucus production) discovered in this study, is the use of longer exposure times *in vivo*. It is also necessary to obtain optimum exposure conditions so the effect can be quantified, as either been a specific response to a chemical or a response to a general stress situation. It would also be necessary to see if one changed the chemical exposure levels during the experiments at different times would this become apparent with the marker. Also field studies would be necessary to validate the findings in this study and any resulting studies.

CHAPTER 8

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CHAPTER 9

APPENDICES

APPENDIX II

2.1 EPC Cell Line

Table I shows the % survival fraction of the EPC cell line exposed to nonoxynol.

Dose (µmol/L)	% Survival Fraction		
0	100	100	100
0.5	96.5 ± 7.2	87.0 ± 2.7	103.0 ± 4.8
1	88.4 ± 1.8	88.5 ± 1.5	92.3 ± 3.8
5	66.7 ± 2.8	60.1 ± 2.9	60.2 ± 3.2
7	50.4 ± 3.1	45.1 ± 2.6	50.8 ± 2.6
8	8.2 ± 2.2	17.9 ± 3.3	-
9	3.4 ± 2.3	0	0.5 ± 0.3

Each value represents the mean ± SEM for n = 3.

Table II shows the % survival fraction of the EPC cell line exposed to prochloraz.

Dose (µmol/L)	% Survival Fraction		
0	100	100	100
0.5	88.6 ± 2.1	79.8 ± 5.1	82.3 ± 0.9
1	68.2 ± 1.4	66.2 ± 4.2	69.1 ± 0.8
5	45.6 ± 0.7	47.5 ± 3.6	46.2 ± 0.9
6	40.4 ± 0.6	41.7 ± 2.9	40.5 ± 0.4
7	30.4 ± 1.0	36.5 ± 1.3	30.7 ± 0.6
8	26.4 ± 2.0	28.1 ± 2.1	26.5 ± 0.6
9	23.3 ± 1.5	22.0 ± 1.5	23.9 ± 0.4
10	16.2 ± 1.4	11.5 ± 0.8	17.6 ± 0.4
11	11.0 ± 0.7	8.4 ± 0.5	13.4 ± 0.7
12	3.4 ± 0.7	3.9 ± 1.3	3.3 ± 0.4

Each value represents the mean ± SEM for n = 3.

2.1 Continued

Table III shows the % survival fraction of the EPC cell line exposed to copper

Dose (µmol/L)	% Survival Fraction		
0	100	100	100
5	89.6 ± 1.2	95.2 ± 4.7	92.2 ± 2.6
10	78.9 ± 1.9	85.9 ± 3.2	84.6 ± 2.9
15	55.7 ± 5.0	79.4 ± 4.2	55.2 ± 2.2
30	49.5 ± 2.9	53.6 ± 4.2	51.7 ± 2.4
40	12.9 ± 2.5	17.8 ± 0.9	14.1 ± 0.7
50	6.2 ± 1.3	10.0 ± 0.7	9.4 ± 2.1
60	6.7 ± 1.4	7.6 ± 0.5	6.0 ± 0.6

Each value represents the mean ± SEM for n = 3.

2.2 DELAYED CELL DEATH (CHSE Cell Line)

2.2.1 Nonoxynol

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
0.5	85.7 ± 5.0	79.8 ± 8.4	68.4 ± 6.0
1	89.9 ± 5.2	64.7 ± 5.3	58.2 ± 3.4
5	75.5 ± 12.5	51.2 ± 4.6	38.7 ± 2.7
6	54.7 ± 7.7	59.7 ± 6.3	32.7 ± 3.0
7	43.6 ± 10.8	52.3 ± 6.9	22.8 ± 2.8
8	28.9 ± 5.5	27.7 ± 5.0	8.0 ± 1.5

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
0.5	87.9 ± 2.9	90.7 ± 2.5	82.3 ± 1.6
1	85.2 ± 2.2	76.2 ± 2.2	64.9 ± 1.4
5	66.9 ± 3.4	58.8 ± 1.5	39.3 ± 0.7
6	55.3 ± 2.9	53.1 ± 1.3	29.4 ± 0.3
7	52.9 ± 6.8	37.8 ± 1.9	20.0 ± 0.9
8	34.5 ± 1.0	25.9 ± 1.2	9.0 ± 0.4

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

2.2.1 Continued

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
0.5	82.1 ± 8.7	85.0 ± 2.9	69.8 ± 1.7
1	84.3 ± 7.9	72.3 ± 2.4	60.9 ± 1.5
5	56.5 ± 4.9	59.0 ± 2.0	33.3 ± 0.9
6	42.3 ± 5.1	57.7 ± 2.7	24.4 ± 1.0
7	34.1 ± 5.7	32.6 ± 1.7	11.1 ± 0.5
8	32.0 ± 2.4	26.5 ± 1.2	8.5 ± 0.4

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

2.2.2 Prochloraz

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	83.6 ± 2.1	98.0 ± 1.7	81.9 ± 1.2
10	43.1 ± 0.4	85.6 ± 1.2	36.9 ± 0.3
11	39.4 ± 1.2	81.6 ± 1.5	32.2 ± 0.9
12	33.0 ± 0.9	75.5 ± 1.2	24.9 ± 0.3
13	28.0 ± 0.4	67.2 ± 1.2	18.8 ± 0.3

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	97.5 ± 1.4	101.6 ± 3.5	99.0 ± 2.1
10	46.9 ± 0.6	86.7 ± 3.3	40.6 ± 0.9
11	41.9 ± 0.9	80.5 ± 2.4	33.7 ± 0.5
12	34.7 ± 0.9	74.6 ± 2.5	25.9 ± 0.6
13	30.2 ± 0.7	67.6 ± 2.2	20.4 ± 0.3

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

2.2.2 Continued

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	83.7 ± 2.9	105.5 ± 4.7	88.3 ± 3.3
10	41.5 ± 1.1	86.7 ± 3.2	36.0 ± 1.0
11	39.3 ± 1.3	82.3 ± 4.2	32.3 ± 1.2
12	34.1 ± 1.3	76.0 ± 3.6	25.9 ± 1.0
13	30.3 ± 2.3	69.1 ± 2.6	20.9 ± 0.6

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

2.2.3 COPPER

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	87.6 ± 3.6	100.1 ± 2.5	87.7 ± 2.0
5	70.2 ± 3.3	98.0 ± 1.4	68.7 ± 0.8
10	53.7 ± 2.1	61.0 ± 1.3	32.7 ± 0.6
15	48.8 ± 2.5	61.8 ± 1.8	30.2 ± 0.9
20	35.8 ± 1.4	62.7 ± 2.0	22.4 ± 0.7
30	30.2 ± 1.2	61.9 ± 1.3	18.7 ± 0.3
40	25.2 ± 1.1	62.3 ± 1.3	15.7 ± 0.3
50	22.4 ± 1.1	60.6 ± 1.3	13.6 ± 0.3

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

Dose Applied (µmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	75.1 ± 5.2	97.5 ± 3.2	73.3 ± 1.9
5	63.0 ± 2.4	96.1 ± 2.5	60.5 ± 1.0
10	44.8 ± 1.9	62.6 ± 2.2	28.0 ± 0.8
15	38.4 ± 2.6	59.5 ± 1.8	22.9 ± 2.5
20	27.2 ± 1.2	62.0 ± 1.8	16.9 ± 0.4
30	25.6 ± 1.4	61.4 ± 1.8	15.7 ± 0.4
40	22.5 ± 1.2	60.8 ± 1.6	13.7 ± 0.2
50	20.0 ± 0.8	59.9 ± 1.9	12.0 ± 0.3

Each f(0) value represents the mean ± SEM for n = 3. Each f(1) value represents the mean ± SEM for n = 9.

