NATIVE NEW ZEALAND FISH: LETHAL AND SUBLETHAL EFFECTS OF PENTACHLOROPHENOL ON EARLY AND ADULT LIFE STAGES

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by

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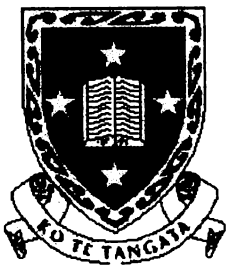

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ABSTRACT

The sensitivity of four New Zealand freshwater fish species to 99.5% pure pentachlorophenol was studied. For one part of the study, four early life stages of smelt (*Retropinna retropinna*), inanga (*Galaxias maculatus*), koaro (*Galaxias brevipinnis*), and common bully (*Gobiomorphus cotidianus*) were exposed to pentachlorophenol, and the acute and sublethal responses were compared to those of the reference species rainbow trout (*Oncorhynchus mykiss*).

Analysis of the acute exposures enabled the determination of 25 LC50 values for the various life stages and fish species examined. In addition, an in depth examination of post-exposure mortality allowed the determination of an additional 16 LC50 values. The acute sensitivities of early life stages of native fish to pentachlorophenol did not differ appreciably when compared to those of rainbow trout. In fact, there was a larger variation between the different life stages within a species, than there was between species. The 48 hour LC50 for pentachlorophenol varied between 31 and 389 ppb (smelt), 30 and 1034 ppb (inanga), 78 and 1043 ppb (koaro), 92 and 191 ppb (common bully), and 77 and 910 ppb (rainbow trout). The larval stages of all fish species examined were more sensitive to acute pentachlorophenol exposure than embryonic stages.

The sublethal parameters of the early life stages that were studied included: growth, the induction of cranial, axial, eye and other deformities, swimming performance, successful hatching rate, time-to-hatch, post-exposure mortality and heart rate. Sublethal effects that were consistently and negatively affected by pentachlorophenol were: post-exposure survival and hatch success, growth, teratology, cranial malformations, swimming performance and heart rates. The parameter time-to-hatch was not consistently altered in either direction, i.e. time-to-hatch after exposure to

pentachlorophenol was just as likely to be either shorter than that observed for the controls or longer.

For the remainder of the study, the adults of two species, inanga and common bully were studied for their sublethal responses to pentachlorophenol. The sublethal responses that were studied included: haematological parameters (lactate, glucose, total red blood cell count, total white blood cell count, differential white blood cell count), liver detoxification enzymes (including ethoxyresorufin-*O*-deethylase and uridine 5'-phosphoglucuronyl transferase) and the induction of 70 kiloDalton heat shock (stress) protein. These parameters were measured at 25, 50 and 75% of the LD50 values for inanga and common bully, which were 0.30 and 0.23 mM, respectively.

Adult inanga and common bully showed surprisingly few physiological changes in response to sublethal doses of pentachlorophenol. Pentachlorophenol-exposed inanga were characterised by a significant decrease in both blood glucose contents and in uridine 5'-diphosphoglucuronic acid transferase activities. Common bully showed no significant changes in any of the experimental parameters examined, although the increase in the level of 70 kiloDalton heat shock protein was comparable to that of the positive heat-shocked controls.

Very little of this type of research with these species had been undertaken previously, and an important aspect of this study was to investigate the feasibility and efficacy of native fish species for toxicology research. The two main technical difficulties encountered were: one, obtaining fertilised eggs for studying the early life stages of the native fish, and two, obtaining sufficient sample for biochemical and haematological analyses of the adult fish. Despite these shortcomings native fish species have been shown to be a valuable new addition for both early and adult life stage toxicity testing and in measuring both acute and sublethal responses to pentachlorophenol.

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AHH	aryl hydrocarbon hydroxylase
ANOVA	analysis of variance
AR	analytical reagent
ATP	adenosine triphosphate
BSA	bovine serum albumin
CTMax	critical thermal maxima
DNA	deoxyribonucleic acid
DNP	dinitrophenol
ECL	enhanced chemiluminescence
ECOD	7-ethoxycoumarin-*O*-deethylase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbant assay
ELS	early life stage
EROD	7-ethoxyresorufin-*O*-deethylase
g	gram
GA	glucuronic acid
GF/A	glass fiber filter of poresize A
GnRH	gonadotropin releasing hormone
grp	glucose-regulated protein
h	hour
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid]
HSF	heat shock factor
hsc	heat shock cognate
hsp	heat shock protein
i.p.	intraperitoneal
i.v.	intra venous
kDa	kiloDalton
LC50	median lethal concentration
LD50	median lethal dose
LHRH	luteinising hormone releasing hormone
LOEC	lowest observed effect concentration
m	meter
MC	methylcholanthrene
MFO	mixed-funtion oxygenase
mm	millimeter

mRNA	messenger ribonucleic acid
MW	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NIWA	National Institute of Water and Atmospheric Research
nm	nanometer
NOEC	no observed effect concentration
PB	phenobarbital
PBS-T	phosphate buffered saline with Tween-20
PCP	pentachlorophenol
PCR	polymerase chain reaction
pH	$-\log[H^+]$
pK_a	$-\log K_a$
PMF	post-mitochondrial fraction
ppb	parts per billion (µg/L)
ppm	parts per million (mg/L)
ppt	parts per thousand (g/L)
pptr	parts per trillion (ng/L)
RBC	red blood cell
RBCC	total red blood cell count
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	standard error of the mean
ßNF	ß-naphthoflavone
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylene diamine
TI	teratogenicity index
Tween	polyoxyethylenesorbitan
UDP	uridine 5'-diphosphate
UDPGA	uridine 5'-diphosphoglucuronic acid
UDPGT	uridine 5'-diphosphoglucuronyl transferase
UK	United Kingdom
USEPA	Unites States Environmental Protection Agency
WBC	white blood cell
WBCC	total white blood cell count
ΔF	delta fluoresence

COMMON AND LATIN NAMES USED IN THIS THESIS

American flagfish	*Jordanella floridae*
Arctic charr	*Salvelinus alpinus*
Atlantic salmon	*Salmo salar*
banded kokopu	*Galaxias fasciatus*
black mudfish	*Neochanna diversus*
bleak	*Alburnus alburnus*
bluegill	*Lepomis macrochirus*
brown mudfish	*Neochanna apoda*
brown shrimp	*Panaeus aztecus*
brown trout	*Salmo trutta*
Canterbury mudfish	*Neochanna burrowsius*
carp	*Cyprinus carpio*
channel catfish	*Ictalarus punctatus*
chinook salmon	*Oncorhynchus tshawytscha*
cod	*Gadus morhua*
coho salmon	*Oncorhynchus kisutch*
common bully	*Gobiomorphus cotidianus*
Crucian carp	*Carassius carassius*
dwarf inanga	*Galaxias gracilis*
Eastern oyster	*Crassostrea virginica*
English sole	*Parophrys vetulus*
fathead minnow	*Pimephales promelas*
flounder	*Platichthys flesus*
giant kokopu	*Galaxias argenteus*
goldfish	*Carassius auratus*
grass shrimp	*Palaemonetes pugio*
herring	*Clupea harengus*
icefish	*Chaenocephalus aceratus*
inanga	*Galaxias maculatus*
killifish	*Fundulus heteroclitus*
koaro	*Galaxias brevipinnis*
lake sturgeon	*Acipenser fulvescens*
lake trout	*Salmo trutta* m. *lacustris*
largemouth bass	*Micropterus salmoides*
longfinned eel	*Anguilla dieffenbachii*

longnose killifish	*Fundulus similis*
medaka	*Oryzias latipes*
mosquito fish	*Gambusia affinis*
pike	*Esox lucius*
pinfish	*Lagodon rhomboides*
rainbow trout	*Oncorhynchus mykiss*
redfinned bully	*Gobiomorphus huttoni*
roach	*Rutilus rutilus*
sandworm	*Neanthes virens*
scup	*Stenotomus chrysops*
sea mussel	*Mytilus edulis*
sea urchin	*Strongylocentrotus purpuratus*
sheepshead minnow	*Cyprinodon variegatus*
shortfinned eel	*Anguilla australis*
shortjawed kokopu	*Galaxias postvectis*
smelt	*Retropinna retropinna*
smooth dogfish	*Mustelus canis*
sockeye salmon	*Oncorhynchus nerka*
spiny dogfish	*Squalus acanthias*
starry flounder	*Platichthys stellatus*
steelhead trout	*Oncorhynchus mykiss*
striped bass	*Morone saxatilis*
striped mullet	*Mugil cephalus*
topsmelt	*Atherinops affinis*
walleye pollock	*Theragra chaleogramma*
white worms	*Enchaetraeus albidus*
whitefish	*Coregonus muksun*
whitespot parasite	*Ichthyophthirius multifilis*
winter flounder	*Pleuronectes americanus*
yellow eel	*Anguilla anguilla*

CHAPTER 1

TOXICITY TESTING OF NEW ZEALAND NATIVE FISH

1.1 Introduction

Pentachlorophenol (PCP) has been utilised in a wide range of applications since its production started on a commercial scale in 1936 (Ahlborg and Thunberg 1980). Its primary usage has been as a wood preservative, but it has also been used as a herbicide, insecticide, molluscicide and as a precursor in the synthesis of other pesticides (Ahlborg and Thunberg 1980). There have been concerns recently about the occurrence of PCP in the New Zealand environment (Gifford et al. 1995), and the effects of this toxicant on the biota. PCP was chosen as the reference toxicant for this study, because its properties have been well studied in the past. Therefore, its toxicity can be used as a benchmark for comparisons between different species.

It is well known that different xenobiotics differ widely in their level of toxicity to individuals of the same species. Toxicants may be classified in a number of ways, one of which is based on their mode of action. These modes can be summarised as follows (Rand et al. 1995):

1) narcosis;
2) polar narcosis (toxicants with polar properties);
3) respiratory uncoupler (e.g. PCP);
4) acetylcholine-esterase inhibitors;
5) membrane irritants;
6) central nervous system convulsant;
7) respiratory blocker;
8) dioxin-like.

The levels at which a xenobiotic is toxic to fish can not be directly extrapolated from the mode of action, but there is a tendency toward higher toxicity in going from group 1 to

group 8 in the above list. The biological activity of toxicants can also be studied using quantitative structure-activity relationships, to assess whether certain chemicals may be either taken up by the organism, eliminated, transformed, or bound to a receptor. Of particular relevance with respect to the processing of a toxicant is which of these steps is rate-limiting (Lipnick 1995). Two factors that are critical in this evaluation include the dissociation constant and the octanol-water partition coefficient of the toxicant (Lipnick 1995).

Toxicity testing may be performed with a variety of aquatic test species, including algae, invertebrates and fish (Rand et al. 1995). Fish are considered an important component of the ecosystem, and they are often incorporated as one of the standard test species, e.g. fathead minnow (*Pimephales promelas*), bluegill (*Lepomis macrochirus*), rainbow trout (*Oncorhynchus mykiss*) and sheepshead minnow (*Cyprinodon variegatus*) (Rand et al. 1995). Of these, rainbow trout is commercially and recreationally important in New Zealand. For part of this thesis rainbow trout is used as a reference test species compared to native fish. A valid and vitally important question is whether rainbow trout is suitably representative in its sensitivity to toxicants compared to native New Zealand fish species. If rainbow trout were proved to be less sensitive than some native fish species, then the native fish could face a substantially greater threat from pollution than what would otherwise be considered harmless by legislated standards for the environment.

Rand et al. (1995) list the properties of appropriate organisms to use for toxicity testing:

1) the species should represent broad ranges of sensitivities;
2) they should be widely available and abundant;
3) they should be indigenous or representative of the ecosystem;
4) they should be recreationally, commercially or ecologically important;
5) routine maintenance, culturing and rearing of the species in a laboratory should be possible;

6) background information should be available regarding physiology, genetics and behaviour of the selected species.

It is well known that different species may have varying sensitivities to the same toxicant (Pascoe 1983; Rand et al. 1995). The differences may be due to (Rand et al. 1995):

1) differences in accessibility of toxicant;
2) differences in ability to metabolise and excrete toxicant;
3) genetic selection of resistant individuals;
4) dietary factors, which influence body composition, physiological and biochemical functions, and nutritional status of organism;
5) life stage differences and differences in body size;
6) general health of the organisms.

Extrapolation of sensitivity from one species to another is not reliable, since toxicity is not predictable based merely on the relatedness of two species. A classic example is the 5000-fold difference in sensitivity to 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin measured with two species: the LD50 for male guinea pigs is 1 µmol/kg, whereas for the golden Syrian hamster it is 5000 µmol/kg (Rand et al. 1995).

The effect of water quality on native New Zealand fish has been studied with reference to thermal pollution, suspended sediment and two toxicants. For instance, the thermal avoidance of inanga (*Galaxias maculatus*) from the Waikato River was studied to determine their response to waste heat discharges from a fossil fuel-power station on the river (Boubée et al. 1991). It was found that water temperature was an important factor in determining upstream migration and distribution of inanga. The thermal tolerance and preference of some native fish were also studied by Richardson et al. (1994). Eels preferred warm temperatures (24.4-26.9°C), whereas galaxiids and common smelt (*Retropinna retropinna*) preferred cooler temperatures (16.1-18.1°C).

Boubée et al. (1997) studied avoidance of suspended sediment by the juvenile migratory stage of six native fish species, and found banded kokopu (*Galaxias fasciatus*) to be the most sensitive species. Koaro (*Galaxias brevipinnis*) and inanga were less sensitive to suspended sediment, whereas shortfinned eels (*Anguilla australis*), longfinned eels (*Anguilla dieffenbachii*) and redfinned bully (*Gobiomorphus huttoni*) were insensitive to suspended sediment.

In one investigation into the effects of toxicants on juvenile inanga, a 96 h LC50 to unionised ammonia (NH_3) was determined to be 1.60 mg/L, which was considered intermediate in comparison to other fish species (Richardson 1991). Further studies with seven indigenous fish species exposed to unionised ammonia found the LC50 for banded kokopu to be the lowest, followed by common bully (*Gobiomorphus cotidianus*), common smelt, redfin bully, inanga, longfinned eels and shortfinned eels (Richardson 1997). The range of the 96 h LC50 values were 0.75 - 2.35 mg/L, which was higher than what has been reported for salmonids (<0.5 mg/L) (Richardson 1997). Cassidy and Lake (1975) determined the 96 h LC50 for inanga exposed to cadmium and found a variation with season, between 1.3 and 10.3 ppm. The variation in response was thought to be due to temperature changes of the stream (Cassidy and Lake 1975).

The question of whether toxicants have had a negative impact on the populations of native fish species is not currently known. It is clear, however, that some native species have a threatened or an endangered status. The species that are of the highest concern are the giant kokopu (*Galaxias argenteus*), the shortjawed kokopu (*Galaxias postvectis*), the dwarf inanga (*Galaxias gracilis*) and three species of mudfish (*Neochanna burrowsius*, *N. apoda* and *N. diversus*) (Williams and Given 1981; McDowall 1990a). Potential reasons for their decline include (McDowall 1990a):

1) exploitation of the fish by humans;

2) the introduction of exotic fishes into the environment;
3) the effect of water management strategies on fish habitats and populations;
4) the effect of land management strategies on water quality and quantity, and thus on fish habitat quality and quantity;
5) the discharge of modified waters into waterways.

A significant amount of research has been done on the negative impact of exotic fish species on native fish populations (Tilzey 1976; Minns 1990; Townsend and Crowl 1991). Only one study has, however, drawn conclusions about direct negative impacts of pollutants on a native fish population (Ward et al. 1987), which will be further reviewed in Section 1.2.1 which reviews relevant aspects of smelt biology.

1.2 The native fish species used in this investigation

This thesis concentrates on the early life stages of four native fish species, which will be introduced in some detail in the following sections (1.2.1 - 1.2.5). The chosen species were common smelt, inanga, koaro and common bully, in addition to rainbow trout which was used as a reference species. The adult stages of inanga and common bully were also used for the investigation of the sublethal effects of PCP.

The use of native fish species for toxicity testing does not completely satisfy the criteria for ideal properties of testing organisms as listed by Rand et al. (1995) above. For example, native fish species do not fulfil the requirements on routine maintenance and background information. In fact, one of the aims of this thesis was to enlarge the information on these points. Whether the chosen species represent a broad range of sensitivities is also not known at present. Points 2-4 in the list of Rand et al. (1995) are, however, well fulfilled: the chosen species are generally widely available, indigenous to New Zealand, and some are ecologically and commercially important. Furthermore, the four native species represent a wide range of habitats, from still lakes and ponds, to slow rivers and fast mountain streams. They are also representative of

the main families of native fish in New Zealand: Galaxiidae, Eleotridae and Retropinnidae. The freshwater native fish groups excluded from this study are eels, lamprey and torrentfish, either due to the unavailability of the early life stages in New Zealand (eels), or because they are represented by only one species (torrentfish and lamprey).