2.2.3 Continued

Dose Applied (μmol/L)	% Survival Fraction		
	f(0)	f(1)	f(0) X f(1)
0	100	100	100
1	87.5 ± 4.0	107.4 ± 4.1	93.9 ± 2.1
5	76.3 ± 3.3	101.0 ± 3.8	77.0 ± 1.6
10	57.0 ± 2.2	66.4 ± 2.4	37.8 ± 0.7
15	50.4 ± 3.3	-	-
20	40.0 ± 1.6	74.3 ± 5.6	29.7 ± 2.1
30	34.0 ± 1.7	70.9 ± 5.1	23.9 ± 1.7
40	32.2 ± 2.3	64.5 ± 4.2	20.8 ± 1.2
50	23.7 ± 1.6	73.0 ± 3.9	39.2 ± 0.8

Each f(0) value represents the mean ± SEM for n=3. Each f(1) value represents the mean ± SEM for n = 9.

APPENDIX III

3.1

% Survival Fraction of Rainbow Trout Epithelial cell cultures exposed for 24h, 48h, 72h and 96h to nonoxynol in serum containing-medium.

Dose (µmol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1.25	93.6 ± 4.4	89.8 ± 3.6	82.8 ± 2.5	93.6 ± 3.7
2.5	80.8 ± 2.9	87.0 ± 1.9	85.4 ± 2.2	99.7 ± 3.4
10	71.6 ± 3.3	77.5 ± 9.9	72.4 ± 4.0	72.0 ± 2.8
12.5	66.3 ± 1.8	78.6 ± 1.9	67.5 ± 5.2	67.6 ± 2.8
25	62.1 ± 3.7	-	30.7 ± 1.3	29.9 ± 1.1
50	8.9 ± 2.7	15.2 ± 3.0	9.4 ± 1.5	10.7 ± 0.8
75	1.9± 1.6	0.9 ± 0.4	1.1 ± 0.5	2.5 ± 0.5

Each value represents the mean ± SEM for n = 3

Dose (umol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1.25	88.4 ± 3.1	76.2 ± 1.1	94.9 ± 2.5	97.7 ± 2.3
2.5	86.8 ± 3.1	72.3 ± 7.2	94.0 ± 2.3	98.6 ± 1.4
10	77.9 ± 5.0	74.3 ± 1.7	79.7 ± 2.9	73.6 ± 1.3
12.5	81.7 ± 4.3	72.1 ± 6.2	81.2 ± 4.8	68.5 ± 1.6
25	30.7 ± 1.1	33.9 ± 1.2	29.9 ± 2.5	29.5 ± 1.5
50	22.7 ± 3.7	9.9 ± 3.2	10.6 ± 3.1	10.0 ± 2.2
75	4.6 ± 2.8	3.2 ± 2.6	1.6 ± 1.4	1.6 ± 0.9

Each value represents the mean ± SEM for n = 3

3.2

% Survival Fraction of Rainbow Trout Epithelial cell cultures exposed for 24h and 48h to nonoxynol in serum-free medium.

Dose (µmol/l)	% Survival Fraction			
	24h	24h	48h	48h
0	100	100	100	100
1.25	97.3 ± 4.6	101.7 ± 6.2	105.5 ± 2.9	92.8 ± 10.9
2.5	79.8 ± 10.7	104.0 ± 9.1	89.3 ± 2.1	84.3 ± 2.3
10	37.7 ± 3.5	68.3 ± 16.4	41.2 ± 3.6	50.6 ± 6.6
12.5	31.2 ± 3.2	19.1 ± 4.5	25.1 ± 3.1	15.5 ± 4.0
25	19.8 ± 6.3	32.8 ± 6.4	19.5 ± 5.1	32.0 ± 4.9
50	4.5 ± 2.2	0	0.9 ± 0.8	9.1 ± 6.0

Each value represents the mean ± SEM for n = 3

3.3

Type of Cells post 24h exposure to nonoxynol in serum-containing medium

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	22.3 ± 1.5	9.1 ± 1.1	0	0
1.25	22.0 ± 1.3	8.9 ± 1.2	0.3 ± 0.2	0
2.5	21.4 ± 1.2	6.7 ± 1.2	1.5 ± 0.8	0.1 ± 0.1
10	13.4 ± 3.6	5.0 ± 1.3	6.5 ± 2.4	0.3 ± 0.3
12.5	12.1 ± 4.0	5.6 ± 1.5	6.0 ± 2.3	0.4 ± 0.3
25	8.0 ± 4.6	2.7 ± 1.7	12.1 ± 4.8	0.8 ± 0.7
50	2.3 ± 1.6	0.4 ±0.3	13.3 ± 2.3	0.8 ± 0.5

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	24.8 ± 1.8	5.1 ± 1.5	0	0
1.25	22.4 ± 2.5	4.8 ± 2.0	1.3 ± 0.9	0.3 ± 0.3
2.5	22.8 ± 2.7	4.2 ± 1.4	3.1 ± 3.1	1.0 ± 0.4
12.5	12.8 ± 2.6	2.4 ± 0.7	7.9 ± 2.8	0.4 ± 0.2
25	6.7 ± 3.8	1.1 ± 0.9	17.2 ± 4.4	1.1 ± 0.6

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.3 Continued

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	22.1 ± 1.7	8.9 ± 1.1	0	0
10	12.8 ± 3.6	3.9 ± 1.2	7.3 ± 2.8	0.3 ± 0.3
50	2.9 ± 2.1	1.6 ± 1.4	17.1 ± 3.3	0.7 ± 0.5

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.4

Type of Cells post 48h exposure to nonoxynol in serum-containing medium

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	25.8 ± 3.1	4.2 ± 1.9	0	0
1.25	23.9 ± 3.0	3.7 ± 1.5	0.1 ± 0.2	0.2 ± 0.2
2.5	20 ± 1.2	4.8 ± 0.8	0.6 ± 0.7	0.3 ± 0.8
12.5	18.5 ± 2.1	2.5 ± 1.1	5.5 ± 2.1	1.3 ± 1.4
25	6.7 ± 3.6	1.3 ± 1.1	16.3 ± 3.5	1.8 ± 0.9

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	21.5 ± 1.3	11.1 ± 1.2	0.1 ± 0.1	0
10	14.8 ± 3.1	8.5 ± 1.2	3.7 ± 1.3	0.4 ± 0.3
50	2.5 ± 1.7	0.3 ± 0.4	12.3 ± 2.3	2.3 ± 0.4

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.4 Continued

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	24.1 ± 2.3	9.1 ± 1.8	0	0
1.25	22.2 ± 0.9	8.7 ± 0.8	0.2 ± 0.2	0.1 ± 0.1
2.5	21.7 ± 1.6	7.1 ± 0.7	1.3 ± 0.4	0.3 ± 0.2
12.5	12.7 ± 3.4	2.1 ± 1.0	8.7 ± 2.2	0.8 ± 0.5
25	6.1 ± 2.8	1.4 ± 0.8	14.1 ± 2.4	2.1 ± 0.7

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	21.2 ± 1.8	9.1 ± 2.0	0.3 ± 0.2	0.1 ± 0.1
10	10.1 ± 2.5	6.8 ± 1.5	5.7 ± 1.9	0.5 ± 0.6
50	3.9 ± 3.5	1.2 ± 0.8	15.5 ± 2.6	1 ± 0.4

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.5

% Survival fraction of rainbow trout epithelial cell cultures exposed for 24h, 48h, 72h and 96h to prochloraz in serum-containing medium.

Dose (µmol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1	97.5 ± 8.3	81.6 ± 8.2	89.6 ± 6.0	70.9 ± 2.2
10	84.5 ± 8.0	95.8 ± 4.5	92.5 ± 6.9	75.5 ± 2.2
25	85.6 ± 5.2	47.8 ± 9.0	73.0 ± 6.4	56.7 ± 3.7
50	25.0 ± 2.8	17.3 ± 2.1	15.4 ± 1.7	12.9 ± 1.0
75	6.1 ± 0.7	8.4 ± 2.0	2.0 ± 0.9	4.2 ± 1.9

Each value represents the mean ± SEM for n = 3

3.5 Continued

Dose (µmol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1	100.3 ± 6.3	91.6 ± 2.2	103.3 ± 6.5	71.9 ± 2.2
10	96.5 ± 11.2	86.8 ± 3.4	82.3 ± 5.4	71.0 ± 2.2
25	59.6 ± 2.9	53.3 ± 2.5	54.4 ± 4.6	53.0 ± 1.1
50	37.1 ± 8.1	31.3 ± 3.4	15.3 ± 2.5	14.1 ± 3.3
75	9.8 ± 2.9	9.3 ± 3.9	6.0 ± 2.0	4.7 ± 0.3

Each value represents the mean ± SEM for n = 3

3.6

% Survival fraction of rainbow trout epithelial cell cultures exposed for 24h and48h to prochloraz in serum-free medium.

Dose (µmol/L)	% Survival Fraction			
	24h	24h	48h	48h
0	100	100	100	100
1	90.6 ± 6.5	101.4 ± 3.3	95.3 ± 4.5	100.5 ± 2.3
10	88.5 ± 5.9	97.2 ± 3.6	85.7 ± 3.0	88.4 ± 2.1
25	47.5 ± 3.8	42.0 ± 1.6	42.8 ± 3.5	20.8 ± 5.5
50	10.9 ± 1.2	9.5 ± 0.6	7.5 ± 2.1	2.5 ± 0.6
75	1.1 ± 0.9	0	0.8 ± 0.7	0

Each value represents the mean ± SEM for n = 3

3.7

Type of cells post 48h exposure to prochloraz in serum-containing medium

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	23.1 ± 1.8	6.8 ± 1.6	0	0
1	24.3 ± 1.5	6.9 ± 1.4	0	0
10	20.6 ± 1.5	5.8 ± 0.7	1.2 ± 0.5	0.4 ± 0.3
25	12.6 ± 2.5	5.3 ± 0.9	5.4 ± 1.5	0.6 ± 0.2
50	5.4 ± 2.6	1.4 ± 1.5	11.2 ± 2.1	1.9 ± 0.6
75	4.1 ± 1.5	0.8 ± 0.5	12.8 ± 2.3	1.0 ± 0.2

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.7 Continued

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	25.9 ± 2.4	7.2 ± 1.8	0.2 ± 0.2	0
1	25.3 ± 1.9	9.5 ± 1.8	0.2 ± 0.2	0
10	22.4 ± 1.7	6.0 ± 0.9	1.6 ± 0.7	0.6 ± 0.3
25	12.4 ± 3.3	6.3 ± 1.3	4.8 ± 1.3	0.6 ± 0.4
50	6.2 ± 2.8	1.2 ± 1.3	11.8 ± 2.7	2.3 ± 0.8
75	2.1 ± 1.1	1.0 ± 0.5	12.6 ± 2.5	2.0 ± 0.4

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

3.8

% Survival fraction of rainbow trout epithelial cell cultures exposed for 24h, 48h, 72h and 96h to copper in serum-containing medium.

Dose (µmol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1	103.0 ± 4.7	100.1 ± 6.1	102.4 ± 1.3	95.8 ± 2.7
10	103.1 ± 3.2	100.3 ± 3.2	102.5 ± 1.5	97.7 ± 3.1
50	94.8 ± 3.8	97.5 ± 5.1	102.4 ± 1.6	96.7 ± 3.2
100	91.5 ± 5.2	99.4 ± 4.1	100.5 ± 1.0	95.4 ± 3.2
500	35.7 ± 6.5	33.4 ± 5.6	34.9 ± 1.5	32.0 ± 2.3
1000	10.4 ± 1.0	10.9 ± 1.1	9.4 ± 0.3	6.7 ± 1.2

Each value represents the mean ± SEM for n = 3

Dose (µmol/L)	% Survival Fraction			
	24h	48h	72h	96h
0	100	100	100	100
1	99.3 ± 2.6	101.8 ± 3.1	100.5 ± 2.7	98.3 ± 1.7
10	101.5 ± 1.8	116.0 ± 2.5	99.7 ± 0.7	100.2 ± 1.4
50	98.8 ± 3.2	106.4 ± 2.2	99.0 ± 1.0	100.3 ± 1.1
100	95.7 ± 4.2	116.0 ± 3.1	97.6 ± 0.7	98.3 ± 1.0
500	34.3 ± 4.9	43.1 ± 8.8	33.2 ± 0.9	35.0 ± 1.8
1000	11.2 ± 2.9	5.1 ± 2.3	8.0 ± 0.2	6.0 ± 0.9

Each value represents the mean ± SEM for n = 3

3.9

% Survival fraction of rainbow trout epithelial cell cultures exposed for 24h and 48h to copper in serum-free medium.

Dose (µmol/L)	% Survival Fraction			
	24h	24h	48h	48h
0	100	100	100	100
1	95.1 ± 8.9	103.7 ± 3.8	110.6 ± 3.4	93.2 ± 4.7
10	90.1 ± 3.6	101.7 ± 2.1	98.7 ± 3.9	98.4 ± 8.2
50	76.5 ± 7.1	94.6 ± 3.4	93.9 ± 2.4	90.7 ± 5.0
100	94.0 ± 7.9	90.8 ± 5.0	80.4 ± 5.0	89.9 ± 10.0
500	82.1 ± 3.0	73.1 ± 5.5	71.1 ± 6.7	64.1 ± 6.0
1000	10.1 ± 1.1	11.4 ± 1.8	12.3 ± 2.2	8.2 ± 1.0

Each value represents the mean ± SEM for n = 3

3.10

Type of Cells post 48h exposure to copper in serum-containing medium

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	21.8 ± 1.1	7.4 ± 1.8	0	0
10	21.3 ± 1.7	7.6 ± 1.3	0.1 ± 0.1	0
50	23.0 ± 1.4	6.3 ± 1.0	0.3 ± 0.1	0
100	20.3 ± 1.2	7.2 ± 1.2	0.2 ± 0.1	0.2 ± 0.2
500	5.9 ± 1.9	2.0 ± 1.5	11.8 ± 1.8	3.7 ± 0.8
1000	1.4 ± 1.2	0.9 ± 0.6	12.4 ± 3.0	6.0 ± 0.5

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

Dose (µmol/L)	No. of Normal Epithelial Cells/Field	No. of Goblet Cells/Field	No. of Necrotic Cells/Field	No. of Apoptotic Cells/Field
0	24.6 ± 1.5	6.5 ± 1.6	0.2 ± 0.2	0
10	25.1 ± 2.1	6.8 ± 1.1	0.1 ± 0.1	0
50	23.2 ± 1.4	6.1 ± 1.4	0.1 ± 0.1	0
100	20.1 ± 1.0	6.4 ± 1.2	0.2 ± 0.1	0
500	5.9 ± 1.5	2.2 ± 1.3	11.4 ± 2.4	2.7 ± 0.4
1000	2.0 ± 1.6	1.3 ± 0.6	12.8 ± 2.6	5.6 ± 0.3

Each value represents the mean ± SEM for n = 3. Five fields counted per culture.