1.2.1 Common smelt

Common smelt are endemic to New Zealand and are found only at low elevations (McDowall 1990a). Where the elevation gradients of rivers are low, the smelt may be found quite far inland, for instance, 160 km along the Waikato River (McDowall 1990a; 1990b). Sea-going populations of smelt actually spend little time in fresh water except when they migrate inland to spawn (McDowall 1990b). Land-locked populations are present in many inland lakes, with some of these populations having been introduced as food for trout (McDowall 1990a; 1990b). Smelt are an important part of the whitebait fishery in the Waikato River, but they have been classified as 'second-class whitebait' (McDowall 1990a, 1990b). They grow to an average of 80 - 100 mm in length (McDowall 1990b), and feed on a variety of aquatic (Diptera, Ephemoptera, Trichoptera) and terrestrial insects (McDowall 1990b).

Spawning is believed to take place on sandy banks in rivers in the summer and autumn (McDowall 1990b). In Lake Taupo, smelt mature at about two years of age, and may spawn more than once in a season (Stephens 1982). Significantly, fifty percent of these smelt were found to die after spawning (Stephens 1982). The eggs hatch in 8 - 10 days depending on the ambient temperature, and are 3 - 6 mm long at time-of-hatch. The hatched larvae are washed out to sea unless they originate from a land-locked population as in Lake Taupo (McDowall 1990b). Smelt eggs have been artificially fertilised and incubated by Mora and Boubée (1993), who found an optimum temperature for development of 15 - 17.5°C.

There are few historical data on the abundance of common smelt in New Zealand, but the population does not appear to have decreased or increased significantly in the last few years. There are, however, a number of environmental factors that may threaten the future of common smelt. For example, alterations in flow patterns and channel conformation may cause changes to spawning habitats utilised by common smelt in the Waikato River (McDowall 1990a). In one study of these types of effects on smelt in Lake Waahi (in the Waikato River catchment area), the abundance of smelt declined drastically between 1975 and 1983 (Ward et al. 1987). Since the recruitment of resident smelt was impaired, it was assumed that the cause of the decline was detrimental conditions for spawning and incubation of smelt eggs. Open pit coal mining in the catchment area was responsible for discharge of silt, boron, phenols, ammonia, sulphide and cyanide, and these together or singly could have caused the decline of the smelt population (Ward et al. 1987).

The choice of early life stage studies of common smelt for this study was based in part on the fact that eggs from this species have been fertilised and collected in the past. In addition, smelt is a common fish species in the Waikato area, and thus there was little concern for causing a decline in their numbers. However, they were deemed unsuitable for studies of adult fish, since they are very sensitive to loss of their scales, and thus experimental manipulation of these fish would have been exceedingly difficult.

1.2.2 Inanga

Inanga occur in western and eastern Australia, Tasmania, Lord Howe Island, Patagonian Chile and Argentina, the Falkland Islands and New Zealand (McDowall 1990a). Inanga are widely abundant in New Zealand in low elevation and low gradient rivers, streams, lagoons and swamps, but they are unable to penetrate very far inland (McDowall 1990a; 1990b). Adult inanga reach to between 100 and 110 mm and occur in shoals (McDowall 1990b). They feed on aquatic organisms as well as terrestrial insects (McDowall 1990b).

Inanga are catadromous, with spawning taking place in estuaries (McDowall 1990a). The inanga spawn at the age of about one year, although some delay spawning until the second or third year. They are not believed to survive spawning (Eldon 1969; McDowall 1990b). Spawning takes place predominantly in the autumn, although it has also been reported to occur from at least September through to June (McDowall 1990b). Downstream migrations of male and female inanga take place when high tides are occurring in the estuaries where they spawn. Eggs are laid on vegetation close to the upper level of the high tide, and the eggs, therefore, incubate in moist air until the following high tide. It has been shown that the eggs are able to develop in a wide range of conditions and temperatures (Carlberg 1993). Furthermore, the eggs have been able to be artificially fertilised using either a dry or a wet method. They are able to tolerate temperatures between 8 and 18°C, with the optimum being about 15°C, and salinities between 0 and 10-20 ppt. The mean incubation time for time-to-hatch at 18°C was 13.3 days at 0 ppt and the mean length of larvae hatched in this study at 0 ppt and 18°C was 7.59 mm (Carlberg 1993). In addition, adult inanga are very tolerant of a diverse range of conditions, with regards to water clarity, pH and forest cover (Chessman and Williams 1975; McDowall 1990b. After hatching in the wild the larvae are washed out to sea, whereafter they return to fresh or brackish water after about 6 months. Inanga is considered to be the most important species in the whitebait catch (McDowall 1990b).

Adult inanga were found to be relatively easy to maintain in an aquarium, as they eat commercially available dried fish foods of sufficiently small size (Eldon 1969; Mitchell 1989). Furthermore, Mitchell (1989) has proposed inanga to be a potentially valuable laboratory species, because they are easy to obtain and adjust readily to laboratory tanks. Inanga reach maturity in laboratory aquaria, but they do not generally spawn spontaneously in captivity. Viable eggs can, however, be obtained using pituitary extracts from salmonids, or luteinising hormone releasing hormone (LHRH) analogues

(Mitchell 1989; Carlberg 1993). The eggs are relatively easy to incubate and their development is readily visible inside the transparent eggs. Similar to the situation with adult inanga, the hatched larvae can be fed commercially available foods. Both the early life stages and adult stages could, therefore, be used for toxicity testing and other research (Mitchell 1989).

Although inanga remain common and abundant there is concern for their decline because of the whitebait fishery (McDowall 1990a). The decline is almost certainly due to habitat degradation, especially of the spawning grounds in estuarine areas. There is also a reduction in both the occurrence and access to lowland swamps and lakes. McDowall (1990a), interestingly, has suggested that excess exploitation by humans may not be a cause for their decline.

Inanga were chosen as a species of both early life stage and adult studies, because it has been relatively well studied and is easily maintained and manipulated in the laboratory. It is also a common fish species in the Waikato region.

1.2.3 Koaro

Koaro are found in south-eastern Australia, Tasmania, the Auckland and Campbell Islands and New Zealand (McDowall 1990a). The species is widespread in New Zealand, and it occurs in the upper reaches of many river systems, due to its ability to climb (McDowall 1990a). Koaro prefer rocky and relatively fast mountain streams covered with forest canopy at high elevations. Adult koaro grow to between 160 and 180 mm in length (McDowall 1990b). The species is normally diadromous, but land-locked populations exist, e.g. in Lake Taupo (McDowall 1990b). In one study on the Omanawa stream on the south side of Mount Pirongia in the Waikato area, koaro were found to be relatively common with an average population density of 11.7 fish/50 m (Swales and West 1991).

Little is known about its reproductive biology, although it is likely that spawning takes place in autumn or early winter (McDowall 1990b). West (1989) found ripe koaro in March and May in the Oparau River and successfully fertilised and incubated the eggs. The incubations took place on moist filter paper at room temperature and at 13°C, or in aerated fresh water at 13°C. The only successful hatchings occurred in the group incubated in aerated fresh water at 13°C after 18 - 33 days, and West (1989), therefore, concluded that it is likely that spawning takes place in the normal adult habitat, i.e. in mountain streams and that koaro eggs are naturally incubated in water. The larvae are believed to be washed out to sea after hatch. Koaro is the second most abundant species in the whitebait fishery (McDowall 1990a). Koaro reach maturity at two or three years of age, and may survive for at least six to eight years (Eldon 1969, McDowall 1990b). Koaro were found to easily adapt to aquaria, which was surprising given their natural habitat (Eldon 1969). Koaro in the wild feed on benthic prey (Kusabs and Swales 1991), but in a tank they become adept at feeding at the surface of the water (Eldon 1969). In their natural habitat, koaro also feed on koaro whitebait, fish ova and terrestrial prey, and these are apparently more important than benthic prey in terms of consumed biomass (Kusabs and Swales 1991). Koaro can tolerate high levels of turbidity, but they are intolerant of acid (pH<6.5) and brown water (McDowall 1990b; Rowe 1991).

Although koaro may be locally abundant, it is obvious that the population as a whole has declined significantly (McDowall 1990a). Koaro can be commonly found in some streams in the Waikato area (e.g. in the Omanawa stream), but overall they have been classified as vulnerable or endangered in this area (Swales and West 1991). Possible causes for their decline include the following:

1) deforestation, since koaro are, as mentioned above, only found in streams covered with forest canopy (McDowall 1984; 1990a);
2) dams and weirs which may stop the inland penetration of koaro (McDowall 1990a);

3) the introduction of trout and other exotic species to inland lakes (McDowall 1990a);
4) water pollution and exploitation, although the potential role has not been determined (Swales and West 1991).

With regard to the effects of introduced fish on koaro, it was found that when there is a dietary overlap between rainbow trout and koaro the densities of koaro were lower (Kusabs and Swales 1991). In addition, rainbow trout were also found to eat small koaro. Due to the competition between koaro and rainbow trout, koaro may be more dependent on availability of habitats which are either unavailable or unusable by rainbow trout (Kusabs and Swales 1991). Finally, the introduction of smelt (although not an exotic species) into some lakes of the central North Island may have displaced juvenile koaro, which normally would occupy the pelagic zones now utilised by smelt (McDowall 1984; 1990b).

The decision to use koaro for early life stage studies was affected by two main considerations: first, the adult fish are not as common in the Waikato region as some of the other chosen species; and, two, the production of fertilised eggs had not been well-studied in the past. However, it was felt that it was important to introduce koaro as a test species, with the proviso that special care had to be taken not to cause any decline in the local population (after egg and milt collection, adults were returned to the streams of origin). Because of its potentially vulnerable status in the Waikato region, koaro were excluded from any tests with adult stages, where it would have been necessary to sacrifice the fish at the end of the experiments.

1.2.4 Common bully

Common bully occurs only in New Zealand, where it remains widespread, apparently in numbers comparable to pre-European times (McDowall 1984; 1990a). Common bully prefer still waters with a pH between 6.0 and 7.0, and are diadromous, although land-locked populations commonly exist (McDowall 1990b; Rowe 1991). Diadromous

bullies normally reach 110 mm in length, whereas land-locked bullies only attain 70-80 mm in length. The males and the females can be distinguished by the different shapes of their genital papillae, and also by the colour and shape of the fish and their fins. Spawning occurs mostly in spring and summer (McDowall 1990b). The males prepare a nest site on the sides or underneath a solid surface and may spawn with several different females. There is also evidence that the females may spawn twice during one season. The eggs are attached to the surface by the females and are guarded by the males until time-of-hatch. The hatched larvae are then washed downstream to the sea (if they are not land-locked) and return to freshwater when they are 15-20 mm in length (McDowall 1990b). Common bully are opportunistic carnivores, eating zooplankton, insect larvae and various molluscs (McDowall 1990b). Although no decline of the population has been recorded, it is likely that such a decline would be the result of habitat deterioration and perhaps barriers to upstream migration (McDowall 1990a).

Common bully is a robust, common and easily maintained fish species, and it was, therefore, deemed suitable for testing with the adult stage. However, little is known about either the possible spontaneous spawning of this species in the laboratory or of its artificial induction.

1.2.5 Rainbow trout

Rainbow trout is an introduced species in New Zealand, and in this thesis it has been used as a reference species for tests with early life stages. Spawning of rainbow trout takes place in late autumn and winter and the eggs are quite large, 3 - 5 mm in diameter (McDowall 1990b). At 13°C, the eggs hatch between 23 and 30 days after fertilisation (Knight 1963). Due to the widespread literature on the biology of rainbow trout, it will not be further expounded upon in this section. Relevant information with regard to its use as a control species will be presented and discussed in appropriate results or discussion sections.

CHAPTER 2

THE BIOLOGICAL EFFECTS OF PENTACHLOROPHENOL

2.1 PCP: properties and its use

The widespread use and the physical characteristics of PCP have led to its release and dispersion into the environment. The sources of PCP include the use of insecticides, the chlorination of phenolic sewage or drinking water and pulp and paper operations (Ahlborg and Thunberg 1980; Canadian Council of Resource Ministers 1987). In the UK, the timber industry is considered to be the largest source of PCP release into the environment accounting for a total of 127 tonnes released per year. A large proportion of this release is due to volatilisation of PCP during treatment of the timber (Wild et al. 1992). Other direct sources of PCP releases are the textile industry resulting in 3 tonnes released per 10 tonnes used, and mushroom farming which generates 18 tonnes of PCP release annually. Sewage sludge and combustion sources are additional significant sources of indirect PCP release, with 6 tonnes per year and unquantified amounts of release, respectively (Wild et al. 1992). The physical characteristics of PCP are listed in Table 2.1.

Table 2.1 Properties of pure pentachlorophenol (C_6HCl_5O).

Molecular weight	266.35 g/mole
Melting point	191°C
Boiling point	310°C
Density	1.987 g/cm^3
Vapour pressure	1.6 x 10^{-4} mm Hg at 25°C
Solubility in water (pure PCP)	20 ppm at 30°C
Solubility in water (sodium salt)	330 ppt
Partition coefficient (octanol/water)	1 x $10^{5.01}$
pK_a	4.71

From Wagner (1983) and Saarikoski et al. (1986)

PCP is persistent in some soils, with a reported half-life of up to 5 years (USEPA 1988). However, it photodegrades in a few hours to a few days in surface waters, dependent on the conditions (Canadian Council of Resource Ministers 1987, USEPA 1988, Wild et al. 1992). In contrast to the role of volatilisation in loss of PCP from the treatment of timber, volatilisation does not play a significant role in removing PCP from the water column (Canadian Council of Resource Ministers 1987). Because of its high octanol/water partition coefficient, PCP is quickly removed from the water column by adsorption to organic-rich sediments (Canadian Council of Resource Ministers 1987). A portion of the biodegradation and the subsequent losses of PCP from the environment is caused by microbes (Uotila et al. 1991; Wild et al. 1992).

2.2 PCP and regulatory legislation

The Japanese government restricted the use of PCP in 1971 because of its high toxicity to fish (Kobayashi 1978) and its use was banned in Sweden in 1978 (Cleveland et al. 1982). Agriculture Canada suspended many of the domestic and commercial uses of wood preservative containing PCP in 1980 (Canadian Council of Resource Ministers 1987) and PCP was deregistered in New Zealand in 1991 (Gifford et al. 1995). In the UK, PCP is included on the Red List of compounds, and its discharges into waterways are being controlled (Wild et al. 1992).

Commercial products containing PCP may contain a number of contaminants, and concern about these compounds, some of which are more toxic than PCP, has been a reason for banning the use of PCP as a pesticide or herbicide in many countries. For example, a technical grade PCP-based fungicide formulation is reported to have had the following impurities: <1 - 2000 ppm chlorinated dioxins, 100 - 1000 ppm chlorinated diphenylethers, 50 - 200 ppm chlorinated dibenzofurans and ~1% chlorinated phenoxyphenols (Wagner 1983). The chlorinated dioxins present in technical grade PCP are mainly the hexachloro-, heptachloro-, and the octachlorodibenzo-*p*-dioxins

and a variety of polychlorodibenzofurans (Wagner 1983). In one study of technical grade PCP, the highly toxic 2,3,7,8-chlorine isomer of chlorinated dioxins was not found (Wagner 1983). Technical grade PCP usually also contains 4 - 12% tetrachlorophenols (Wagner 1983). Due to the potential complicating effects of these and other impurities in technical grade preparations, the emphasis in this study with respect to the biological effects of PCP exposure will primarily be data derived from studies that utilised pure PCP, which in this thesis will be defined to be AR-grade PCP or to have >97% purity. In addition, in the experimental part of this thesis, only pure PCP (99.5%) was used.

2.3 PCP and its use as a reference toxicant

It has previously been suggested that pentachlorophenol should be used as a reference toxicant to ensure that the conditions of toxicity tests or the animals of different laboratories are similar (Davis and Hoos 1975; Adelman and Smith 1976; Lee 1980). The characteristics of an ideal reference toxicant according to Lee (1980) are:

1) previous and continued use as a reference toxicant;
2) recognised as an environmental contaminant;
3) should be a general metabolic stressor;
4) toxicity at low concentrations;
5) information available on its toxicity, stability and analytical techniques;
6) availability at a consistent purity;
7) usable in both continuous-flow and static bioassays;
8) readily quantified in aqueous solution at concentrations used in bioassays;
9) rapid lethality;
10) nonselectiveness (i.e. non-specific and toxic to both invertebrates and fish);
11) stability (i.e. not changing significantly in potency in static bioassays);

PCP satisfies criteria 1 - 3 in that it has been previously used as a reference toxicant, is recognised as an environmental contaminant, and its mode of action is that of a general metabolic stressor (Lee 1980; Rand et al. 1995). Other advantages of PCP as a reference toxicant include a relatively high solubility in water (of the sodium salt), toxicity to fish at low concentrations, virtually complete ionisation in solution at pH 7.0, stability in solution at pH 7.0 - 8.5, and its availability as a dry solid of high purity (Alderdice 1963, as detailed by Davis and Hoos 1975). In one study using fish, Lee (1980) found that PCP was fast acting and reached the lethal threshold concentration before 48 h with fathead minnows. In another investigation, PCP was judged to be 'good' or 'very good' as a reference toxicant because of consistency of fish response, detection of abnormal (unhealthy) fish, rapid attainment of threshold LC50, use in both static and flow-through bioassays, and general ease of handling (Adelman and Smith 1976).