APPENDIX IV

4.1 HEAT SHOCK PROTEIN

4.1.1 Nonoxynol

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	564 ± 32	16.8	0	10.3	3.5	17.6	1.9	14.9 ± 1.9	1.8 ± 0.9
2	506 ± 88	27.9	5.4	5.7	0	15.3	0	16.3 ± 5.3	1.8 ± 1.5
3	510 ± 128	15.1	4.1	17.4	0	9.6	0	14.0 ± 1.9	1.4 ± 1.2
AVERAGE POSITIVITY								**15.1 ± 2.0**	**1.7 ± 0.7**

12.5 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	364 ± 50	41.6	16.4	41.4	25.7	53.5	10.6	45.5 ± 3.4	17.6 ± 3.6
2	367 ± 63	50.5	12.6	30.9	51.8	30.7	39.0	37.4 ± 5.4	34.5 ± 9.5
3	400 ± 34	65.9	4.5	70.3	11.5	45.0	8.6	60.4 ± 6.4	8..2 ± 1.7
AVERAGE POSITIVITY								**47.8 ± 4.4**	**20.1 ± 5.0**

Table 4.1.1 - continued

25 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	285 ± 33	65.9	24.0	58.2	9.6	31.5	25.0	51.8 ± 8.6	19.5 ± 4.1
2	326 ± 57	66.1	25.2	59.9	6.4	73.2	11.0	66.4 ± 3.2	14.2 ± 4.6
3	324 ± 108	48.7	25.2	44.8	41.5	25.9	53.2	39.8 ± 5.8	40.0 ± 6.7
AVERAGE POSITIVITY								**52.7 ± 5.1**	**24.6 ± 4.8**

35 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	225 ± 18	60.4	39.6	66.3	32.6	43.5	56.5	56.8 ± 5.6	42.9 ± 1.8
2	238 ± 47	56.1	28.7	67.4	15.6	34.2	65.9	52.6 ± 8.0	36.7 ± 12.3
3	299 ± 76	30.2	41.6	99.0	1.0	82.4	5.4	70.5 ± 17.0	16.0 ± 10.5
AVERAGE POSITIVITY								**59.9 ± 7.0**	**31.9 ± 6.9**

4.1.2 Prochloraz

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	336 ± 56	33.0	0	19.0	0	31.5	0	27.8 ± 3.7	0
2	274 ± 39	26.3	52.0	48.6	0	12.6	0	29.2 ± 8.6	17.3 ± 14.2
3	243 ± 4	54.0	6.4	18.4	0	46.4	0	39.6 ± 8.9	2.1 ± 1.8
AVERAGE POSITIVITY								**32.2 ± 4.7**	**6.5 ± 5.4**

10μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	356 ± 58	54.7	0.6	70	30	51.6	12.4	58.8 ± 4.7	14.3 ± 7.0
2	451 ± 73	75.6	0	51.5	0	42.2	2.3	56.4 ± 8.12	0.8 ± 0.7
3	217 ± 24	45.5	0	19.6	73.2	65.3	33.2	43.5 ± 10.8	35.5 ± 17.3
AVERAGE POSITIVITY								**52.9 ± 5.3**	**16.9 ± 7.9**

Table 4.1.2 - continued

25 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	449 ± 28	85.8	2.2	52.9	10.6	51.5	3.4	63.4 ± 9.2	5.4 ± 2.2
2	372 ± 52	33.7	53.2	91.5	8.1	60.9	22.4	62.0 ± 13.7	27.9 ± 10.9
3	358 ± 45	70.2	6.1	69.5	1.2	44.8	52.4	61.5 ± 6.9	19.9 ±13.4
AVERAGE POSITIVITY								**62.3 ± 6.0**	**17.7 ± 6.6**

50 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	427 ± 69	42.0	29.5	91.3	8.7	60	29.7	64.4 ± 11.8	22.6 ± 5.7
2	368 ± 5	97.5	2.5	87.9	0	59.7	8.8	81.7 ± 9.3	3.8 ± 2.2
3	337 ± 15	12.0	85.4	66.1	0	57.8	42.2	45.3 ± 13.8	45.5 ± 20.2
AVERAGE POSITIVITY								**63.8 ± 8.4**	**23.0 ± 8.8**

4.1.3 Copper

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	335 ± 87	1.0	0	0	0	14.4	0	5.1 ± 3.8	0
2	358 ± 27	0	0	5.5	0	19.2	0	11.0 ± 4.9	0
3	437 ± 10	4.4	0	0	0	-	-	2.2 ± 1.6	0
AVERAGE POSITIVITY								**5.6 ± 2.5**	**0**

100 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	271 ± 53	81.9	14.1	59.4	18.4	28.1	71.9	56.5 ± 12.8	34.8 ± 15.2
2	195 ± 15	21.8	78.2	35.8	48.3	41.3	58.7	33.0 ± 4.8	61.7 ± 7.2
3	268 ± 39	37.6	3.3	45.8	21.7	34.9	0	39.4 ± 2.7	8.3 ± 5.6
AVERAGE POSITIVITY								**42.9 ± 5.7**	**35.0 ± 9.36**

Table 4.1.3 - continued

400 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	626 ± 182	83.6	15.0	62.1	37.9	41.0	46.9	62.2 ± 10.1	33.3 ± 7.8
2	320 ± 47	68.3	28.8	46.8	14.9	86.4	12.6	67.2 ± 16.2	18.8 ± 4.14
3	212 ± 16	59.3	40.7	76.3	5.2	19.8	58.8	51.8 ± 13.7	34.9 ± 12.9
AVERAGE POSITIVITY								**60.4 ± 6.8**	**29.0 ± 5.8**

700 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	455 ± 99	17.6	80.9	10.4	59.5	30.7	68.6	19.5 ± 4.9	69.6 ± 5.1
2	179 ± 56	0	100	1.8	98.2	30.7	41.2	10.8 ± 8.2	79.8 ± 15.8
3	250 ± 51	5.3	94.7	52.8	47.2	12.7	87.3	23.6 ± 12.1	76.4 ± 12.1
AVERAGE POSITIVITY								**18.0 ± 5.5**	**75.3 ± 7.0**

4.2 PAS STAIN FOR MUCUS

4.2.1 Nonoxynol

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	342 ± 153	6.6	3.6	1.4	0	4.2	0	4.1 ± 1.3	1.2 ± 1.0
2	638 ± 172	7.3	1.3	3.8	0	5.9	1.8	5.7 ± 0.9	1.0 ± 0.5
3	523 ± 86	3.9	1.6	5.8	4.6	3.6	1.8	4.6 ± 0.6	2.7 ± 0.8
AVERAGE POSITIVITY								**4.7 ± 0.6**	**1.7 ± 0.6**

12.5 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	540 ± 153	22.2	2.4	7.4	0	-	-	14.8 ± 5.3	1.2 ± 0.7
2	469 ± 124	12.0	1.3	7.2	3.6	9.6	2.6	9.6 ± 1.8	2.5 ± 1.73
3	781 ± 52	2.4	2.4	8.5	1.5	4.3	2.2	5.1 ± 1.5	2.1 ± 0.3
AVERAGE POSITIVITY								**9.2 ±2.0**	**2.0 ± 0.4**

Table 4.2.1 - continued

25 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	492 ± 12	18.8	5.6	20.5	5.5	15.3	3.7	18.2 ± 1.3	4.9 ± 0.6
2	383 ± 34	14.7	1.3	18.3	6.1	10.7	5.3	14.6 ± 1.8	4.2 ± 1.2
3	561	15.3	2.5	-	-	-	-	15.3	2.5
AVERAGE POSITIVITY								**16.3 ± 1.2**	**4.3 ± 0.7**

35 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	179 ± 26	26.3	1.5	33.5	0	13.2	5.4	24.4 ± 4.9	2.3 ± 1.4
2	180 ± 16	17.5	5.5	13.3	5.7	16.0	1.8	15.6 ± 1.1	4.4 ± 1.1
3	177 ± 15	12.4	16.7	23.2	4.0	12.6	6.3	16.0 ± 3.0	9.0 ± 3.2
AVERAGE POSITIVITY								**18.7 ± 2.4**	**5.2 ± 1.6**

4.2.2 Prochloraz

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	554 ± 150	8.5	0.3	9.4	1.5	-	-	8.9 ± 0.3	0.9 ± 0.4
2	505 ± 77	5.4	0	10.5	1.7	10.0	1.9	8.6 ± 1.4	1.2 ± 0.5
3	167 ± 13	10.7	0	2.2	0	1.5	0	3.5 ± 2.1	0
AVERAGE POSITIVITY								**7.3 ± 1.3**	**0.7 ± 0.3**

10μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	498 ± 74	19.8	4.5	7.8	1.1	14.3	4.7	14.0 ± 2.9	3.4 ± 1.0
2	583 ± 46	3.6	1.0	14.1	1.5	9.3	4.1	9.0 ± 2.5	2.2 ± 0.8
3	395 ± 186	13.2	1.8	0.8	0	-	-	7.0 ± 4.5	0.9 ± 0.7
AVERAGE POSITIVITY								**10.4 ± 2.1**	**2.3 ± 0.6**

Table 4.2.2 - continued

25 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	312 ± 23	16.9	0.3	16.5	0	16.2	1.5	16.5 ± 0.2	0.6 ± 0.4
2	414 ± 42	8.6	0	12.6	5.7	18.6	3.5	13.2 ± 2.4	3.1 ± 1.4
3	357 ± 18	8.0	1.9	14.0	4.5	28.8	9.5	16.9 ± 5.1	5.3 ± 1.8
AVERAGE POSITIVITY								**15.6 ± 2.0**	**3.0 ± 1.0**

50 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	302 ± 14	17.9	1.9	14.5	0	21.3	12.5	17.9 ± 1.6	4.8 ± 3.2
2	319 ± 51	21.7	2.1	8.5	8.5	-	-	15.1 ± 4.7	5.3 ± 2.3
3	336 ± 34	15.7	2.3	11.8	4.3	12.4	1.7	13.3 ± 1.0	2.8 ± 0.7
AVERAGE POSITIVITY								**15.5 ± 1.6**	**4.2 ± 1.4**

4.2.3 Copper

Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	439 ± 171	4.1	0	5.9	0.1	9.3	0.6	6.4 ± 1.3	0.2 ± 0.2
2	481 ± 48	10.9	1.1	-	-	7.5	0.7	9.2 ± 1.3	0.9 ± 0.13
3	306 ± 67	3.8	0	4.5	0.3	-	-	4.1 ± 0.26	0.1 ± 0.1
AVERAGE POSITIVITY								**6.6 ± 1.0**	**0.4 ± 0.2**

100 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	494 ± 112	2.5	0.2	6.6	5.8	11.1	2.1	6.7 ± 2.1	2.7 ± 1.4
2	601 ± 149	6.2	11.5	4.4	15.4	-	-	5.3 ± 0.7	13.4 ± 1.4
3	534 ± 138	7.2	4.7	7.5	2.2	9.6	0	8.1 ± 0.7	2.3 ± 1.1
AVERAGE POSITIVITY								**6.9 ± 1.0**	**5.2 ± 1.9**

Table 4.2.3 - continued

400 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	651 ± 133	8.7	2.5	8.9	1.2	8.2	0.9	8.6 ± 0.2	1.5 ± 0.4
2	503 ± 75	8.8	11.4	13.0	8.4	13.9	1.7	11.9 ± 1.3	7.2 ± 2.4
3	514 ± 165	11.0	0.4	10.8	2.1	-	-	10.9 ± 0.1	1.3 ± 0.7
AVERAGE POSITIVITY								**10.4 ± 0.8**	**3.6 ± 1.4**

700 μmol/l

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
1	284	19.0	2.1	-	-	-	-	19.0	2.1
2	213 ± 36	5.4	10.5	9.0	18.6	8.0	17.1	7.5 ± 0.9	15.4 ± 2.1
3	542 ± 95	10.1	11.7	6.9	0.4	4.8	1.5	7.3 ± 1.3	4.5 ± 3.0
AVERAGE POSITIVITY								**9.0 ± 1.7**	**8.8 ± 2.6**

APPENDIX V

The following are the weight and lengths of all the fish that were killed and explants cultured from for this study. The length of tube going from the tank to the pump is also included (s= short, t=long). The relevant dose is also recorded (P100 = 100ppb Prochloraz, P10 = 10ppb Prochloraz, M = Prochloraz/Nonoxynol mixture, 0 = tanks only exposed to 2.7% ethanol).

Time 0 (before any chemical was added)

These fish were used as controls for the experiment.

Fish No.	Tank + length of tube	Weight (g)	Length (cm)
9B	13 s	45.3	20.2
9	14 s	59.6	21.3
8	16 l	37	19
7	17 l	33.2	18.2
12B	24 l	57.3	21.4
1	1 s	45.9	16.7
2	2 s	42.1	15.4
3	4 l	47.8	16.2

Time +18hrs (18hours exposure to the chemical)

Fish No.	Tank +length of tube	Weight (g)	Length (cm)
12	21 (Mixture) s	42.6	19.5
4	9 (P10) s	49.9	20.4
5	15 (P100) s	66.3	18.9

Day 5 – 5 days post exposure

Fish No.	Tank +length of tube	Dose	Length (cm)	Weight (g)
12	21 s	M	19.8	59
11	20 s	M	21.4	57.4
10	24 l	M	21.3	58.3
9	14 s	P100	20.8	59.1
8	13 s	P100	20.6	49.2
7	16 l	P100	19.3	43.9
6	9 s	P10	18	59.7
5	8 s	P10	17.9	55.4
4	12 l	P10	16.6	55.4
3	2 s	0	16.3	49.1
2	1 s	0	21.2	54.5
1	4 l	0	22.4	67.2

Day 12 - 12 days post exposure

Fish No.	Tank +tube length	Dose	Length (cm)	Weight (g)
12	21 s	M	21.5	56.8
11	20 s	M	21.9	62.3
10	24 l	M	21.2	62.5
9	14 s	P100	22.3	75.4
8	13 s	P100	20.4	52.1
7	16 l	P100	20.9	54.7
6	9 s	P10	22.3	68.4
5	8 s	P10	20.7	66.4
4	12 l	P10	20.4	48.3
3	2 s	0	20	52.8
2	1 s	0	21.1	55.3
1	4 l	0	22.3	65.2