2.4 PCP in the abiotic and biotic environment

PCP has been found in both abiotic and biotic environments, and nearby sources or spills of PCP can often be detected. Although now deregistered, past use of PCP is of concern in the New Zealand environment. In the catchment area of Lake Rotorua in New Zealand there is a major sawmill which has used PCP for at least 35 years (Gifford et al. 1995). Soil samples from the saw mill area were shown to contain high levels of PCP, between <0.35 - 3620 ppm. PCP was also found to have dispersed into the ground water, where PCP-levels of <1 - 5180 ppb were detected. The Waipa Stream, a tributary of the Puarenga Stream, was also found to be contaminated and contained <0.01 - 655 ppb PCP (NTG 1992). Subsequently, the PCP concentrations of the biotic and abiotic compartments of Lake Rotorua were compared to sites known to be pristine. The water of Lake Rotorua was shown to have PCP concentrations less than 0.04 ppb and streams flowing into it less than 3.62 ppb. Muscle tissue of rainbow or brown trout (*Salmo trutta*) caught in Lake Rotorua had less than 6.4 ppb PCP (wet weight), whereas bile had PCP concentrations of 1745 and 2819 ppb PCP (wet

weight). In comparison, rainbow or brown trout caught at pristine sites in New Zealand had muscle tissue concentrations of <2 ppb and bile concentrations of <25 ppb PCP. In this same study, freshwater mussels from Lake Rotorua had PCP concentrations between nondetectable and 38 ppb (dry weight) and mussels from pristine remote lakes had concentrations of less than 4 ppb (dry weight). Finally, PCP concentrations in sediments of Lake Rotorua and its tributaries varied between <1 and 418 ppb (dry weight), whereas remote lake sediments had PCP concentrations between 0.6 and 1.3 ppb (dry weight). The authors concluded that localised contamination could be detected near areas of past use of PCP, but that there was not a widespread contamination of the environment of New Zealand (Gifford et al. 1995).

The levels of PCP found in the New Zealand environment are comparable to levels found in some overseas studies. Levels of PCP in fresh surface and ground water found in connection with known PCP spills, a wood treatment facility and a pulp and paper mill have ranged from 0.54 to 3350 ppb (Pierce et al. 1977; Pierce and Victor 1978; Canadian Council of Resource Ministers 1987). Similarly, the levels of PCP in some fish after a known spill have been shown to reach 2500 ppb (dry weight) and in sediments, 1300 ppb (dry weight) (Pierce et al. 1977). In contrast to the PCP concentrations at or near contaminated sites, background levels of PCP in fresh surface water have been reported to be 0.005-1.4 ppb in the Great Lakes (Canadian Council of Resource Ministers 1987), and 0.41 ppb in the IJssel River in the Netherlands (Wegman and Van Den Broek 1983). In the Gulf of Mexico in Texas, the aqueous PCP concentration was reported to be 8.2 pptr (Murray et al. 1981).

The mode of action of PCP toxicity is that of a general metabolic stressor and is widely regarded as being due to the uncoupling effect on mitochondrial oxidative phosphorylation (Weinbach 1954; Ahlborg and Thunberg 1980). Uncoupling agents allow electron transport to occur in mitochondria, but they prevent the phosphorylation of ADP to ATP by uncoupling the linkage between electron transport and ATP

synthesis. The free energy released by the electron flow generates heat rather than resulting in a net production of ATP. It has been suggested that uncoupling agents can short-circuit oxidative phosphorylation by transporting protons through the lipid bilayer of the mitochondria (Lehninger 1982). The partial uncoupling of oxidative phosphorylation at low levels of PCP may be followed by complete inhibition of it at higher concentrations (Desaiah 1978). Another well-known uncoupler of oxidative phosphorylation is 2,4-dinitrophenol. The sublethal effects of PCP on early life stages and adult fish will be discussed in detail in Chapter 3.

An important characteristic of a toxicant is the LC50, that concentration which causes 50% mortality among the tested organisms for the duration of the toxicity test. The LC50 predicted for PCP which has a log octanol-water partition coefficient (log K_{ow}) of 5.01 (Table 2.1) by Rand et al. (1995) (log LC50 (mM) = -1 x log K_{ow} + 1.7) is 130 ppb, which is close to the LC50 values actually found in a variety of aquatic species (Table 2.2).

The bioconcentration factor (BCF) of PCP varies between 0.26 and 278 for various aquatic organisms (Table 2.3). This can be compared to chemicals with high BCFs, including chlordane with a BCF of 14 000, dichlorodiphenyl trichloroethane (DDT) with 54 000 and polychlorinated biphenyls (PCBs) with 100 000 (Gerba 1996). The BCF predicted from the equation log BCF = 0.79 log K_{ow} - 0.40 is calculated to be 3613 for PCP for small fish with a lipid content of 7.6% (Spacie et al. 1995), which is 10 to 100-fold higher than actual measured BCF values for fish. This may be related to the ability of aquatic organisms to rapidly metabolise PCP (Ahlborg and Thunberg 1980; Canadian Council of Resource Ministers 1987). The metabolism of PCP will be further discussed in Section 3.4.2.

Table 2.2 LC50 values for fish exposed to pure PCP

Duration of test	LC50, ppb	Species	Comment	Reference
96 h	58-140	Atlantic salmon (*Salmo salar*)	juvenile	Burridge and Haya 1990
96 h	47-106	Rainbow trout	tested at different laboratories	Davis and Hoos 1975
96 h	32 and 92	Coho salmon (*Oncorhynchus kisutch*)	tested at different laboratories	Davis and Hoos 1975
96 h	50 and 130	Sockeye salmon (*Oncorhynchus nerka*)	tested at different laboratories	Davis and Hoos 1975
96 h	3000 1300 3000 480 32 18	Rainbow trout	0 h eggs 24 h eggs early eyed eggs late eyed eggs sac fry early fry	Van Leeuwen et al. 1985
96 h	223-392	Sheepshead minnow	1 day - 6 week old fry	Borthwick and Schimmel 1978
96 h	470	Fathead minnow		Cleveland et al. 1982
96 h	210	Fathead minnow		Adelman and Smith 1976
96 h	220	Goldfish (*Carassius auratus*)		Adelman and Smith 1976
21 day	1490	Medaka (*Oryzias latipes*)	eggs	Helmstetter and Alden 1995
96 h	136-287	Largemouth bass (*Micropterus salmoides*)	14 - 84 days old	Johansen et al. 1985
96 h	300	Bluegill		Pruitt et al. 1977
96 h	53	Pinfish (*Lagodon rhomboides*)		Schimmel et al. 1978

Table 2.2 (continued) LC50 values for fish exposed to pure PCP.

Duration of test	LC50,ppb	Species	Comment	Reference
96 h	>306	Longnose killifish (*Fundulus similis*)		Schimmel et al. 1978
96 h	112	Striped mullet (*Mugil cephalus*)		Schimmel et al. 1978
96 h	218	American flagfish (*Jordanella floridae*)	juvenile	Smith et al. 1991
48 h	78	Bleak (*Alburnus alburnus*)		Oikari 1987
96 h	66	Bleak		Oikari 1987
96 h	38	Roach (*Rutilus rutilus*)		Oikari 1987
96 h	45	Pike (*Esox lucius*)	newly hatched	Oikari 1987
48 h	87	Crucian carp (*Carassius carassius*)		Oikari 1987
96 h	54	Lake trout (*Salmo trutta m. lacustris*)	fry	Oikari 1987
48 h	65	Whitefish (*Coregonus muksun*)	newly hatched	Oikari 1987
96 h	43	Whitefish	newly hatched	Oikari 1987

Table 2.3 Bioconcentration factors (BCFs) of PCP for various aquatic organisms.

BCF	Species	Reference
64.4	Sea urchin (*Strongylocentrotus purpuratus*)	Anderson et al. 1994
278 ± 182	Topsmelt (*Atherinops affinis*)	Benner and Tjeerdema 1993
134-190	Striped bass	Gates and Tjeerdema 1993
174	Fathead minnow	Huckins and Petty 1983
132	Mosquito fish (*Gambusia affinis*)	Lu et al. 1978
38	Striped mullet	Schimmel et al. 1978
30	Longnose killifish	Schimmel et al. 1978
0.26	Brown shrimp (*Panaeus aztecus*)	Schimmel et al. 1978
1.7	Grass shrimp (*Palaemonetes pugio*)	Schimmel et al. 1978
41-78	Eastern oyster (*Crassostrea virginica*)	Schimmel et al. 1978
53	Longnose killifish	Trujillo et al. 1982

PCP accumulates in different tissues to varying degrees. The 24 h total BCF for striped bass (*Morone saxatilis*) exposed to 60 ppb pure PCP was found to be between

134 and 190 (Gates and Tjeerdema 1993). The greatest concentration of PCP was found in the heart, followed by, in order of decreasing tissue concentration, the liver, viscera/gonad, gill, head and muscle. Skeletal muscle, however, retained the largest fraction of total residues, due to its large mass (Gates and Tjeerdema 1993). Glickman et al. (1977) exposed rainbow trout to 25 ppb PCP, and found rapid uptake into the tissues, with the highest levels found in liver tissue. Similarly, Pruitt et al. (1977) found the greatest concentration of PCP in the liver of PCP-exposed bluegill, followed by the digestive tract, gills and muscle. Kobayashi (1978) found that PCP accumulated in goldfish until a level of about 100 μg/g body weight was reached, whereafter the fish died from the toxic effects of PCP.

The half-lives of PCP in different animals varies between 5.2 and 112.8 h and is summarised below in Table 2.4. The half-life is a reflection of the relative rates of excretion and biotransformation (detoxification) through kidney and bile (Rand et al. 1995). Terrestrial animals generally possess greater capabilities in excreting PCP than aquatic animals, which is due to differing metabolic capabilities (Benner and Tjeerdema 1993). The metabolism of PCP by fish will be dealt with in greater detail in Section 3.4.2, and the lethal and sublethal effects of PCP will be discussed in conjunction with discussions on the endpoints used in this study (Sections 3.2 - 3.4.3).

Table 2.4 The half-life of PCP in different animals exposed to PCP.

Half-life	Species	Reference
10 h	Goldfish	Kobayashi 1978
6.2-23 h (in different tissues)	Rainbow trout	Glickman et al. 1977
112.8 h	Longnose killifish	Trujillo et al. 1982
5.2 h (i.v. exposure) 5.8 h (oral exposure)	B6C3F1 Mice	Reigner et al. 1992

CHAPTER 3

END POINTS OF THIS STUDY

3.1 Review of maturation and ovulation in fish

The study of pollutant effects on fish may be performed on all the different life stages of the fish, including reproductive and embryonic stages. In one part of this study, the effects of PCP on the embryonic stages and newly hatched larvae of four native fish species and rainbow trout were investigated.

One of the advantages of using early life stages as test organisms is the relative homogeneity of every batch of eggs. The eggs are of identical age and have been raised under identical conditions. The degree of genetic variability depends, in part, on the number of females and males that were used for the production of eggs. With respect to the technical feasibility of performing toxicity tests, the use of early life stages requires smaller quantities of incubation and testing solutions than those utilising adult life stages.

Fertilised eggs may be obtained for toxicity testing utilising 3 different strategies:

1) by using adult, sexually mature fish captured in the field for artificial fertilisation;
2) by collecting eggs naturally spawned in the field, or occasionally in laboratory aquaria;
3) by artificially inducing ovulation of adult fish maintained in the laboratory through either environmental or hormonal manipulations.

Even though some fish species are known to spawn spontaneously in the laboratory, many species achieve maturity but ultimately fail to undergo ovulation (Barnabé 1994). Therefore, it is usually necessary to manipulate the fish to achieve the desired degree of ovulation. The induction of spawning, achieved by use of environmental variables (e.g. light, temperature, presence of spawning substrate), requires detailed knowledge

of the requirements of each fish species. A simpler and more reliable method incorporates the use of ovulation-inducing hormones.

Ovulation can be hormonally induced by the following methods (Barnabé 1994):

1) injection with crude fish hypophyseal (i.e. pituitary) extracts (hypophysation);
2) injection of substances with gonadotropic activity, e.g. human chorionic gonadotropin;
3) injection with releasing hormone analogues, e.g. gonadotropin releasing hormone (GnRH).

These methods of inducing ovulation all act on different stages of the hypothalamic-pituitary-gonadal axis which is responsible for the maturation of gonads in fish. The natural sequence of maturation can be described as follows (Jobling 1995):

The internal or external environment stimulates the hypothalamus in the brain to produce either inhibiting factors or releasing hormones, which affect gonadotropic cells in the pituitary gland. The pituitary then releases gonadotropic hormones, which stimulate the ovary or testis to produce oestrogens or androgens, respectively. Androgens initiate the production of sperm, and oestrogen mediates the production of oocytes and vitellogenin, which is required for yolk build-up. Both androgens and oestrogen act in a negative feedback fashion on the pituitary and hypothalamus.

The releasing hormone analogues, therefore, simulate the action of the hypothalamus on the pituitary gland. The use of releasing hormone analogues have several advantages over the use of the relatively crude extracts in hypophysation techniques (Zohar 1986):

1) GnRH analogues stimulate the secretion of species-specific gonadotropin in the fishes;
2) They can be easily synthesised and obtained in pure form;

3) Their use in µg/kg body weight doses is economical;

4) They represent a low degree of species specificity;

5) Being small peptides, they are non-immunogenic.

In this study, a synthetic GnRH (Section 4.3) with an increased residence time in the fish was used. When using GnRH, it is necessary to also use an antagonist to dopamine, a hypothalamic inhibiting factor (Zohar 1986).

In one investigation of the ability of hormones to induce ovulation in fish, Carlberg (1993) studied the use of a luteinising hormone releasing hormone (LHRH) analogue to induce the maturation and ovulation of inanga eggs. The author found that a single dose of 150 µg/kg gave optimum yield in terms of percentage of ovulated fish. The LHRH dose was within what is normally used in fish (1 - 600 µg/kg) (Zohar 1986).

3.2 Toxicity tests

When studying the acutely toxic effects of xenobiotics on fish, the most commonly used method is the determination of LC50, i.e. the aqueous concentration that causes mortality to half of the tested organisms within the stated time period. Commonly used periods for toxicity testing are 48 and 96 hours. Concern about the chronic effects of exposure to pollutants has, however, lead to the conduction of toxicity tests covering the entire life cycle of fish. These tests usually begin with fertilised eggs from the F1-generation, and continue with the sexual maturation and production of eggs of these individuals. The tests are completed with the development of embryos and larvae of the F2 generation (Nagel 1993). Conducting these types of tests require at least 6-12 months, which makes them both costly and labour intensive. Some researchers (Woltering 1984; Suter et al. 1987; Nagel 1993) maintain that whole life cycle tests are the only type of test to provide a good understanding of chronic toxicity. Chronic life cycle tests are also capable of detecting detrimental effects at concentrations lower than with acute toxicity tests. In contrast, other investigators (McKim 1985; Giesy and

Graney 1989; Bresch 1993; Walker et al. 1994) have proposed that only the early life stages of fish be used.

The main reason for utilising early life stages is thought to be their increased sensitivity to xenobiotic exposure when compared to later life stages of fish. For instance, McKim (1985) studied the effect of pollutants on different life stages of fish, and found that the lowest observed effect concentrations (LOECs) of early life stages were the more sensitive or equally sensitive to the whole life cycle LOECs in 83% of the reviewed tests. However, if the early life stage LOEC values were different from the LOEC of the whole life cycle tests, they differed only by a factor of two. For this study, it was unlikely that whole life cycle tests could be conducted with a high degree of reliability, since the production and maintenance of successive generations of native New Zealand fish species remains largely unstudied.

3.3 Sublethal effects of toxicants on early life stages

3.3.1 Teratology

Teratology is the study of how adverse conditions or xenobiotics may cause abnormalities in developing embryos. A teratogenic agent causes irreversible effects that do not cause direct lethality but which result in structural or functional anomalies in live offspring. Developmental toxicity covers any detrimental effect produced by exposures to developing organisms during embryonic stages of development.

Manson and Wise (1991) reviewed the timing of teratogenesis in mammalian species, and found that the critical phase for inducing anomalies in individual organ systems can either be as short as one day or can extend throughout organogenesis. Consequently, the exact time of exposure during the development of the embryos can have a marked effect on the final pattern of malformations (Manson and Wise 1991).

The major effects of exposure on developing embryos are embryo-lethality, malformations and growth retardation. The dose-response pattern for each type of toxicant is different: highly teratogenic agents cause malformations at much lower dosages than those required to induce lethality; embryo-lethal agents may cause a mixture of lethality, malformations and growth retardation; and embryo-toxic agents may cause growth retardation in surviving individuals, but this is quickly followed by lethality at higher doses (Manson and Wise 1991).

The most common abnormalities that are found in developing fish embryos are yolk-sac, eye, otic capsule, jaw and axial deformities (Rosenthal and Alderdice 1976). The effects are not considered to be specific to certain compounds, but rather it appears that fish embryos only have a limited number of ways of responding to a toxic insult (von Westernhagen 1988). The lack of differentiated responses may also be a reflection of the lack of techniques to study more subtle and primary changes in developing embryos (von Westernhagen 1988).

Another feature of teratogenesis is that many lesions that are initiated at an early developmental stage, only become observable at a later life stage (delayed toxicity). Some effects may, therefore, be most efficiently studied at the time of hatching rather than shortly after fertilisation (Rosenthal and Alderdice 1976). This consideration affected the approach taken in this study in the determination of the teratological responses of PCP-exposed eggs, i.e. only mortality was recorded prior to the hatching of the eggs, and teratological changes were only studied in hatched larvae.

There are several additional points that are important to consider when conducting toxicity tests with fish eggs and these include the following:

1) Many compounds cause either developmental arrest or retardation; this in turn may prolong exposure at sensitive stages (Laale 1981; Weis and Weis 1989);

2) The yolk sac may serve as a toxicant sink for lipophilic hydrocarbons and heavy metals, and thus, exposure to a toxicant may continue even after transfer of eggs to clean water (Sharp et al. 1979; Longwell et al. 1992);
3) The duration of exposure may not be as important as the timing of exposure, i.e. some stages of development may be more sensitive to toxicants than others (Sharp 1990);
4) Since suboptimal rearing conditions (with regards to oxygen tension, salinity and incubation temperature) may cause abnormalities in developing eggs, it cannot be assumed that all terata are a result of toxic agents, unless there are appropriate controls (Cameron et al. 1992);
5) The relevance of abnormalities for the survival of the individual and ultimately for the whole cohort of larvae must be taken into consideration. Some terata are obviously relevant for swimming and prey capture, but others are more subtle, and the relevance for future survival may not be readily apparent (McKim 1985; Cameron et al. 1992).

Abnormalities of fish eggs and larvae have been reported in field studies. For instance, they have been found when collecting eggs in field situations where the terata were shown to be correlated to the presence of pollutants or of polluted sites (Cameron et al. 1992; Longwell et al. 1992). The incidence of terata in eggs collected in the field, in general, decreased as the eggs developed, which suggested that malformed eggs may not be able to survive for long in a natural situation (Cameron et al. 1992; Longwell et al. 1992).

Laboratory-based studies which have examined the teratogenic effects of PCP on fish have also been described. For example, PCP caused interocular haemorrhage, fin erosion and mild cranial malformations in embryonic rainbow trout (Dominguez and Chapman 1984). In an additional study, eggs of medaka exposed to PCP suffered developmental abnormalities such as swollen abdomens, developmental arrest,

pericardial edema and associated secondary tube heart (Helmstetter and Alden 1995). Finally, early life stages of fathead minnows exposed to PCP developed severe pericardial edema (Holcombe et al. 1982).

Interestingly, Mayura et al. (1991) found that PCP was not a potent teratogen when studying the effects of PCP on the early development of rats. However, in another investigation, skeletal anomalies (lumbar spurs and vertebrae with unfused centra) were found in weanling rats exposed to PCP during the embryonic period (Schwetz et al. 1978). Furthermore, in an earlier study on rats, PCP also caused fetal anomalies such as subcutaneous edema, delayed ossification of the skull, lumbar spurs, in addition to anomalies in the ribs, vertebrae and sternebrae (Schwetz et al. 1974). From this earlier study it was concluded that PCP was embryo-lethal and embryo-toxic, but not teratogenic, since the terata were seen at doses higher than the LD50.

In addition to the above studies investigating PCP-induced abnormalities, dinitrophenol (DNP) which, like PCP, is an uncoupler of oxidative phosphorylation, caused anophthalmia, bilateral microphthalmia and dedifferentiation of the optic anlage in developing fish eggs (Rosenthal and Alderdice 1976). In another study, Middaugh et al. (1988) found that DNP caused a reduction in head size, defects in cardiac structure and function, and bending and stunting of *Menidia* embryos kept in 560 to 3200 ppb DNP until death or hatch.

3.3.2 Embryonic growth

The growth of toxicant-exposed fish is an often measured and affected parameter of the early life stages of fish. Sprague (1971) has supported the use of growth as an endpoint in sublethal tests, because it is easily measured and it is a criterion for success in fish populations in nature. Von Westernhagen (1988), however, claimed that larval length was not important for larval fitness, finding that length at hatch was correlated to the amount of yolk left. The author, therefore, suggested that it was the ontogenic

stage in comparison to the yolk reserves that was relevant. Woltering (1984) has also opposed the use of growth as an endpoint for sublethal tests, because this parameter was inconsistent (e.g. dependent on feeding rate) and was not suggested to be critical for the establishment of no observed effect concentrations (NOECs) in 86% of 173 reviewed cases.

Growth is likely to be, nonetheless, a critical endpoint for the study of PCP-induced effects, since it is postulated that the primary effect of PCP is on the energy production of exposed animals. The reduction in length due to PCP exposure has been proposed to be the result of either reduced energy supplies available for growth or the failure of some elements of the trunk to form during embryogenesis (Rosenthal and Alderdice 1976; Giesy and Graney 1989). Although growth is often measured as length, this endpoint has been suggested to lack consistency as a reliable indicator, and thus, dry weight has been proposed to be a better parameter (Blaxter 1988).

Despite these proposed potential limitations to growth being employed as an indication of PCP exposure, growth has been reduced in response to PCP exposure in almost all studies where this parameter has been measured. Thus, this effect has been seen with both rats (Knudsen et al. 1974; Schwetz et al. 1974; Schwetz et al. 1978; Mayura et al. 1991) and fish (Webb and Brett 1973; Hodson and Blunt 1981; Cleveland et al. 1982; Holcombe et al. 1982; Dominguez and Chapman 1984; Johansen et al. 1987; Samis et al. 1993). Only one study showed no effect on growth of juvenile American flagfish when exposed to less than 179 ppb PCP (Smith et al. 1991). The decline in growth of PCP-exposed fish has been suggested to take place mainly through two different mechanisms: first, the uncoupling of oxidative phosphorylation (equivalent to energy starvation); and, second, the reduction in food intake (Samis et al. 1993). In early life stages of fish, there is also evidence for a decreased yolk sac resorption efficiency at high PCP concentrations (Hodson and Blunt 1981). Finally, the food conversion

efficiency has been found to be negatively affected by PCP in juvenile largemouth bass (Johansen et al. 1987), and sockeye salmon (Webb and Brett 1973).

3.3.3 Successful hatch

When studying the effects of pollutants on the hatching of fish eggs, it is important to distinguish between the total hatch and viable hatch. Hatchability includes all hatched larvae, whether viable or not, and the proportion of viable hatch is, therefore, a more sensitive indicator of the pollutant's effect (von Westernhagen 1988).

Generally, exposure of embryos before closure of the blastopore and during neurulation will have more severe consequences for the hatch success rate of the eggs than exposure at later embryonic stages (von Westernhagen 1988).

3.3.4 Time-to-hatch

Time-to-hatch is less frequently studied as a response to toxicants than is the degree of successful hatch. Although one would expect exposure to lengthen the time-to-hatch, this is not necessarily the case as toxicants have been found to either shorten or lengthen the incubation time (Rosenthal and Alderdice 1976).

Delayed hatching may be the result of two different mechanisms, either a delay in development or an impaired hatching process (von Westernhagen 1988). Hatching does not necessarily, however, occur at a specific ontogenic stage (Blaxter 1988).

The hatching process may be adversely affected by several conditions:

1) The hatching enzyme, which is responsible for the digestion of the chorion, may not be distributed throughout the perivitelline fluid because of inactivity of the embryo (von Westernhagen 1988). Inactivity of fish embryos is often seen as a result of toxicant exposure (Rosenthal and Alderdice 1976). An indicator of only

localised proteolytic activity is the presence of a punctiform hole in the egg shell after hatching (von Westernhagen 1988).

2) The embryo may not be able to break the outer part of the egg shell even though the inner part of the zona radiata has been digested (von Westernhagen 1988).
3) The proteolytic hatching enzyme may be directly inhibited by toxicants (von Westernhagen 1988).

When incubating fish eggs in the laboratory, hatching may be delayed until the appropriate external stimulus for doing so is given. One such stimulus can be the agitation of eggs by water movement (Næsje and Jonsson 1988).

With regard to the specific effects of PCP on hatching success, late hatching has been observed in rainbow trout in two studies (Hodson and Blunt 1981; Dominguez and Chapman 1984). In addition, PCP may have also caused unhatched eggs in American flagfish and medaka (Smith et al. 1991; Helmstetter and Alden 1995).

3.3.5 Swimming performance

Swimming ability is vitally important for hatched larvae for both the capture of prey and the avoidance of predation. Erratic or inhibited swimming may be a result of either the narcotic effects of some toxicants, induced neurological (brain, eye, otolith) defects, or because of failure to inflate the swimbladder (von Westernhagen 1988; Weis and Weis 1989; Marty et al. 1990).

Swimming performance is often measured as swimming speed using a variety of techniques (Sprague 1971; Little et al. 1993). Little et al. (1993) have suggested that behavioural parameters may be very sensitive indicators, with the potential for detecting effects at 0.1 - 5% of the LC50 values of toxicants. The same authors detected a significantly reduced swimming activity when juvenile rainbow trout were exposed to 4% of their 96 h LC50 of PCP (92% pure) (Little et al. 1990). Other studies have also

found effects on the activity of fish after PCP exposure. For instance, PCP-exposed juvenile largemouth bass performed fewer feeding acts and had a lower rate of prey capture than control fish, although the exposed fish spent more time swimming than control fish (Brown et al. 1987). A lower swimming activity level of hatched rainbow trout fry exposed to PCP was observed by Hodson and Blunt (1981), of medaka larvae by Helmstetter and Alden (1995), and of fathead minnows by Holcombe et al. (1982). Swimming performance (measured as swimming speed) was, however, not affected when under-yearling sockeye salmon were exposed to PCP (Webb and Brett 1973).

3.3.6 Heart rates

The heart rates of fish embryos increase with age up to time-to-hatch, and may decrease as a response to toxicants. Therefore, it is important to distinguish whether a change in heart rate is due simply to the age of the embryo or alternatively, due to exposure to a toxicant (von Westernhagen 1988). Although the value of heart rate as an indicator of PCP exposure is not disputed, there is some disagreement on whether heart rate is a more sensitive indicator of toxicant exposure than hatch success (Rosenthal and Alderdice 1976; Sharp et al. 1979). A reduced heart rate has been suggested to indicate an impaired energy metabolism (von Westernhagen 1988).

Decreases in the heart rate of embryos have been noted when fish embryos have been exposed to cadmium, zinc, mercury, dinitrophenol, sulphuric acid and emulsifiers (Rosenthal and Alderdice 1976; von Westernhagen 1988). In one additional study, PCP caused a reduced heart rate and circulation of medaka (Helmstetter and Alden 1995).

3.4 Adult stages

3.4.1 Haematology as an indicator of toxicant exposure

When studying the effects of pollutants on adult fish stages, it is desirable to find observable changes that are more sensitive than mortality. Mortality is certainly a meaningful endpoint ecologically, but it is thought to be more important to detect alterations in the physiology of fish prior to mortality becoming evident as a change in the structure of whole populations in the field. The effects of stress and pollutants on the haematology of fish have been included in many investigations when monitoring fish 'health'.

Haematological changes in fish have been reported in response to lindane (Ferrando and Andreu-Moliner 1991), pulp mill effluents (Förlin et al. 1995), endosulphan and phosphamidon (Gill et al. 1991), tetrachloroguaiacol (Johansen et al. 1994), resin acids (Kennedy et al. 1995), cypermethrin (Philip et al. 1995), bis(tri-n-butyltin)oxide (Schwaiger et al. 1994) and fenvalerate (Shakoori et al. 1996), to name just a small proportion of the numerous published studies. A summary of studies on the effects of xenobiotics on the blood chemistry of fish is provided by Folmar (1993).

The types of haematological measurements that have been undertaken vary widely. They include composition of electrolytes, enzyme activities and metabolic indicators such as total protein, cholesterol, triglycerides, bilirubin, glucose and creatinine concentrations. Other parameters include haematocrit, leucocrit, haemoglobin and levels of hormones (Folmar 1993).

The parameters measured in this study were total red blood cell count (RBCC), total white blood cell count (WBCC), differential white blood cell count, whole blood glucose and whole blood lactate. These will be introduced in some detail below.

3.4.1.1 Red and white blood cells

Red blood cells (RBCs) are nucleated in fish, and distribute oxygen to the tissues with the aid of haemoglobin. Although the total count of RBCs can sometimes be correlated to haemoglobin and haematocrit values (Clark et al. 1979), caution must be applied since swelling of RBCs is thought to occur in response to stress (Neilson 1996). Increases in the total number of erythrocytes occur because of contraction of the spleen after an adrenergic response to stress (Folmar 1993). Decreases may occur as a result of haemodilution because of either an impaired renal function (Gill et al. 1991) or an increased breakdown of RBCs (Johansen et al. 1994). The normal life span of piscine erythrocytes is thought to be somewhat longer than the 120 days for anucleated mammalian red blood cells (Fänge 1992).

The leucocytes (WBCs) of fish can be divided into three main categories: the lymphocytes, the thrombocytes and the granulocytes. The piscine classification is often based on the morphological similarity to mammalian leucocytes, even though functional and ontogenic relationships should also be established (Ellis 1977).

Fish lymphocytes are produced in the thymus and anterior kidney, and are thought to be nonphagocytic cells with immunological functions (both cell-mediated and humoral immune response) similar to that of mammals, even though the functional equivalents of T and B lymphocytes have not yet been ascertained in fish (Ellis 1977; Fänge 1992). Thrombocytes are thought to originate in the spleen of fish, and their function is to aid in the clotting of circulating fluids. Granulocytes can be divided into neutrophils, eosinophils and basophils. The most numerous of these in fish are the neutrophils. The majority of granulocytes are phagocytically active (Fänge 1992). Some workers have also reported the presence of monocytes in the circulation of fish, and these are thought to account for approximately 0.1% of the leucocyte population. Fish monocytes are capable of migrating through tissues to form phagocytic macrophages (Ellis 1977).

The number of RBCs and WBCs may vary with sex, maturity and season as has been shown by Slicher (1961) with killifish (*Fundulus heteroclitus)*, by McCarthy et al. (1975) with rainbow trout, and by Cameron (1970) with pinfish and striped mullet.

The effects of PCP on red and white blood cells in various animals have been investigated in a few studies. For instance, Sprague-Dawley rats exposed to pure PCP showed no change in haemoglobin levels, packed cell volume, total RBC count or total and differential white blood cell counts (Schwetz et al. 1978). In another study, the RBC count increased in male rats after being maintained on food for 11 weeks which was contaminated with 50 and 200 ppm PCP, and the WBC counts were decreased only at the 50 ppm dose (Knudsen et al. 1974).

3.4.1.2 Glucose and lactate

The levels of glucose and lactate represent two important biochemical indicators of energy metabolism in fish, and they are often included in the study of physiological responses to xenobiotics. Significantly, the blood levels of glucose in individual fish are known to fluctuate more than the levels in mammals (Wells et al. 1986; McDonald and Milligan 1992). The variation among fish species is relatively large (0.2 - 15 mM), and is thought to be related to the activity level of the species (McDonald and Milligan 1992). Handling stress may increase the levels in fish significantly, in addition to increases that can result from osmotic, thermal and exercise stress (McDonald and Milligan 1992; Hontela et al. 1995, Roche and Bogé 1996). Other factors influencing glucose and lactate levels include the size, age, nutritional status and sexual maturity of the fish (McDonald and Milligan 1992).

Like mammals, the storage form of glucose in the liver and muscle is glycogen. In the liver, adrenaline activates glycogen phosphorylase (via an intracellular messenger), which results in the conversion of glycogen to glucose (Lehninger 1982). Other messengers stimulating the production of glucose are glucagon, glucocorticoids,

adrenocorticotrophic hormone, and growth hormone, which may induce glucose production from glycogen, acetyl coenzyme A and pyruvate in the liver, from protein in the muscle, or from fat deposits in adipose tissue (Campbell and Smith 1982).

Anaerobic conditions in the muscles promote the breakdown of glycogen to lactate and ATP because pyruvate is prevented from entering the citric acid cycle (Lehninger 1982; McDonald and Milligan 1992). The lactate produced by the muscles is subsequently transported to the liver to be reconverted to glucose and glycogen.

The effects of PCP on the central metabolic pathways in fish have been found to be variable. In one investigation using juvenile chinook salmon (*Oncorhynchus tshawytscha*) that had been exposed to PCP at 50% of the LC50, the fish had decreased plasma glucose levels compared to both those exposed to 5% of the LC50 and the control group (Iwama et al. 1986). In another study of the effects of PCP on yellow eel (*Anguilla anguilla*), major metabolic pathways were significantly affected by 100 ppb PCP after exposure for 4 days. The pentose phosphate shunt, the citric acid cycle, and the respiratory chains, together with hexokinase were found to be activated, whereas lactate dehydrogenase and pyruvate kinase activities were suppressed. It was postulated that an increased mitochondrial respiration rate caused an increased demand for substrate from the citric acid cycle, which may have been fuelled by an increase in the utilisation of fat reserves. It was also suggested that catecholamines may have caused an increased supply of glucose and lipids to be present as substrates (Boström and Johansson 1972).

The variable effects of PCP on central metabolic pathways has also been found when studying rats. PCP caused an increase in serum glucose concentrations in male rats after being fed 50 and 200 ppm PCP for 6 weeks, and 200 ppm for 12 weeks, whereas glucose levels were unaffected in female rats exposed at the same dosages (Knudsen et al. 1974).

Thus, the effects of PCP on the lactate and glucose concentrations in fish and rats have been variable, and the rationale for investigating these parameters for this study was due to the known effects of PCP on central metabolic pathways. Because PCP causes an increase in the rate of oxidative phosphorylation without a concurrent increase in the production of ATP, glucose levels may increase as a result of the utilization of energy stores. If energy stores are depleted, a decrease in glucose may be observed. Increased levels of lactate may be a sign of anaerobic metabolism caused by internal hypoxia, which in turn may be caused by an increase in the metabolic rate.