5.1 SURVIVAL OF PRIMARY EPITHELIAL CULTURES

CONTROL Day 0

Fish No.	Tank	Weigh t (g)	Lengt h (cm)	% Residual of Initial Day 3 Growth						
				Day3	Day5	Day7	Day 9	Day11	Day 14	
9B	13s	45.3	20.2	100	100	104.4	130	117.2	144.5	
9	14s	59.6	21.3	100	166.7	133.3	C	C	142.9	
7	17l	33.2	18.2	100	100	125.8	230.8	130.4	0	
12B	24l	57.3	21.4	100	112	135.5	118.9	183.3	66.7	
1	1s	45.9	16.7	100	94.1	100	130	88	140	
2	2s	42.1	15.4	100	107.7	113.3	225	60.9	170	
3	4l	47.8	16.2	100	87.5	121.9	120	C	C	
AVERAGE				**100**	**109.7 ±9.3**	**119.2 ±4.9**	**159.1 ±19.9**	**116.0 ±18.5**	**132.8 ±15.6**	

+ 18 Hours Exposure

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day 3	Day 5	Day 7	Day 9	Day 11	Day14
12	21Ms	42.6	19.5	100	136.4	240	330	281	221.4
4	9(P10)s	49.9	20.4	100	138.9	191.7	216.7	168.2	234.4
5	15 p100s	66.3	18.9	100	115.2	65	64.3	154.8	174.4
AVERAGE				**100**	**130.0± 15.5**	**165.6± 42.6**	**203.7± 62.9**	**201.3± 32.7**	**210.1± 14.9**

5.1 Continued

DAY 5 EXPOSURE

Prochloraz >45ppb

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Day 3 Growth					
				Day 3	Day5	Day7	Day 9	Day 11	Day 14
9	14	20.8	59.1	100	162.5	43.5	92.1	62.5	60
8	13	20.6	49.2	100	77.8	70.6	144.5	c	143.5
AVERAGE				**100**	**120.4± 30.0**	**57.0 ±9.6**	**118.3± 18.6**	**62.5±0**	**101.7± 29.6**

Prochloraz 1-45ppb

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Day 3 Growth					
				Day 3	Day5	Day7	Day9	Day11	Day 14
7	16	19.3	43.9	100	121.4	158.3	227.3	138.5	300
6	9	18	59.7	100	100	115.8	50	152.9	C
5	8	17.9	55.4	100	100	142.9	108.3	150	238.5
4	12	16.6	55.4	100	120	133.3	196	202.9	200
Average				**100**	**110.4± 5.2**	**137.6± 7.8**	**145.4± 35.2**	**161.1± 12.4**	**246.2± 23.8**

2.7% Ethanol

Fish No.	Tank	Weight	Length	% Residual of Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day14
3	2	16.3	49.1	100	100	100	69.2	134.6	400
2	1	21.2	54.5	100	100	115.4	50	31.6	C
1	4	22.4	67.2	100	115.4	100	125	62.5	C
				100	**105.1 ±4.2**	**105.1 ±4.12**	**81.4 ±23.4**	**76.2 ±25.0**	**400**

5.1 Continued

Mixture (high)

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day14
12	21	19.8	59	100	114.2	65	64.3	154.8	174.4
11	20	21.4	57.5	100	100	103.5	184.6	152.4	36.7
				100	**107.2 ±5.0**	**84.2 ±13.6**	**124.5 ±42.6**	**153.6 ±0.9**	**105.6 ±48.7**

Mixture (Low)

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day14
10	24	21.3	58.3	100	192.3	300	318.2	190.9	150

12 Days Exposure

Prochloraz >45ppb

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day21
9	14	22.3	75.4	100	118.2	103.9	73.8	29.0	29.6
8	13	20.4	52.1	100	96.2	78.6	41.7	14.8	15.8
Average				**100**	**107.2 ±7.8**	**91.1 ±9.0**	**57.7 ±11.4**	**21.9 ±5.1**	**22.7 ±4.9**

Prochloraz 1-45ppb

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day21
7	16	20.9	54.7	100	47.4	67.7	30.4	27.3	17.9
6	9	22.3	68.4	100	131.3	82.4	48	C	C
5	8	20.7	66.4	100	76.9	80	80	100	22.7
4	12	20.4	48.3	100	100	100	38.1	72.2	20.0
Average				**100**	**88.9±15.4**	**82.5 ±5.8**	**49.1 ±9.5**	**66.5 ±17.3**	**20.2 ±1.2**

5.1 Continued

2.7% Ethanol

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Initial Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day21
2	1	21	55.3	100	100	100	67.5	71.4	0
3	2	20	52.8	100	92.9	57.1	60	83.3	45.5
1	4	22.3	65.2	100	107.7	106.3	100	C	81.8
Average				**100**	**100.2 ±3.5**	**87.8 ±12.6**	**75.8 ±10.1**	**77.4 ±4.3**	**63.6 ±12.9**

Mixture - High

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day21
12	21	21.5	56.8	100	105.3	66.7	79.0	0	33.3
11	20	21.9	62.3	100	83.3	95.2	61.3	92.3	29.2
Average				**100**	**94.3 ±7.8**	**81.0 ±10.1**	**70.1 ±6.3**	**92.3**	**31.2 ±1.5**

Mixture-Low

Fish No.	Tank	Weight (g)	Length (cm)	% Residual of Day 3 Growth					
				Day3	Day5	Day7	Day9	Day11	Day21
10	10	21.2	62.5	100	94.1	85.7	105	83.3	22.2

5.5 PERIODIC ACID SCHIFF STAIN

CONTROL Day 0

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No.2		Flask No.3		Average	
		% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.
9B	1255±167	7.7	0.1	13.9	0.3	5.5	0.1	9.0 ± 2.1	0.2 ± 0.1
9	945±389	-	-	6.6	0.3	5.2	0.3	5.9 ± 0.5	0.3 ± 0.1
7	639±112	11.7	7.7	16.8	3.5	8.1	0.4	12.2 ± 2.1	3.9 ± 1.8
8	748±115	10.9	0.3	6.7	0.3	3.6	0.8	7.1 ± 1.8	0.5 ± 0.2
12B	1554±482	12.5	0.8	8.6	0.4	9.3	0.3	10.2 ± 1.0	0.5 ± 0.2
1	661±186	6.9	6.9	10.6	7.9	2.4	0.9	6.6 ± 2.0	5.2 ± 1.8
2	613	-	-	-	-	6.7	0.7	6.69	0.65
3	572±44	7.9	0.2	21.8	3.9	6.2	0.2	8.6 ± 3.7	1.4 ± 1.1
AVERAGE POSITIVITY								9.0 ± 1.0	1.7 ± 0.6

+ 18 Hours Exposure

Fish No.	Av. No. of cells scored per Sample	Flask No. 1		Flask No. 2		Flask No. 3		Average	
		% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.
12	937±180	4.8	0.3	3.4	0.3	-	-	4.1 ± 0.6	0.3 ± 0.1
4	849±411	8.7	0	5.3	0.6	9.9	0	8.0 ± 1.2	0.2 ± 0.2
5	870±40	3.2	0.1	5.6	0.2	-	-	4.4 ± 0.9	0.2 ± 0.1
AVERAGE POSITIVITY								5.8 ± 0.9	0.2 ± 0.1