3.4.2 Detoxifying enzymes

An increasingly utilised parameter for the assessment of the biological effects of pollution on fish are detoxification enzymes, which have been shown to be induced in many studies in both field and laboratory situations (Kleinow et al. 1987; Payne et al. 1987). The specific advantages of detoxifying enzymes are given by Jimenez and Stegeman (1990) as:

1) a more sensitive indicator of pollution than other variables;
2) rapid response;
3) induced activity can persist for many weeks;
4) a direct indicator of organic chemical exposure.

For simplicity, detoxification of xenobiotics is often divided into two phases, phase I and phase II. In the first phase the toxicant undergoes mainly oxidative but also hydrolytic or reductive reactions (Lech and Vodicnik 1985; Di Giulio et al. 1995). In the second phase, an endogenous water soluble moiety is attached to the toxicant, thus increasing the water solubility and the excretability of the metabolite (Jimenez and Stegeman 1990).

One of the most important enzyme systems responsible for phase I reactions is the cytochrome P450 monooxygenase system (Buhler and Williams 1988). There are multiple cytochrome P450 isozymes (Buhler and Williams 1988; Okey 1990), and in this review the emphasis will be placed on cytochrome P4501A, because this enzyme is inducible by environmental toxicants (Stegeman and Hahn 1994). Mammals possess two copies of the *CYP1A* gene (*CYP1A1* and *CYP1A2*) which encode for the cytochrome P4501A enzymes (Celander and Förlin 1991). Fish were originally thought to have only one copy of the gene (*CYP1A1*), until the presence of two copies of the gene was recently confirmed in rainbow trout (Berndtson and Chen 1994). The induction of cytochrome P4501A is mediated by the aryl hydrocarbon (Ah) receptor (Okey 1990; Di Giulio et al. 1995). The receptor combines with the substrate, which then diffuses to the nucleus and initiates transcription of the cytochrome P450-encoding genes (Di Giulio et al. 1995). The Ah receptor has been identified in 7 fish species: rainbow trout, brown trout, killifish, winter flounder (*Pleuronectes americanus*), scup (*Stenotomus chrysops*), smooth dogfish (*Mustelus canis*) and spiny dogfish (*Squalus acanthias*), but not in jawless fish or eight species representing four invertebrate phyla (Stegeman and Hahn 1994).

The generalised reaction catalysed by cytochrome P450 is:

$$RH + NADPH + O_2 + H^+ \rightarrow ROH + NADP^+ + H_2O$$

where RH is the substrate and ROH is the hydroxylated product (Stegeman and Hahn 1994). Other components required for supporting the reaction *in vivo* are NADPH-cytochrome P450 reductase, the phospholipid membrane of the endoplasmic reticulum, and for some reactions, NADH and NADH-cytochrome b_5 reductase (Di Giulio et al. 1995). Cytochrome P450 enzyme activity is also referred to either as mixed-function oxygenase (MFO) or monooxygenase activity (Di Giulio et al. 1995).

The level of cytochrome P4501A is most commonly measured as the activity towards certain model substrates. In the present study it was measured as 7-ethoxyresorufin *O*-

deethylase (EROD) activity, but it may also be measured as aryl hydrocarbon hydroxylase (AHH) or 7-ethoxycoumarin *O*-deethylase (ECOD) activity (Goksøyr and Förlin 1992). The relative activity with different substrates partially reflects the presence of the isozymes of cytochrome P450. The induction of monooxygenases may also be investigated either as the quantity of cytochrome P450 on SDS-PAGE gels or as cytochrome P450-specific mRNA identified with the aid of specific cDNA probes (Goksøyr and Förlin 1992).

Phase II reactions are catalysed by uridine 5'-diphosphoglucuronyl transferases (UDPGT), sulphotransferases, glutathione-S-transferases and acetylases (Jimenez and Stegeman 1990). Here the emphasis will be on UDPGT, which is one of the enzyme activities measured in this study. Similar to EROD, UDPGT is also located in the endoplasmic reticulum and induction of UDPGT activity is mediated by the Ah receptor (Di Giulio et al. 1995).

The overall reaction catalysed by UDPGT is:

$$R\text{-}X + UDPGA \rightarrow R\text{-}X\text{-}GA + UDP$$

where UDPGA is the cofactor uridine diphosphate glucuronic acid and X is either a OH, COOH or NH_2 group of the substrate R (Lech and Vodicnik 1985). UDPGT activity can be measured with many different substrates, which can include bilirubin, 1-naphthol, phenolphthalein and 4-nitrophenol (Clarke et al. 1991).

The activities of detoxifying enzymes are generally lower in fish than in mammals (Chambers and Yarbrough 1976), which, in part, is responsible for their lower ability to excrete toxicants than terrestrial animals. There is, however, a wide range of activities among fish species. For example, EROD activities have been found to vary up to 200-fold, with the average EROD activity level being 180 pmol min^{-1} mg^{-1} protein for fish (Sijm and Opperhuizen 1989). In addition, there are other factors known to affect the level of enzyme activities in fish. For instance, temperature, hormones, sex,

season, diet and age can all have profound effects on the measured activities (Sijm and Opperhuizen 1989). Females during the spawning season have been shown to have lower activities than males of the same species, an effect attributed to repression by estradiol (Andersson and Förlin 1992). The influence of both environmental and physiological variables must, therefore, be taken into account when investigating the responses of detoxifying enzyme activities in fish.

In addition to these factors affecting detoxifying enzyme activities, the level of activity also depends on the tissue being analysed. Liver is most commonly analysed because it usually has the highest levels of detoxifying activity and, therefore, is the least difficult to measure. There are also examples where the induction process has been found to be tissue-selective, with induced activities in, for example, the kidneys but not in liver. In these types of cases it is more appropriate to analyse a tissue other than liver (Kleinow et al. 1987).

Mammalian cytochrome P4501A is induced by both phenobarbital (PB) and 3-methylcholanthrene (MC)-type inducers, with the chemicals that cause induction being referred to as either PB- or MC-type inducers. The PB-type of induction is indicated to be rare in fish (Chambers and Yarbrough 1976; Binder et al. 1984; Kleinow et al. 1987). Substrates for cytochrome P4501A sometimes induce this enzyme and thus their own detoxification, but not all substrates are inducers (Chambers and Yarbrough 1976; Okey 1990). The enzymes belonging to the MFO system are fairly unspecific, reacting on functional groups rather than the whole molecule (Lech and Vodicnik 1985). Compounds that are metabolised by cytochrome P450 include polycyclic aromatic hydrocarbons, aromatic amines, aromatic amides, pesticides, azo compounds, flavonoids, drugs and endogenous substrates such as prostaglandins, steroid hormones, fatty acids and bile salts (Di Giulio et al. 1995). Inducers of the cytochrome P4501A protein include polycyclic aromatic hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and ß-

naphthoflavone (Di Giulio et al. 1995). ß-naphthoflavone is often used as a prototype inducer of cytochrome P4501A protein (Andersson and Koivusaari 1985; Celander et al. 1993).

Induction or inhibition of cytochrome P450 by one toxicant may have profound consequences for the distribution, excretion and toxicity of other chemicals. Effects that have been observed when a fish had been treated initially with one chemical acting as either an inducer or an inhibitor, and then exposed to another toxicant include (Kleinow et al. 1987):

1) change in the level of excretion into the bile and change in the retention levels in body tissues;
2) change in toxicity measured as LC50 or LD50;
3) change in the bioconcentration factor;
4) change in the profile of metabolites.

Similar to cytochrome P450, piscine UDPGT is refractive to PB-type induction (Clarke et al. 1991). UDPGT (with 4-nitrophenol as a substrate) is, however, induced by MC-type inducers such as ß-naphthoflavone, 3-methylcholanthrene, Aroclor 1254® (a polychlorinated biphenyl mixture), Clophen A50® (a polychlorinated biphenyl mixture), Vapam® (*N*-methyldithiocarbamate) and octachlorobiphenyl, but UDPGT is often less reactive than the monooxygenases (Andersson et al. 1985; Clarke et al. 1991). An imbalance between induced phase I and phase II enzyme activity due to differential induction or repression may have important ramifications for toxicity when the intermediate metabolites from phase I are more toxic than the parent compound (Okey 1990). This 'toxifying' action of the phase I enzymes is known to occur in a few cases, for instance, when benzo(a)pyrene-7,8-diol is metabolised to the carcinogen benzo(a)pyrene-7,8-diol 9,10-epoxide (Ioannides et al. 1984; Di Giulio et al. 1995).

The above considerations draw attention to the biological significance of induction of detoxifying enzymes: is induction beneficial or deleterious to the organism and does it have further biological significance other than merely being a marker of exposure? There is a certain degree of controversy with regard to the answer to this question. Okey (1990), however, addressed these concerns and has made a summary of the consequences of high levels of phase I enzyme activity as follows:

(a) it is beneficial when it is coupled with high phase II enzyme activities, and when the toxicant is given orally or as an intraperitoneal injection, so that it first passes the liver with its high detoxifying activity;

(b) it is deleterious to the organism when it is coupled with low levels of phase II enzyme activity or when the toxicant is applied directly to a target tissue (e.g. skin or lungs).

Possible physiological effects of MFO induction can be appreciated when considering that these enzymes are linked either directly or indirectly to the metabolism of a multitude of important physiological control systems including: haem, heat shock proteins, steroid receptors, antioxidant enzymes, metallothioneins, oncogene and tumour suppression genes, and the synthesis and binding of gaseous biological messengers (NO and CO) (Stegeman and Hahn 1994). For example, special concern has been shown for the effect of induced cytochrome P450 enzymes on the hormone balance of fish (Payne et al. 1987). In one study, the fertilisation success, embryological success and viable hatch of starry flounder (*Platichtys stellatus*) all correlated inversely with the log of hepatic AHH activity, which in turn was induced in fish from a polluted site in the San Francisco Bay area compared to fish from the relatively unpolluted reference site (Spies et al. 1985). In another study of English sole (*Parophrys vetulus*) from Puget Sound, Washington, it was found that the level of AHH activity was the best predictor of whether or not a female would enter vitellogenesis, and AHH activity was negatively correlated with estradiol levels (Johnson et al. 1988). In a nonhormonal finding, induction of EROD was correlated

with fat accumulation and cholangiohepatitis of the liver of lake sturgeon (*Acipenser fulvescens*) at a polluted site (Rousseaux et al. 1995).

In contrast to the above examples of links between MFO induction and demonstrated physiological responses, there have been cases where induction of monooxygenases was the only variable to change in fish exposed to polluted water, and no association was found with any observable pathology of the fish (Kloepper-Sams et al. 1994). Similarly, Munkittrick et al. (1994) found no correlation between increased MFO activity and steroidal depressions in fish. Thus, some authors have suggested that it may not be entirely clear whether induction of detoxifying enzymes should be considered as a definitive marker of biological effect or exposure in fish (Kloepper-Sams et al. 1994).

However, as mentioned earlier, detoxifying enzymes have been clearly demonstrated to be sensitive indicators of pollution both in the field and the laboratory. In the field, increased MFO activities in fish exposed to pulp mill effluents has been a common result (Andersson et al. 1988; Rogers et al. 1989; Lindström-Seppä and Oikari 1990; Ahokas et al. 1994; Kloepper-Sams et al. 1994). Polycyclic aromatic hydrocarbons and polychlorinated biphenyls have also been correlated with induced enzymes (Collier et al. 1995). Some examples of induction identified in the laboratory include: fish enzymes or MFO-specific protein levels have been induced by pulp mill effluents (Andersson et al. 1987; Martel et al. 1995); ß-naphthoflavone (Andersson et al. 1985; Kloepper-Sams and Stegeman 1989; Celander et al. 1993; O'Hare et al. 1995); Clophen A50® (Besselink et al. 1996) and Clophen A40® (Boon et al. 1992).

In New Zealand, the levels of EROD activities have been measured in the shortfinned eel, longfinned eel and brown trout captured, or caged, at various pristine and polluted sites (Jones et al. 1995). Induction was found in caged (4.3-fold) and captured (6.4-fold) eels in a river polluted by chemithermo-mechanical pulp effluent, but eels from a

site known to be contaminated with PCP and dioxins showed no induction, and one site with no known pollutants had eels with increased EROD activities (Jones et al. 1995). Tasmanian inanga have also been utilised for one laboratory-based study of detoxifying enzymes. The fish were exposed to pulp mill effluent, and induction of EROD (3.5-fold) was found when the fish had been exposed through both food and water (Woodworth et al. 1996).

The effect of PCP on the induction of detoxifying enzymes has been investigated in a number of other studies, but the results have not shown a clear correlation between exposure and induction. For example, Ahotupa et al. (1981) found a small increase in ECOD activity when exposing rats to 30 mg/kg of reagent grade PCP on three consecutive days, but a single large dose caused either no change in AHH activity or inhibition of ECOD and UDPGT activities one hour after administration. *In vitro*, PCP at concentrations between 1 x 10^{-6} and 100 x 10^{-6} mol/L caused a direct inhibition of UDPGT and phase I enzymes (Ahotupa et al. 1981). Repression of UDPGT activity in fish exposed for 4 days to PCP at 20% of the LC50 values was also observed by Castrén and Oikari (1987). Support for an inhibitory effect of PCP has also been provided by Arrhenius et al. (1977), who found that PCP selectively inhibited C-oxygenation of aromatic amines in favour of N-oxygenation. C-oxygenation requires intact electron transport between a flavin enzyme and cytochrome P450, whereas N-oxygenation can take place completely independently of the cytochrome P450 system (Arrhenius et al. 1977).

In contrast to the above apparent lack of correlation between PCP exposure and inducibility, PCP has been found to induce AHH and ECOD activities in calves fed 10 mg/kg pure PCP for 6 weeks (Shull et al. 1986). PCP also induced hepatic ECOD and EROD activities in rats fed PCP-contaminated food for 14 days (Vizethum and Goerz 1979). Cattle exposed to pure PCP for 160 days at 15 or 20 mg/kg/day showed an increase in AHH activity, but, interestingly, not in UDPGT activity (McConnell et al.

1980). In another study of rat responses to PCP exposure, rats fed 20-500 ppm pure PCP for 8 months showed no increase in AHH activity, but did have a significant increase in UDPGT activity (Goldstein et al. 1977).

Detoxifying enzymes play an important role in the excretion of PCP. In general, PCP is excreted as sulphate and glucuronide conjugates, or as the original parent compound (Ahlborg 1978; Ahlborg and Thunberg 1980). In mammals, microsomal enzymes mediate the hydrolytic and reductive dechlorination of PCP to form tetrachloro-*p*-hydroquinone or trichloro-*p*-hydroquinone (Ahlborg 1978; Ahlborg and Thunberg 1980). Other PCP-derived metabolites found in rat include tetrachlorophenols, tetrachlorocatechol, tetrachlororesorcinol and benzoquinones (Renner and Hopfer 1990).

Fish are able to metabolise PCP in a manner similar to that in mammals. Thus, PCP may be excreted as the parent compound or as the sulphate or glucuronide conjugates (Glickman et al. 1977; Kobayashi 1978; Lech et al. 1978; Lu et al. 1978; Huckins and Petty 1983; Benner and Tjeerdema 1993; Gates and Tjeerdema 1993; Cravedi et al. 1995). The sulphate conjugate is excreted via the urine, whereas the glucuronide is excreted into the bile. Intestinal microbial action on the glucuronide conjugate may, however, release free PCP which can then be reabsorbed, thus prolonging its retention (Benner and Tjeerdema 1993). The qualitative and quantitative excretion of PCP and its conjugates varies with fish species (Stehly and Hayton 1989a). In line with the concepts of Kleinow et al. (1987), where inhibition of detoxifying enzymes was shown to cause an increase in the retention of toxicants, inhibition of detoxifying enzymes by salicylamide increased the bioconcentration factor in rainbow trout exposed to 5 ppb PCP (Stehly and Hayton 1989b). Induction of detoxifying enzymes also increased the proportion of PCP that was excreted as PCP-derived metabolites (Ahlborg 1978).

In summary, detoxifying enzymes have been successfully applied as a biomonitoring tool to a range of fish species and pollutants, and they have been shown in many cases to be one of the most sensitive indicators of exposure to xenobiotics. There is, however, some residual doubt regarding the biological significance of induced activities of these enzymes. In addition, some studies have shown PCP to cause a mixed response in phase I and phase II enzymes in fish and mammals. The only two published studies undertaken with fish species native to New Zealand are with the shortfinned eel, longfinned eel (Jones et al. 1995), and Tasmanian inanga (also called common Jollytail in Australia) (Woodworth et al. 1996). It would, therefore, be extremely useful to add to the knowledge of the effects of PCP on detoxifying enzymes, and to determine the level of detoxifying enzymes in other New Zealand native fish.

3.4.3 Heat shock proteins

The research on heat shock proteins (hsps) has grown during the last two decades to impressive proportions. The basic observations made by Ritossa (1962) on *Drosophila* polytene chromosomes proved to have wide ranging implications. Ritossa exposed *Drosophila* to increased temperatures and observed new polytene puffs on their chromosomes in response to the heat treatment. In the 1970s it became evident that the chromosome puffs were one of the signs of increased expression of a certain set of proteins, which were called heat shock proteins (Morimoto et al. 1990; Black and Subjeck 1991). Further research has shown that hsps are present not only in *Drosophila*, but in virtually all organisms so far studied, from archaebacteria and other prokaryotes, to plants, yeast and higher eukaryotes (Lindquist and Craig 1988; Welch 1990; Black and Subjeck 1991). Hsps are also present in cells as their cognates (i.e. constitutively expressed) under normal physiological conditions and are in many cases crucial to the survival of the cells and organisms. In the context of this thesis, hsps are of interest because they have been proposed as indicators of environmental pollution (Sanders 1988; Dyer et al. 1993).

Ritossa made some observations on the survival of *Drosophila* exposed to heat shock and noted that the ones that were initially exposed to a mild heat shock treatment displayed higher survival rates after a second more severe heat shock, when compared to a control group that had been transferred directly to the more severe heat shock environment. Subsequently, it was shown that all organisms possess this ability to condition themselves for a normally lethal heat shock by pre-exposure to a mild heat shock. The state induced by the preconditioning heat shock was termed thermotolerance (Welch 1990; Black and Subjeck 1991). Much evidence points to the hsps as being able to provide thermoprotection. For example, thermotolerance and its decay is coincident with hsp synthesis and the turnover of hsps, respectively. In addition, organisms that are deficient in hsp synthesis cannot tolerate heat shock (Welch 1990). Furthermore, thermotolerant cells are able to recover more quickly and more efficiently from heat shock than control cells (Laszlo 1992). There is, however, some conflicting evidence, whereby thermotolerance may seemingly develop without hsps, and hsps may not always provide thermotolerance (Amaral et al. 1988, Smith and Yaffe 1991).

Hsps are often called stress proteins, since it has become clear that many other stressors besides heat shock may induce these proteins (Black and Subjeck 1991). These other stressors have also been shown to induce thermotolerance, and thus make exposed organisms or cells more resilient to a severe heat shock. The converse is also true; a prior heat shock may confer higher tolerance to other stressors (Lindquist and Craig 1988; Black and Subjeck 1991; Lee et al. 1991). Stressors that have been shown to induce hsps are listed in Table 3.1.

It is surprising that so many different types of stressors may elicit a common response, and the question is whether there is a common denominator for the early effects of all

these agents. Welch (1990) has noted some of the early biochemical changes in a stressed cell:

Table 3.1 Stressors that have been shown to induce hsps in a variety of organisms and cells (Morimoto et al. 1992).

General stress type	Specific stressors
Environmental stress	heat shock amino acid analogues transition heavy metals inhibitors of aerobic energy metabolism (e.g. 2,4-dinitrophenol)
Physiological stressors	cell cycle growth factors development and differentiation oncogenes and proto-oncogenes
Pathophysiological state	fever and inflammation hypertrophy oxidant injury ischemia (inadequate flow of blood to part of the body) anti-neoplastic chemicals viral and bacterial infection neuronal injury ageing

1) Changes in the cholesterol content and, therefore, the fluidity of cellular membranes in thermotolerant cells.
2) Changes in intracellular pH and calcium levels in hyperthermic cells, but these changes do not seem necessary for hsp induction.
3) Interestingly, with respect to PCP, a known inhibitor of oxidative phosphorylation, there are morphological and biochemical changes in stressed cells that indicate alterations in the integrity of mitochondria. These changes are accompanied by decreased intracellular ATP level, and an increase in the reliance on anaerobic metabolism. In addition, stressed cells may show an increased dependence on the glycolytic pathways of energy metabolism after heat shock. However, it is difficult

to ascertain whether such changes are important to the initiation of hsp synthesis, or are a secondary result of stress.

4) A mechanism often proposed as being the most important event in the induction of hsps is the occurrence of partially denatured proteins in the cell (Ananthan et al. 1986; Morimoto et al. 1990). The term proteotoxicity was introduced by Hightower (1991) for the perturbation of the normal configurations and associations of proteins caused by agents listed in Table 3.1.

The induction of hsp synthesis by the presence of intracellular abnormally folded proteins is well correlated with the function of hsps in the cell. The different types of hsps commonly found in cells are listed in Table 3.2, along with some of the key roles each one participates in. It should be noted that hsps are often labelled according to their molecular weight, and that hsps which are essentially the same, or belong to the same family of hsps, may have different names depending on the organism or the cellular compartment where they are found. Table 3.2 summarises the demonstrated and proposed functions of the multitude of hsps that are reported in the literature.

Hsp70 will be the focus of one portion of this thesis, and a close examination of its roles in normal and stressed cells is warranted and follows. There are four different types of hsp70: the constitutively expressed hsp70 (often called a cognate or hsc70), the heat-inducible hsp72, grp78 and p72 (Black and Subjeck 1991; Ellis and van der Vies 1991). Grp78 is not heat-inducible, but it is inducible by other stressors, and it is present in the endoplasmic reticulum (Black and Subjeck 1991; Hightower 1991). The hsp p72 is expressed at high levels in growing cells (Lindquist and Craig 1988). The cognate hsp70 and the heat-inducible hsp72 do not differ biochemically or biologically, but they are, in fact, two different gene products (Welch 1990). The hsc70 and the hsp72 will here, for simplicity, be referred to as hsp70. The primary function of hsp70 is to associate with nascent polypeptides, and aid in the transport and/or translocation of these to other cellular compartments (the endoplasmic reticulum and the mitochondria).

In the endoplasmic reticulum, grp78 (also called binding protein or BiP) takes over the 'chaperone' activity of hsp70. In mitochondria, mitochondrial hsp70 associates with

Table 3.2 Major groups of hsps and their basic functions (Lindquist and Craig 1988; Morimoto et al. 1990; Black and Subjeck 1991; Hershko and Ciehanover 1992).

Hsp family	Functions
hsp90	• binds to specific polypeptides, and either silences their function (e.g. glucocorticoid receptor), and/or escorts them to their proper cellular compartment • associated with the cytoskeleton • essential for the survival of cells
hsp70	• dissociates some protein aggregates; maintains some polypeptides in an unfolded state, thus facilitating their translocation across membranes, and/or accelerating proper folding and oligomerisation; binds to specific polypeptides • acts as a clathrin uncoating ATPase • essential for normal growth and gene regulation
hsp60	• maintains some polypeptides in unfolded state, thus facilitating their translocation across membranes, and/or accelerating proper folding and assembly (by preventing misfolding?) • present in the Bacteria, Archaea, mitochondria and chloroplasts
Small hsps (15-40 kDa)	• eukaryotic function largely unknown • little homology between species • number of small hsps varies between species • associated with regulation of intracellular calcium • primitive viral elements or a form of selfish DNA?
Ubiquitin	• conjugates to and "tags" unfolded polypeptides destined to be destroyed by the intracellular 26S protease complex

newly imported polypeptides, and together with hsp60 and hsp10, aids in the final folding of the proteins. Polypeptides may also reach their final configuration in the cytoplasm, or be targeted by hsp70 for lysosomal breakdown in case of irreversible damage to the proteins (Gething and Sambrook 1992).

Folding of proteins has traditionally been regarded as a spontaneous event, which would be dependent on the distribution of hydrophobic or hydrophilic regions of the amino acid sequence as they are translated. Experiments with proteins both *in vitro* and *in vivo* have, however, shown that misfolding and aggregation *in vitro* is an often-encountered problem, whereas folding *in vivo* takes place with high accuracy and speed (Ellis and van der Vies 1991; Gething and Sambrook 1992). It has been suggested that the role of hsp70 is probably to prevent unsuitable associations within and between polypeptides, since hsp70 in itself does not catalyse the final secondary structure (Gething and Sambrook 1992).

The regulation of hsp70 is thought to be via an autoregulatory loop mediated through the heat shock factor, abbreviated HSF (Morimoto et al. 1992). HSF in a normal unstressed cell is usually associated with hsp70. However, in the presence of increasing amounts of abnormal proteins produced during stress, hsp70 dissociates from HSF to bind to these improperly folded proteins, thus producing free HSF. Existing associations between hsp70 and abnormal proteins may also last longer, thus slowing the dissociation rate of the hsp70-abnormal protein complexes (Hightower 1991). The HSF that is released when hsp70 becomes increasingly bound to proteins will then bind to DNA (to the heat shock element) which will then promote the transcription of hsp70-encoding genes (Hightower 1991). When hsp70 is present at levels in excess of denatured cytoplasmic proteins then the free hsp70 can bind to HSF, which will then dissociate from the heat shock element resulting in a reduction in transcription of the hsp70 genes (Morimoto et al. 1992).

As mentioned previously, hsps are of interest because they have been proposed as indicators of environmental pollution (Sanders 1988; Dyer et al. 1993). The induction of hsp70 and other hsps has been observed in a large number of studies in response to pollutants, both in the laboratory and from specimens collected in the field. A summary of recent studies where induction was shown is provided in Table 3.3.

It should, however, be pointed out that a degree of caution must be applied when attempting to use this parameter as a biomonitoring tool. Research into the environmental applications of hsps is still in its infancy, and it has been suggested that the relevance of hsps as indicators of detrimental biological effects needs to be questioned even when it is clear that the hsps are an indication of exposure (Bradley 1993). There have also been examples of studies where hsps were insensitive to the known presence of xenobiotics. For instance, cadmium, copper, lead and zinc failed to induce hsp70 in adult rainbow trout (*Oncorhynchus mykiss*), even though it did induce hsp/hsc70 in juvenile gill tissue exposed to the metals through both diet and water (Williams et al. 1996). In another study, ethanol and dinitrophenol (at nonlethal levels) failed to induce any hsps in Reuber H35 rat hepatoma cells, even though arsenite and cadmium did induce some hsps (Wiegant et al. 1994). Finally, sea mussels (*Mytilus edulis*) collected at a polluted site and exposed to an additional heat shock, had repressed hsp70 synthesis compared to controls, and the response of 9 other hsps was variable (either repressed or induced) (Veldhuizen-Tsoerkan et al. 1991).

Table 3.3 Studies where the induction of hsps has been reported in response to xenobiotics.

Agent/site	Cell/organism	Induced hsp(s)	Reference
arsenite	Fathead minnow	hsp70 and 4 others	Dyer et al. 1993
chromate	Fathead minnow	hsp68, 70 and 20	Dyer et al. 1993
lindane	Fathead minnow	hsp70 and 72	Dyer et al. 1993
diazinon	Fathead minnow	hp70 and 10 others	Dyer et al. 1993
cadmium copper	fish cell cultures	hsp70 and 27	Ryan and Hightower 1994
polluted sites	*Mytilus edulis* *Collisella pelta*	hsp60 and 70	Sanders 1988
PCP	*Escherichia coli*	2 hsps	Blom et al. 1992
lead	soil invertebrates	hsp70	Köhler et al. 1992
SDS AgCl	*Daphnia magna*	hsp70	Bradley 1993
arsenite	*Tetrahymena pyriformis*	70-75 kDa 25-29 kDa	Amaral et al. 1988
zinc cadmium	fish cell lines	hsp70	Misra et al. 1989
arsenite ethanol	*Xenopus laevis* A6 cells	hsp70 mRNA	Heikkila et al. 1987
PCP	*Euglena gracilis*	40 kDa	Barque et al. 1996
cadmium	*Euglena gracilis*	hsp40, 55, 70 and 90	Barque et al. 1996
arsenite cadmium	Reuber H35 rat hepatoma cells	hsp70 and 5 other hsps	Wiegant et al. 1994
cadmium copper lead zinc	rainbow trout juveniles	hsp/hsc70	Williams et al. 1996

The method by which hsps are analysed may have implications for the interpretation of results. The most commonly used method is the metabolic labelling of newly synthesised proteins during stress or heat shock. The tissues or cells are subjected to one or two-dimensional gel electrophoresis, which is then followed by autoradiography

or fluorography (Amaral et al. 1988; Blom et al. 1992; Barque et al. 1996). When hsps are induced during heat shock, other proteins are synthesised in reduced amounts (Morimoto et al. 1990; Welch 1990), and the results often show prominent bands for newly synthesised hsps with a reduction in the number and intensity of non-hsp bands. The advantage of this method is that the entire profile of hsp induction can be studied in the same autoradiograph or fluorogram, and newly synthesised radiolabelled proteins are easier to study than total amounts of protein. The disadvantages are that the use of radioactivity is required and the fact that organisms exposed to contaminants for long periods may already have accumulated high levels of hsps and thus may not show an increase in newly synthesised hsps (Sanders 1993). In addition, although radiolabelling can demonstrate *de novo* synthesis of hsps it does not provide any direct information on the turnover of the hsps. At the time of analysis, the level of a particular hsp subtype is always controlled both by the degree of new synthesis and the turnover of that hsp. The half-life of hsp70 has been shown to be between only 1 - 2 h for *Drosophila* and >10 h for the rat (Craig 1985).

Other methods for measuring induction of hsps rely on the use of antibodies specific for certain hsp subtypes. For this technique, the proteins are first separated on either a one- or two-dimensional gel and then transferred to a nitrocellulose or other membrane. After transfer, the membrane is incubated with the primary antibody specific for the hsp-subtype which is subsequently followed by detection with an appropriate visualisation system (Bradley 1993; Bagchi et al. 1996). Other methods based on the use of antibodies are dot blotting and enzyme-linked immunosorbant assay, i.e. ELISA (Sanders et al. 1991; Dietz and Somero 1993; Fader et al. 1994; Yu et al. 1994). These methods measure the total amount of antibody-identified hsps rather than newly synthesised hsps. This may reveal more about the long-term effects of stress on cells, but may also be slightly less sensitive than studying *de novo* synthesis of hsps. In addition to antibody-based methods, the polymerase chain reaction (PCR) has also been used for studying hsps (Cochrane et al. 1994). The use of PCR is of limited value,

however, as hsp mRNAs are short-lived when an organism is exposed to continuous stress, and yet the hsps themselves can remain elevated for a long time (Sanders 1993). Northern blotting has also been used to study transcription of hsp genes (Bagchi et al. 1996).

In summary, hsps are an important new addition to the biomonitoring tools available for assessment of pollutant effects on fish. As seen in Table 3.3, PCP has induced some hsps in *E.coli* and *Euglena gracilis*, but the response to PCP has not been thoroughly studied. In addition, the measurement of hsps in New Zealand native freshwater fish has not been applied as a biomonitoring tool in the past.

3.5 Aims of this investigation

The utilisation of native New Zealand fish species for toxicity testing remains to this day relatively unstudied. In addition, the majority of the tests that have been published have been performed using standard reference species such as rainbow trout. Although a limited quantity of toxicity testing has been performed previously with native New Zealand fish, most of these studies have used adult life stages, even though early life stages have been repeatedly shown to be the most sensitive. Furthermore, all previous testing has utilised end points such as avoidance or lethal concentration, and no attempts have been made to measure sublethal physiological responses of either early life stages or adult native fish. This study seeks to assess the effectiveness of using native fish for such studies.

The results of the study were compared to those obtained with a reference species, rainbow trout, and used PCP as the reference toxicant. PCP is environmentally important in New Zealand, and its liberation and prevalence in the New Zealand environment has been quantified at some locations. Furthermore, the toxic properties, biochemical effects and detoxification mechanisms of PCP have been well characterised in past studies. The approach taken in this study, in order to gain a further

understanding of the feasibility and utility of using both early life stage and adult native fish for toxicity testing, was to divide the experiments into the following two parts:

For the first part of the study (Chapters 4, 5 and 8), fertilised eggs of four native fish species (smelt, inanga, koaro and common bully) were obtained using several techniques. Subsequently, four early life stages (24 h, early eyed, late eyed and larvae) were tested for their sensitivity to PCP in order to determine LC50 values, and the results were compared to those of the same life stages of rainbow trout. Following the acute testing, the survivors were maintained in clean water, and the sublethal effects of the previous PCP exposure were studied. The end points examined were post-exposure survival and hatching success, time-to-hatch, teratology, growth, swimming performance and heart rate, as reviewed in this chapter (Sections 3.3.1 - 3.3.6)

For the second part of the investigation (Chapters 6, 7 and 9), the adult life stages of two species, inanga and common bully, were assessed for their sublethal responses to 25, 50 and 75% of their respective LD50 values. This was achieved through intraperitoneal injection of PCP. The end points studied were RBCC, WBCC, differential white blood cell count, blood glucose, blood lactate, EROD, UDPGT and hsp70, as reviewed in this chapter (Sections 3.4.1 - 3.4.3).

CHAPTER 4

MATERIALS AND METHODS FOR EARLY LIFE STAGES

4.1 Assessment of the maturity of adult fish

The collection and fertilisation of eggs of all fish species, except for common bullies, required an accurate assessment of the maturity of the gonads. The staging of maturity, as outlined by Pollard (1972), was used as a guide during these assessments. This guideline was designed to be used in classifying inanga, but it is applicable to other species as well. The stages were:

Stage I (Immature virgin) - Testes and ovaries thin and threadlike, translucent and colourless; sexes usually indistinguishable.

Stage II (Developing virgin and recovering spent) - Testes thin and strap like, translucent and greyish, sometimes with melanophores. Ovaries more rounded, translucent and colourless, eggs not evident to the naked eye.