Table 5.5 - continued

5 DAYS EXPOSURE

2.7 % Ethanol

Fish No.	Av. No. of cells scored per Sample	Flask No. 1		Flask No.2		Flask N0. 3		Average	
		% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.
3	724±65	4.2	0	10.7	1.7	8.3	0.2	7.7 ± 1.6	0.6 ± 0.5
2	850±171	8.3	0	9.6	0.2	4.5	0	7.5 ± 1.3	0.1 ± 0.1
1	435±118	6.3	0.9	6.0	0.7	4.9	1.1	5.7 ± 0.6	0.9 ± 0.1
AVERAGE POSITIVITY								7.0 ± 0.8	0.5 ± 0.2

1-45ppb Prochloraz

Fish No.	Av. No. of cells scored per Sample	Flask no. 1		Flask .No. 2		Flask No. 3		Average	
		% Wk. Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
7	986±175	4.5	0	12.0	0.5	9.0	0.4	11.6 ± 1.2	0.6 ± 0.2
6	1135±159	8.8	2.8	6.7	0.7	11.1	1.0	8.9 ± 1.1	1.5 ± 0.5
5	956±213	7.4	0	16.5	1.5	11.7	0.1	11.9 ± 2.2	0.5 ± 0.4
4	1029±242	5.0	0	13.9	0.7	18.4	0.7	12.4 ± 3.2	0.5 ± 0.2
AVERAGE POSITIVITY								11.2 ± 1.3	0.8 ± 0.2

Table 5.5 - continued

>45ppb Prochloraz

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
9	1125±366	16.1	5.5	30.8	7.4	17.7	0.5	21.6 ± 3.8	4.5 ± 1.7
8	891±177	14.4	0.4	7.7	4.4	13.9	1.3	12.0 ± 1.8	2.0 ± 1.0
AVERAGE POSITIVITY								16.8 ± 2.9	3.2 ± 1.6

High dose Mixture

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
12	1193±241	10.7	2.8	21.8	2.3	23.9	0.1	18.8 ± 3.4	1.7 ± 0.7
11	1082±196	11.5	0.1	6.6	0.2	7.1	0.8	8.4 ± 1.3	0.4 ± 0.2
AVERAGE POSITIVITY								13.6 ± 2.8	1.0 ± 0.5

Low dose Mixture

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
10	570±73	10.0	0.7	5.8	0	9.0	0	8.3 ±1.1	0.3 ± 0.6

Table 5.5 - continued

12 DAYS EXPOSURE

2.7 % Ethanol

Fish No.	Av. No. of cells scored per Sample	Flask No. 1		Flask No.2		Flask N0. 3		Average	
		% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.	% Wk Pos.	% INT Pos.
3	985±176	14.8	0.6	7.1	0	3.3	0	8.4 ± 2.8	0.2 ± 0.2
2	493±133	7.7	0.3	3.5	0	8.0	0	6.4 ± 1.2	0.1 ± 0.1
1	742±83	10.2	0.1	10.5	0.9	8.6	0.2	9.8 ± 0.5	0.4 ± 0.2
AVERAGE POSITIVITY								8.2 ± 1.2	0.2 ± 0.1

1-45ppb Prochloraz

Fish No.	Av. No. of cells scored per Sample	Flask no. 1		Flask .No. 2		Flask No. 3		Average	
		% Wk. Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
7	1045±145	12.3	1.4	25.3	0.8	8.4	0.3	15.3 ± 4.2	0.8 ± 0.3
6	1135±159	10.9	0.3	4.4	0.3	12.9	0.2	9.4 ± 2.1	0.3 ± 0.1
5	1292±273	10.6	2.3	9.4	0.2	-	-	10.0 ± 0.5	1.3 ± 0.8
4	1975±536	13.8	0	14.0	0.2	3.9	0.1	10.6 ± 2.7	0.1 ± 0.05
AVERAGE POSITIVITY								11.4 ± 1.7	0.6 ± 0.2

Table 5.5 - continued

>45ppb Prochloraz

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
9	1243±135	24.2	2.9	5.2	0.8	12.6	1.5	14.0 ± 4.6	1.7 ± 0.6
8	1243±198	17.3	0.8	13.4	0	15.8	4.1	15.5 ± 1.0	1.6 ± 1.1
AVERAGE POSITIVITY								14.8 ± 2.3	1.7 ± 0.6

High dose Mixture

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
12	974±39	10.8	1.5	9.7	1.0	6.4	0	9.0 ± 1.1	0.8 ± 0.4
11	1208	6.9	0.3	-	-	-	-	6.87	0.33
AVERAGE POSITIVITY								8.4 ± 1.0	0.7 ± 0.3

Low dose Mixture

Fish No.	Av. No. of cells scored per sample	Flask No. 1		Flask No. 2		Flask No.3		Average	
		% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos	% Wk Pos	% INT Pos
10	1181±280	10.8	0.5	7.8	0	14.6	0.7	11.1 ± 1.6	0.4 ± 0.2

APPENDIX VI

The following are the weight and length of the fish used in this experiment.

Day 0

Fish No.	Length (cm)	Weight (g)
19(1)	15.8	51.5
19(2)	17.5	66.6
6(1)	17.4	68.4
6(2)	18	74.4
23(1)	18.3	67.8
23(2)	17.1	64.8

Day 9

Fish No.	Length (cm)	Weight (g)
19	18.1	74.2
6	21.2	63.6
23	22	56.2
21	19.9	46.2
16	21.6	56.3
18	23.4	69.3
10	21.1	74.0
22	23.5	72.6
17	21.0	72.2
14	19.9	68.8
11	21.6	69.3
13	22.1	67.6
20	19.1	51.5
24	21.6	60.2
15	22.1	60.3

6.1 Survival of Epithelial Primary Cell Cultures

Control - Day 0

Fish No.	Day 3	Day 5	Day 7	Day 9
23(1)	100	134.30± 9.6	249.7 ± 15.9	168.7 ± 43.4
23(2)	100	117.54± 13.4	119.6 ± 25.6	101.2 ± 4.4
6(2)	100	63.9 ± 12.9	141.4 ± 11.5	184.5 ± 34.6
19(1)	100	115.1 ± 2.5	125.7 ± 38.4	369.9 ± 180.5
6(1)	100	143.5 ± 23.1	125.8 ± 17.7	294.3 ± 49.1
19(2)	100	111.1 ± 9.1	155.9 ± 29.7	107.2 ± 10.5
Average	**100**	**114.2 ± 8.1**	**153.0 ± 12.8**	**204.3 ± 36.2**

Each Figure represents the mean ± SEM for n = 3

9 Days Exposure

Tap Water Control

Fish No.	Day 3	Day 5	Day 7	Day 9
19	100	110.5 ± 8.6	104.3 ± 22.7	81.6 ± 11.0
23	100	107.1 ± 7.7	121.2 ± 8.4	98.4 ± 14.1
6	100	150.0 ± 35.4	100	138.1 ± 25.5
Average	**100**	**122.5 ± 11.7**	**108.5 ± 10.9**	**106.2 ± 13.1**

Each Figure represents the mean ± SEM for n = 3

Tap Water + 100μg/l Nonylphenoldiethoxylate

Fish No.	Day 3	Day 5	Day 7	Day 9
10	100	89.6 ± 5.3	116.9 ± 12.8	83.3 ± 24.6
20	100	147.4 ± 21.0	211.0 ± 12.4	175.9 ± 48.0
17	100	109.8 ± 12.6	109.9 ± 94	117.8 ± 5.2
Average	**100**	**115.6 ± 11.6**	**145.9 ± 16.8**	**125.7 ± 25.5**