Stage III (Developing) - Testes thickening, opaque and greyish white, smooth texture. Ovaries thickening, opaque and pale yellowish, eggs small but visible to the naked eye.

Stage IV (Maturing) - Testes enlarged, opaque and whitish, smooth texture. Ovaries enlarged, opaque and yellowish, eggs large.

Stage V (Mature) - Testes fill most of body cavity, opaque and creamy white, smooth texture. Ovaries fill most of body cavity, opaque and yellow, eggs large.

Stage VI (Ripe) - Testes fill body cavity, opaque and pure white, smooth and crumbly texture, milt extruded by pressure on abdominal wall. Ovaries distend body cavity, translucent pale golden eggs large and extruded by pressure on abdominal wall.

Stage VII (Spent) - Testes thin and flaccid, greyish, sometimes white areas (residual sperm). Ovaries thin and flaccid, translucent and colourless to pale yellowish, sometimes contain large yellow opaque residual eggs.

Of these stages, the ripe stage, stage VI, was most often recognised simply by squeezing the abdominal area of a fish. If eggs or milt were extruded, then the ripeness of the fish had to be ascertained; only flowing milt and translucent eggs were used for fertilisation. For artificial induction of final maturation (ovulation), the stage of the fish had to be at least mature (stage V).

4.2 Collection of smelt eggs

Fertilised smelt eggs were obtained from mature adults caught in the Waikato River by beach seining. Upon capture, the fish were examined for their degree of maturity by gentle pressure along their abdomens towards the vent. Ripe females released clear golden or beige eggs, and ripe males released running milt. The females and the males were briefly stored in river water in separate buckets until all fish had been sorted.

Fertilised eggs were obtained by holding male and female fish vent to vent, and squeezing their gonadal products out, mixing them as they were released. After contact for 5 - 15 seconds the eggs were allowed to fall into or were rinsed into plastic bags containing river water. They were then rinsed with river water to clean the milt from the eggs.

After collection in the field the smelt eggs were maintained in the laboratory in a 100 L water bath. The temperature was kept at 18°C with a Julabo F10 water bath, which was close to the optimum temperature for smelt eggs as determined by Mora and Boubée (1993). The incubation water was charcoal filtered, dechlorinated tap water and the oxygen saturation was maintained close to 100% by aeration with two air stones. The incubation containers consisted of PVC tubing with a diameter of 5 cm and a depth of 4 cm with nylon mesh (425 µm) glued to the bottom of the tubes. A polystyrene sheet with holes 5 cm in diameter was used to float the egg containers in the water bath. At the top of the egg containers, 'claws' consisting of steel wire were used to ensure that the containers did not fall through the holes into the water bath. The

water bath was covered at all times with a plastic lid and a black cloth, to protect the eggs from laboratory lighting. The level of illumination for the eggs was 0.8 $\mu E/cm^2/s$ during working hours and near-total darkness after working hours.

Dead or diseased eggs were removed daily in order to reduce the potential for fungal infections, but no further treatment or prevention of disease was undertaken.

The availability of fertilised smelt eggs was unreliable even when apparently ripe adults were caught on a regular basis. In 1994, the Waikato River was beach seined in Hamilton, Huntly, and Ngaruawahia 16 times between March 28th and July 13th, but in only three attempts were fertilised eggs produced: in Huntly on June 2nd and July 1st, and in Ngaruawahia on July 5th.

In 1995, eggs were donated by Nigel McCarter (NIWA), who had beach seined the Waikato river at Tuakau, Mercer, Huntly and Hamilton between February 10th and June 9th. Fertilised eggs were obtained on the 11th and 19th of April at Tuakau, and on the 27th of April in Huntly and Hamilton (McCarter et al. 1995).

4.3 Collection of inanga eggs

Adult inanga were captured by beach seining between July and September in both 1994 and 1995 in the Waikato River at Hamilton. Alternatively, they were beach seined or electrofished in streams in the Port Waikato or Raglan areas in July to September, 1995. Adult inanga were often absent in the Waikato River at Hamilton, and they could be more reliably found in streams closer to the sea. Beach seining was the preferred method of fishing, but some sites were not suitable for this type of fishing, and electrofishing was performed instead.

Mature inanga were brought to the laboratory and maintained in aerated, dechlorinated and charcoal-filtered tap water. The temperature was maintained at 18°C with a Julabo

FT200 cooler. The fish were fed daily with frozen Chironomid larvae or trout pellets ground to an appropriate size and the tank was cleaned daily of debris.

Male fish were running ripe and did not require any intervention for fertilisation of eggs. The female fish were mature, but they did not ovulate or spawn spontaneously. Final maturation of the females was achieved by intraperitoneal injection with Ovaprim (Syndel product code 13430). Ovaprim contains 20 μg/mL of the salmon gonadotropin releasing hormone (GnRH) analogue (D-Arg^6, Trp, Leu, Pro ethylamide) and 10 mg domperidone/mL, which is a dopamine antagonist. Ovaprim was diluted 10-fold with MILLI-Q® water and injected intraperitoneally with a Terumo U-100 1.0 mL syringe fitted with a 27 G x 13 mm needle at a dosage rate of 10 μg GnRH analogue/kg fish. Prior to injection the fish were anaesthetised with phenoxyethanol at 0.22 mL/L for 1-2 min.

The females usually ovulated within 2-3 days from injection. The eggs were stripped and fertilised in the same manner as was described for smelt, and washed with dechlorinated tap water.

Two batches of eggs from 2 females and 1 or 3 males were incubated as recommended by Mitchell (1989) and N. McCarter (pers. comm.). Glass petri dishes were autoclaved and various substrates were cut to size and placed in the petri dishes. The substrates were Whatmans GF/A filter paper placed on top of nylon scouring pads or blue wipe cloths. After rinsing, the eggs were placed onto the substrates and initially sprayed with a solution of zinc-free malachite green (2 mg/L) in dechlorinated tap water to prevent growth of fungus, and thereafter, sprayed daily with dechlorinated tap water. The petri dishes were rinsed daily, and the filter papers was replaced daily. Lids were placed on top of the petri dishes, ensuring that the lids did not touch the eggs. The petri dishes were kept in an incubation cupboard at 18°C in the dark. This was done to mimic the conditions for developing inanga eggs naturally spawned amongst vegetation

in tidal zones. Concurrently with this trial, eggs from the same pool of fertilised egg were also maintained in synthetic soft water (USEPA 1991) in the same type of containers as described for smelt. Soft synthetic water was made using MILLI-Q® and reagent grade chemicals and the pH and conductivity was checked as a quality control (USEPA 1991). According to USEPA (1991), the final pH of this water should be 7.2-7.6, the hardness 40-48 mg/L (as $CaCO_3$), and the alkalinity 30-35 mg/L (as $CaCO_3$).

The survival of eggs maintained in petri dishes was poor for both lots of eggs. For one batch of fertilised eggs the survival rate during 2-4 days post-fertilisation was only 4.5%. The survival for eggs incubated in soft synthetic water was only marginally better (20% during days 2-4). After these initial trials it was decided to change the planned protocol from incubating eggs on artificial substrates to incubating them in dechlorinated, aerated and filtered tap water. The survival rates had been poor for eggs incubated both in petri dishes and in water, and this was thought to reflect the quality of the eggs (i.e. over-ripe eggs) rather than unfavourable incubation conditions. The primary reason for changing the protocol was the cumbersome procedure for cleaning the petri dishes and for checking the survival of eggs. The best way to assess the development and survival of eggs was with a stereo microscope with the light source underneath the eggs, and this involved removing the eggs from the solid substrates, a procedure which may have been stressful for the eggs. An additional reason was that the toxicity tests had to be performed in water solutions, and it was thought that it would be beneficial to be consistent and maintain the eggs in water during the whole incubation period as well as during toxicity tests.

Eggs that were used for toxicity tests were kept in aerated, charcoal-filtered and dechlorinated water at 18°C in the same containers and water bath as described for smelt. This method of incubation was eventually successful, as judged by the fact that the eggs thrived and developed normally, provided they were initially normal and

viable, and kept free of fungal growth by removal of dead and diseased eggs. Of the 11 attempts to fertilise inanga eggs in 1994 and 1995, 8 attempts were successful. Of these 8 batches of fertilised eggs, 2 batches of eggs had a large proportion of eggs develop abnormally, 1 batch had almost a 100% mortality rate within 4 days, and one batch apparently started developing normally, but no developed embryos could be seen within 5 days. Thus, only four batches of inanga eggs were deemed viable, as judged by their ability to progress through defined embryological stages to hatch.

4.4 Collection of koaro eggs

Adult and mature koaro were caught in the Hinemaia stream in March, 1995, and in the Omanawa stream between March and May, 1995, and again in March, 1996, by minnow trapping or electrofishing. They were brought to the laboratory and kept in dechlorinated tap water, which was chilled to 13°C with a Julabo FT200 water cooler. This temperature was chosen because the Omanawa stream was 13.5°C in March 1995, and West (1989) had successfully incubated koaro eggs at 13°C in the past. The volume of the fish aquarium was 280 L. The water was filtered with a Fluval 303 charcoal filter. The fish were fed, *ad libitum*, live white worms (*Enchaetraeus albidus*) or frozen Chironomid larvae and the tank was cleaned daily.

The koaro caught in the Hinemaia stream in 1995 lacked appetite and showed signs of whitespot, and they were, therefore, treated with 17 mL formalin and 0.2 g zinc-free malachite green/100 L water for one hour (Bylund and Fagerholm 1987). They did not, however, recover from the disease even after the above treatment, and were then sacrificed. Upon dissection of the fish, it was found that there were a number of sexually mature males among these individuals, but no mature females.

Among the fish caught in the Omanawa stream, sexually mature males were numerous, but mature females were rare. Mature females were injected intraperitoneally with Ovaprim after anaesthesia with phenoxyethanol, as detailed for inanga, except that the

Ovaprim was not diluted. It was necessary to inject females twice within an interval of a week, whereas running-ripe males were not injected. In 1995, 12 fish were brought to the laboratory from the Omanawa stream, and of these, 6 were running ripe males and only one female ovulated after two injections with Ovaprim. On May 10th, 1995, 16 koaro were caught in the Omanawa stream, but all the fish had spawned except for a few males. In 1996, 11 koaro were brought to the laboratory; of these there were 7 ripe males and three females that ovulated after two injections with Ovaprim.

After ovulation, the eggs were fertilised as detailed for smelt. The eggs were stripped, fertilised and maintained in 13°C dechlorinated and charcoal-filtered tap water, in the same containers as for smelt. The egg batch obtained in 1995 was kept in a 13°C temperature controlled room in 15 L aquariums, and the batches obtained in 1996 were kept in the 100 L water bath as was used for incubation of smelt eggs.

The eggs adhered strongly to each other, forming a tight mat that did not break easily. It was possible, however, to gently detach the eggs from each other without damaging them. This manipulation was necessary when removing dead or diseased eggs. Because manual removal of dead eggs was a cumbersome procedure, a small number of eggs were treated for fungus with zinc-free malachite green at 2 mg/L for 1 hour (Bylund and Fagerholm 1987), but these died within a day. This method of fungal control was therefore abandoned, and no other koaro eggs were treated chemically for disease.

4.5 Collection of common bully eggs

Common bully have been known to spawn spontaneously in aquaria, provided they are fed and the males are kept separate from each other (D. West, R. McDowall, personal communication). It was, therefore, decided to capture bullies and return them to the laboratory to determine whether they could produce fertilised eggs without any intervention. Adult common bullies were caught by beach seining, electrofishing or

minnow trapping in the Waikato River, in eastern lowland streams of Mt. Pirongia, and in the University of Waikato ponds between November, 1994 and January, 1995. In November, 1995, bullies were caught by electrofishing in streams near Port Waikato.

In the laboratory, each male bully was maintained with 3-4 females in separate 30 L aquaria. The males and the females were distinguished by the shape of the genital papillae (McDowall 1990b). The 18°C dechlorinated tap water was charcoal filtered, and debris was siphoned off daily. The fish were fed daily, *ad libitum*, with frozen Chironomid larvae. A variety of rocks and pots were provided as potential nesting sites.

Mature males and females were found when dissecting sacrificed or dead bullies. The males tended to occupy a nest site (usually a pot). Eggs were, however, deposited only twice, once on the side of the aquarium, and once inside a pot. None of these eggs were viable, as they were either not fertilised or had died shortly after fertilisation.

Two attempts (in December, 1994 and January, 1995) were made to induce spawning by injection with Ovaprim as detailed for inanga (Section 4.3). Intraperitoneal injection of bullies with Ovaprim was not successful, however, in the production of fertilised eggs.

Lake Rotoiti is known to be inhabited by only one species of bully, the common bully (D. Rowe, personal communication). This lake was, therefore, chosen for collecting natural spawns of bully, to ensure that all eggs collected were of this species.

Artificial substrates for spawning were placed in Lake Rotoiti on October 26th, 1995, near the natural lake vegetation close to shore, at a depth of approximately 1 meter. The substrates were white plastic 2 L bottles filled with polystyrene to keep them afloat. The bottles were attached to cement blocks with a rope and when placed in the lake, the

bottles floated about 20-50 cm beneath the surface. The artificial substrates were checked for bully eggs on 9 occasions between November 2nd and December 18th, 1995. Natural substrates such as nearby logs were also examined for the presence of common bully nests.

On 7 of the 9 occasions bully eggs were found on substrates in Lake Rotoiti. The nests were positioned underneath floating logs or boards, inside the hollow area of the cement blocks, or in the handle area of the plastic bottles. On three occasions a male common bully was seen guarding the nest.

Fertilised bully eggs were detached from the various surfaces with a sharp object or very fine forceps while keeping the eggs continually submerged in water. In the laboratory, the eggs were kept in the same containers and same conditions as described for maintaining smelt eggs. The laboratory incubation temperature, 18°C, was the same as the temperature of the lake at the time of egg collection. Dead and diseased eggs were removed daily, but no further treatment of disease was undertaken.

4.6 Collection of rainbow trout eggs

Fertilised rainbow trout eggs were obtained from the Ngongotaha Hatchery, Ngongotaha, Rotorua in July, 1994. They were wrapped in moist cheesecloth and transported in a cooler to the laboratory. They were maintained in floating incubation trays with a 1 mm mesh glued to the bottom of the trays. The incubation temperature was 13°C, and the incubation conditions were otherwise identical to that of smelt. Dead eggs were removed daily.

4.7 Exposing eggs to pentachlorophenol

4.7.1 Pentachlorophenol formulation

The pentachlorophenol formulation used for all toxicity tests in this thesis had a purity level of 99% (Aldrich). To confirm this and to determine what other contaminants

might have been present, the PCP was analysed by gas chromatography-mass spectroscopy (5890 GC Hewlett Packard and 5970 Mass Selective Detector) by Ms J. Sims, Department of Chemistry, University of Waikato. It was confirmed that the formulation was essentially pure (99.54% pentachlorophenol), with the remainder being 2,3,4,6-tetrachlorophenol (0.46%).

PCP-stock solutions were prepared in 100% ethanol (Analytical Reagent grade, AR) to either 1 or 10 g/L and stored in the dark at 4°C in glass containers covered with aluminium foil. When performing toxicity tests, the ethanol concentration never exceeded 0.5 mL/L in the test solutions (Parrish 1985).

4.7.2 Toxicity tests

Toxicity tests were performed at four different developmental stages of the five species that were studied. The stages were 24 h, early eyed, late eyed and newly hatched larvae. The use of fish eggs for toxicity testing was approved by the Ethics Committee on the Welfare of Experimental Animals at the University of Waikato.

At least one static range finding toxicity test was initially performed to obtain a narrower range of PCP concentrations for subsequent definitive flow-through toxicity tests.

Soft synthetic water for dilution of PCP was made using MILLI-Q® and reagent grade chemicals (USEPA 1991). As quality control, the pH and the conductivity of the soft synthetic water was checked prior to adding PCP. The average pH (± standard deviation, SD) was 7.35 ± 0.23, and the average conductivity (± SD) was 166 ± 16 µS/cm.

In static tests, the test PCP solutions were added to 50 mL glass beakers. The beakers were then placed on a tray in a temperature controlled water bath (Julabo F10), and

when the test temperature was reached inside the beakers, the eggs or larvae were placed in the containers in random order. The test temperatures were identical to that used for incubations of the eggs of each species. No aeration was provided, except for rainbow trout eggs, due to their much larger size.

For flow-through tests, the test solutions were prepared directly in 5 or 10 L glass containers. The glass containers were protected from light with a black cloth to prevent any photolysis of PCP. The test solutions were gravity fed (height 42 cm) through polythene tubing to gang valves, which divided the flow between 2-10 replicate glass beakers (25 or 50 mL) for each concentration, which served as the exposure containers. The flow rates in the beakers were checked six times, and always provided a minimum of 5 x 90% changes of solution per 24 h, fulfilling the recommended requirements for flow rates in a flow-through test (USEPA 1991). Similar to the method for controlling temperature in static tests, the temperature of the beakers was also controlled by placing them on a tray in a temperature controlled water bath (Julabo F10). The test solutions overflowed into the water bath, and from there to waste. When conducting tests with larvae, which had a tendency to swim at the surface of the solutions, it was necessary to fit a plastic siphon to every beaker, which prevented the larvae from floating over the edges of the beakers. A white plastic lid with a black cloth on the water bath protected the eggs and the larvae from laboratory lighting; the illumination in the water bath during working hours was 0.99 $\mu E/cm^2/s$.