Each Figure represents the mean ± SEM for n = 3

Tap Water + 400μg/l Nonylphenoldiethoxylate

Fish No.	Day 3	Day 5	Day 7	Day 9
22	100	113.0 ± 5.4	58.8 ± 30.9	91.9 ± 5.0
11	100	107.6 ± 4.0	148.7 ± 5.8	94.3 ± 17.9
14	100	106.7 ± 5.5	86.2 ±18.7	57.8 ± 10.7
Average	**100**	**109.1 ± 3.1**	**97.9 ± 17.5**	**81.3 ± 9.1**

Each Figure represents the mean ± SEM for n = 3

Table 6.1 - continued

River Water Control

Fish No.	Day 3	Day 5	Day 7	Day 9
18	100	-	137.5 ± 26.6	100
16	100	125.4 ± 18.8	248.0 ± 51.9	184.4 ± 16.5
21	100	77.8 ± 18.2	111.3 ± 26.6	170.4 ± 57.1
Average	**100**	**101.6 ± 20.9**	**165.6 ± 31.7**	**151.6 ± 28.2**

Each Figure represents the mean ± SEM for n = 3

River Water +400μg/l Nonylphenoldiethoxylate

Fish No.	Day 3	Day 5	Day 7	Day 9
13	100	136.0 ± 30.2	137.9 ± 7.9	134.2 ± 27.7
15	100	151.7 ± 27.6	95.2 ± 3.9	104.0 ± 15.0
24	100	163.2 ± 6.0	182.1 ± 58.1	258.6 ± 70.1
Average	**100**	**150.3 ± 14.3**	**138.4 ± 19.2**	**165.6 ± 30.4**

Each Figure represents the mean ± SEM for n = 3

6.2 PERIODIC ACID SCHIFF STAIN FOR MUCUS

Control Day 0

Fish No.	Av. No. of Cells Scored per Sample	Flask No. 1		Flask No. 2		Flask No. 2		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
6-2	469±121	9.7	0.7	8.6	0.3	5.8	1.0	8.0 ± 1.0	0.6 ± 0.2
19-2	363±44	11.0	1.4	1.3	0.3	-	-	6.2 ± 3.4	0.9 ± 0.4
23-1	459±75	1.0	0.2	1.9	7.7	8.9	0.7	3.9 ± 2.1	2.9 ± 2.0
23-2	368±94	2.6	1.3	4.6	0.4	7.8	0.3	5.0 ± 1.2	0.6 ± 0.3
6-1	431±47	10.1	7.3	9.1	3.8	1.1	0	6.8 ± 2.3	3.7 ± 1.7
19-1	471±11	2.4	2.4	20.6	9.5	11.0	2.6	11.3 ± 4.3	4.8 ± 1.9
AVERAGE POSITIVITY								**6.9 ± 1.2**	**2.3 ± 0.7**

9 DAYS EXPOSURE

Tap Water Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
23	474±128	18.9	0	23.0	1.7	18.8	1.4	20.2 ± 1.1	1.0 ± 0.4
6	411±51	9.9	5.3	18.7	7.5	14.4	1.5	14.3 ± 2.1	4.7 ± 1.4
19	356±47	17.4	0	26.6	2.5	25.6	1.3	23.2 ± 2.4	1.3 ± 0.6
AVERAGE POSITIVITY								**19.3 ± 1.7**	**2.3 ± 0.8**

Table 6.2 - continued

Tap Water + 100µg/l Nonoxynol

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
20	556±111	24.1	8.9	16.9	3.4	10.6	1.1	17.2 ± 3.2	4.5 ± 1.9
10	406±87	19.2	7.8	7.3	1.6	7.7	3.8	11.4 ± 3.2	4.4 ± 1.5
17	310	21.6	0	-	-	-	-	21.61	0
AVERAGE POSITIVITY								**15.3 ± 2.4**	**3.8 ± 1.2**

Tap Water + 400µg/l Nonoxynol

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
22	551±117	25.2	1.2	20.1	14.9	20.6	8.1	21.9 ± 1.3	8.0 ± 3.2
14	343±50	14.9	14.0	16.5	21.6	18.4	6.8	16.6 ± 0.8	14.2 ± 3.5
11	599±93	17.6	0.2	31.6	7.3	20.6	1.7	23.3 ± 3.5	3.1 ± 1.8
AVERAGE POSITIVITY								**20.6 ± 1.6**	**8.4 ± 2.3**

Table 6.2 - continued

River Water Control

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
21	567±117	20.6	0.3	10.9	2.4	6.0	1.8	12.5 ± 3.5	1.5 ± 0.5
18	369	12.2	6.2	-	-	-	-	12.20	6.23
16	467±61	16.8	9.2	14.8	5.7	11.0	9.5	14.2 ± 1.4	8.1 ± 1.0
AVERAGE POSITIVITY								**13.2 ± 1.7**	**5.0 ± 1.3**

River Water + 400ppb Nonoxynol

Fish No.	Av. No. of Cells Scored per Sample	Flask No.1		Flask No. 2		Flask. No. 3		Average	
		% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos	% Wk. Pos.	% Int. Pos
24	540±152	20.0	2.8	17.4	0.4	14.9	1.3	17.5 ± 1.2	1.5 ± 0.6
13	631±72	10.6	2.2	13.4	12.5	14.9	4.6	12.9 ± 1.0	6.5 ± 2.6
15	793±110	12.4	3.3	10.6	3.3	-	-	11.5 ± 0.7	3.3
AVERAGE POSITIVITY								**14.3 ± 1.1**	**3.8 ± 1.2**

CHAPTER 10

PUBLICATIONS FROM THIS WORK

PUBLICATIONS

Dowling K. & Mothersill C. (1999): The development of rainbow trout primary skin epithelial cell cultures as a diagnostic tool in ecotoxicolgy risk assessment. A study with Nonoxynol. *Environmental Toxicology and Chemistry* **18** (12), 2846-2850.

Dowling K. & Mothersill C. (1997): European Community Environmental Program: Diagnostic Ecotoxicology, Cell based methodology to develop markers for early sublethal effects assessment. Contract **ENV4-CT96-0223**. Year-End Report.

Dowling K. & Mothersill C. (1998): European Community Environmental Program: Diagnostic Ecotoxicology, Cell based methodology to develop markers for early sublethal effects assessment. Contract **ENV4-CT96-0223**. Year-End Report.

POSTERS PRESENTED AT CONFERENCES

Dowling K., Mothersill C. The potential use of mucus production in fish epithelial primary cultures as a pollution biomarker. 9th Annual Meeting of Setac-Europe Leipzig, Germany, 25-29 May 1999

Dowling K., Howe O., Mothersill C. A study on the growth and survival of rainbow trout, *Onchorynchus mykiss*, primary epithelial cultures, treated *in vivo* with the aquatic pollutant, prochloraz. V111 International Symposium on Fish Physiology. Uppsala Sweeden, 15-18 Aug. 1998.

Dowling K., Mothersill C. The use of rainbow trout primary epidermal cell cultures as an alternative to immortalised cell lines in toxicity assessment. A study with Nonoxynol. V111 International Symposium on Fish Physiology. Uppsala Sweeden, 15-18 Aug. 1998.

Dowling K., Mothersill C. Toxicity of two organic pollutants nonoxynol and prochloraz to primary cultures of rainbow trout and a fish cell line. 7th International Conference on Environmental Mutagens, Toulouse France, 7-12 Sept. 1997.