For range finding static toxicity tests, 10 - 30 eggs or larvae per concentration were tested. For flow-through definitive tests, 20 - 100 eggs or larvae per concentration were tested. The loading of eggs never exceeded 0.5 g/L for static tests, or 1 g/L for flow-through tests (Parrish 1985). The 4 - 5 test concentrations were in an arithmetic series, except when testing rainbow trout eggs, where the dilution factor between test concentrations was 50%. All PCP concentrations were nominal. The two controls included a soft synthetic water and an ethanol control.

Before the tests were initiated, the eggs or larvae were sorted under a Zeiss microscope (10 -1000 x magnification), and healthy and normal ones were chosen. An egg was deemed normal if it had no fungus or other disease, no malformations, and the developmental stage was normal for its age. The eggs were then randomly and evenly distributed to the different beakers. The design of the flow-through test system prevented a random placement of the test beakers in the temperature controlled water bath.

The duration of the static and flow-through tests was 48 h. The temperature and the dissolved oxygen concentration were checked daily in at least the control beakers with a YSI model 55 handheld dissolved oxygen monitor. The temperatures stayed within ± 1°C of the chosen temperatures, and the oxygen content in the individual beakers was in excess of 75% saturation. The pH in all the beakers was measured at the end of each test with a Fisher Accumet model 220 pH meter. The pH values within each test were tested (ANOVA or Kruskal-Wallis) to detect significant differences between the different concentrations, and no significant differences were found.

4.7.3 Follow-up of acute flow-through toxicity tests

Surviving eggs from the replicates of acute flow-through toxicity tests were pooled and returned to filtered, aerated and dechlorinated tap water and incubated under the same conditions as prior to the tests being initiated. The eggs were examined daily and dead ones were removed. The time-to-hatch for each egg was recorded, and from this, both median hatch (50%) hatch and average hatch (with standard deviation) were calculated. The median hatch value gave only one value without the range, and no statistical analyses could be calculated on these values, whereas average hatch could be analysed using standard ANOVA procedures. The heart rates of 10 - 30 individual eggs or larvae were counted during incubation of smelt. In addition, upon hatching, the larvae

were visually checked for swimming performance (of common bully and koaro) and developmental abnormalities.

Using Sharp (1990) as a guide, the malformations were classified as:

1) axial (kyphosis, scoliosis, lordosis, no axial development);
2) eye (optic convergence, synophthalmia, cyclopia, microphthalmia, anophthalmia);
3) cranial (micrencephaly, microcephaly, synarthrosis, absence of jaw development);
4) caudal (deformity of caudal fin and caudal region of notochord).

These abnormalities are illustrated photographically in Figure 4.1A-H.

Not included in the description of malformed larvae were:

1) Half-hatched eggs which were left in the incubation medium until they either hatched or died;
2) Size of yolk sac, which could vary considerably depending on the time-of-hatch and stage of development of the larvae;
3) Length of larvae. Dwarfism is a type of malformation, but the size distribution of the larvae is better described by measuring the hatched larvae (see below).

Malformations were also evident during incubation after exposure to PCP and before hatch (Figure 4.2), but many of these embryos failed to hatch and these deformities were, therefore, not quantified.

Photos of anaesthetised live eggs and larvae were taken using a Nikon AF F-801s camera set on automatic exposure and fitted to a Nikon SMZ-U microscope. The film was Kodak Ektar 25 PHR 135-36. The larvae were then sacrificed with an overdose of benzocain, and preserved in 10% neutral phosphate-buffered formalin (pH 7.0). When testing larvae, the survivors were kept for only two days after the test, before being anaesthetised and preserved in formalin solution.

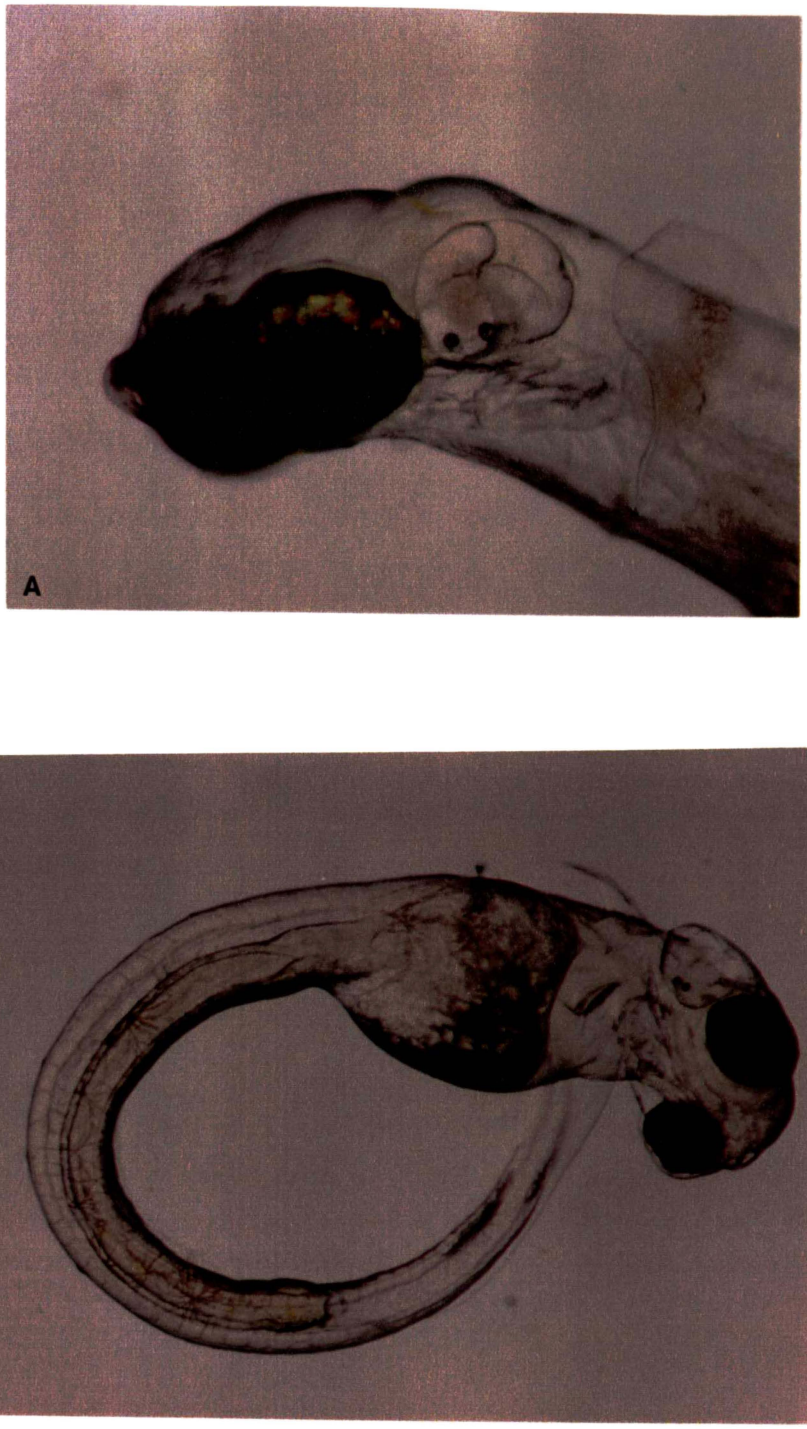
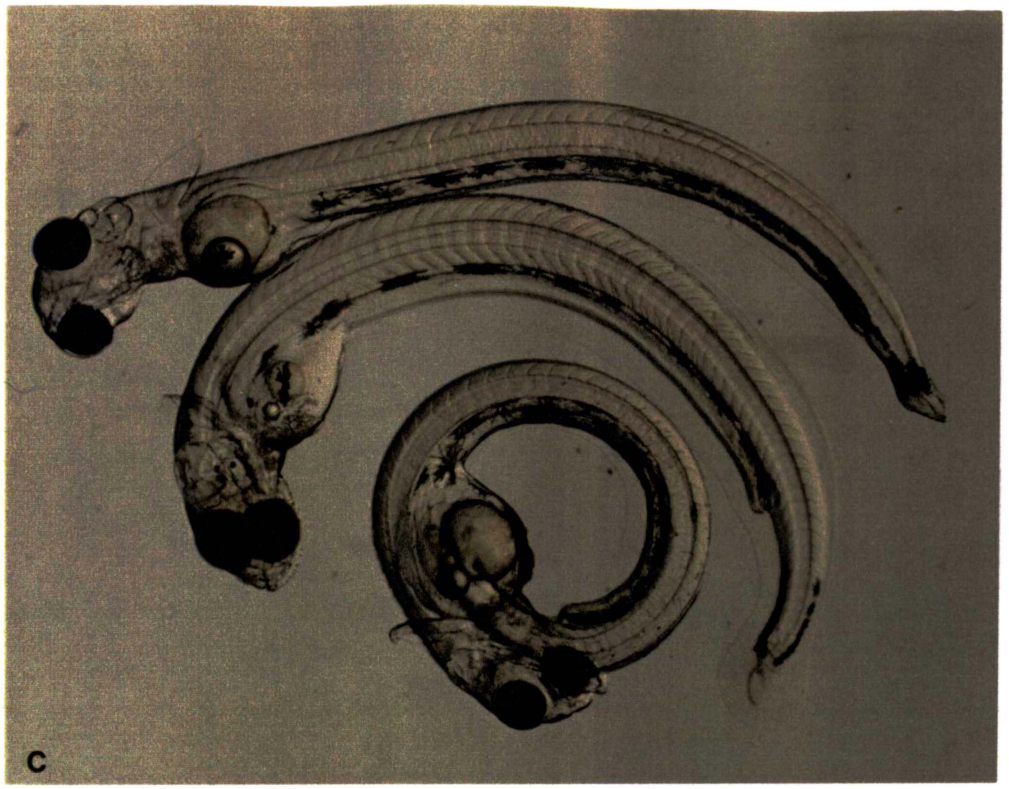


Figure 4.1 Terata produced by PCP in the four native fish species. (A) absent jaw (smelt) (B) scoliosis (smelt).



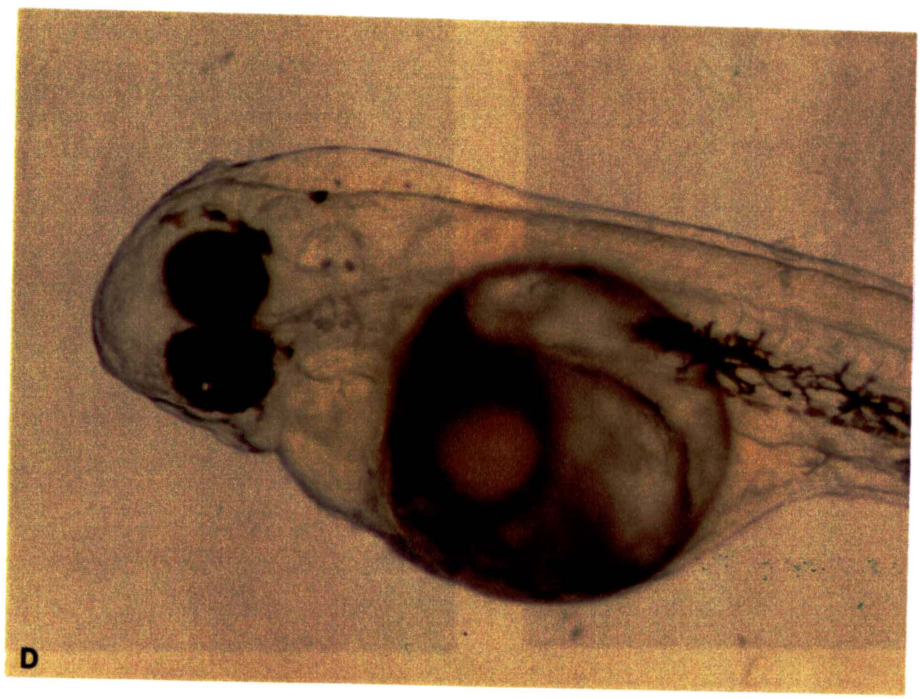


Figure 4.1 (continued) Terata produced by PCP in the four native fish species. (C) varying degrees of ventral lordosis, caudal fins abnormal (koaro) (D) synophthalmia, microphthalmia, edema, no jaw (common bully).

Figure 4.1 (continued) Terata produced by PCP in the four native fish species. (E) severe ventral lordosis, synophthalmia (koaro) (F) dwarfism, scoliosis, edema, caudal fin malformation, absence of jaw formaton (inanga) (G) ventral lordosis, microphthalmia, microcephaly, pericardial edema, pigmentation lacking (except eyes) (koaro) (H) kyphosis (smelt).

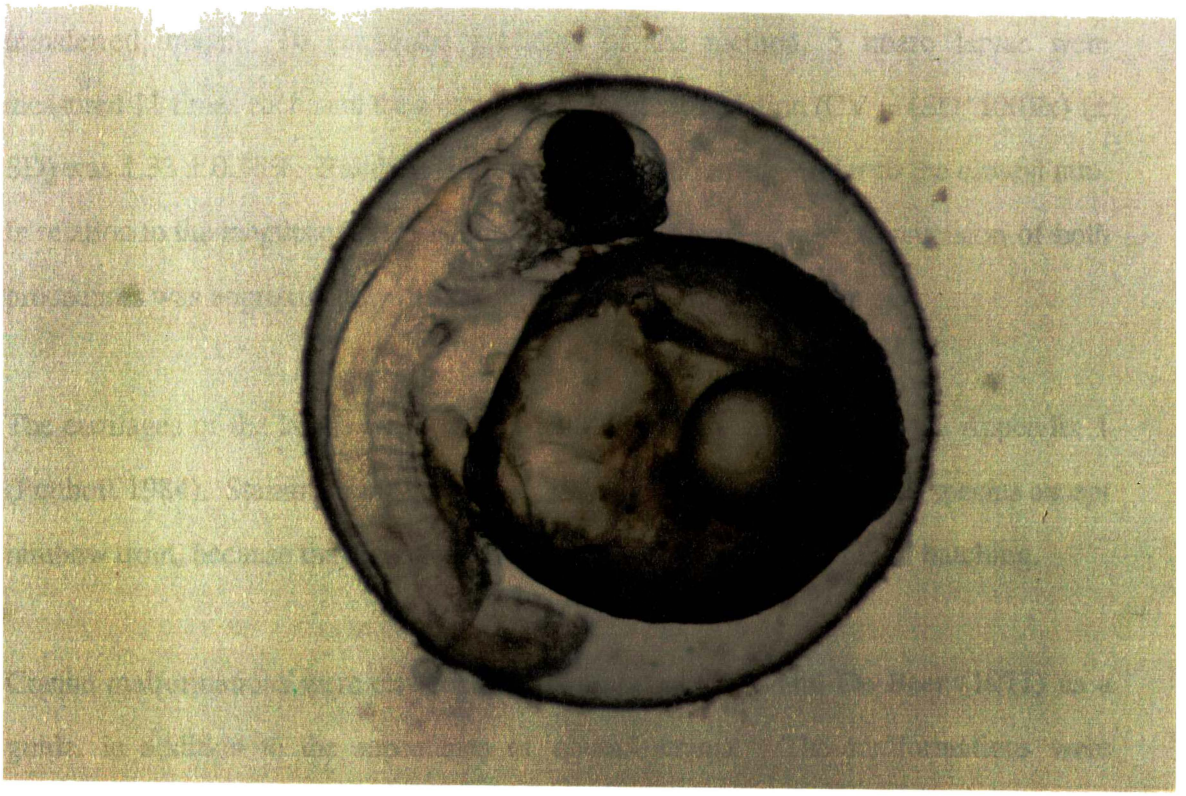


Figure 4.2 PCP-exposed inanga embryo which was grossly malformed and never hatched.

The lengths of up to 30 preserved larvae of the native species (depending on availability) were measured using an image analysis system, consisting of a SMZ-U Nikon microscope, linked to IPLAB Spectrum™ version 3.0.1 software. The measurements were made from the front of the upper cranium to the caudal end of the notochord (standard length ± 100 µm). The caudal fins were not clearly visible in the transferred image. To gauge the precision of the method, 5 koaro larvae were measured 11 times each, and the average coefficient of variation (CV = (SD*100)/x) (± SD) was 1.33 ± 0.38%. Rainbow trout were measured using a ruler to the closest mm. In relation to the lengths of the fish larvae and fry, the level of relative precision of both procedures was approximately the same (3 - 5%).

The cartilages of the larvae were stained with alcian blue as described in Appendix 1 (Potthoff 1984). Staining bone tissue with alizarin red was omitted in all species except rainbow trout, because they had not developed bone tissue at the time of hatching.

Cranial malformations were classified using Benzie (1968) and De Beer (1971) as a guide, in addition to the appearance of control crania. The malformations were classified as:

1) shape/size (for malshaped, large or small cartilages);
2) asymmetry/organisation (dorsal and ventral, or frontal and caudal, or lateral misalignment of the crania, or disorganised craniums);
3) missing structures (when cartilages were missing);
4) eye (shape, size, position of eyes abnormal, excluding abnormal pigmentation, which could be artefactual, due to the staining procedure).

Examples of the above disturbances can be seen in Figure 4.3A-K.

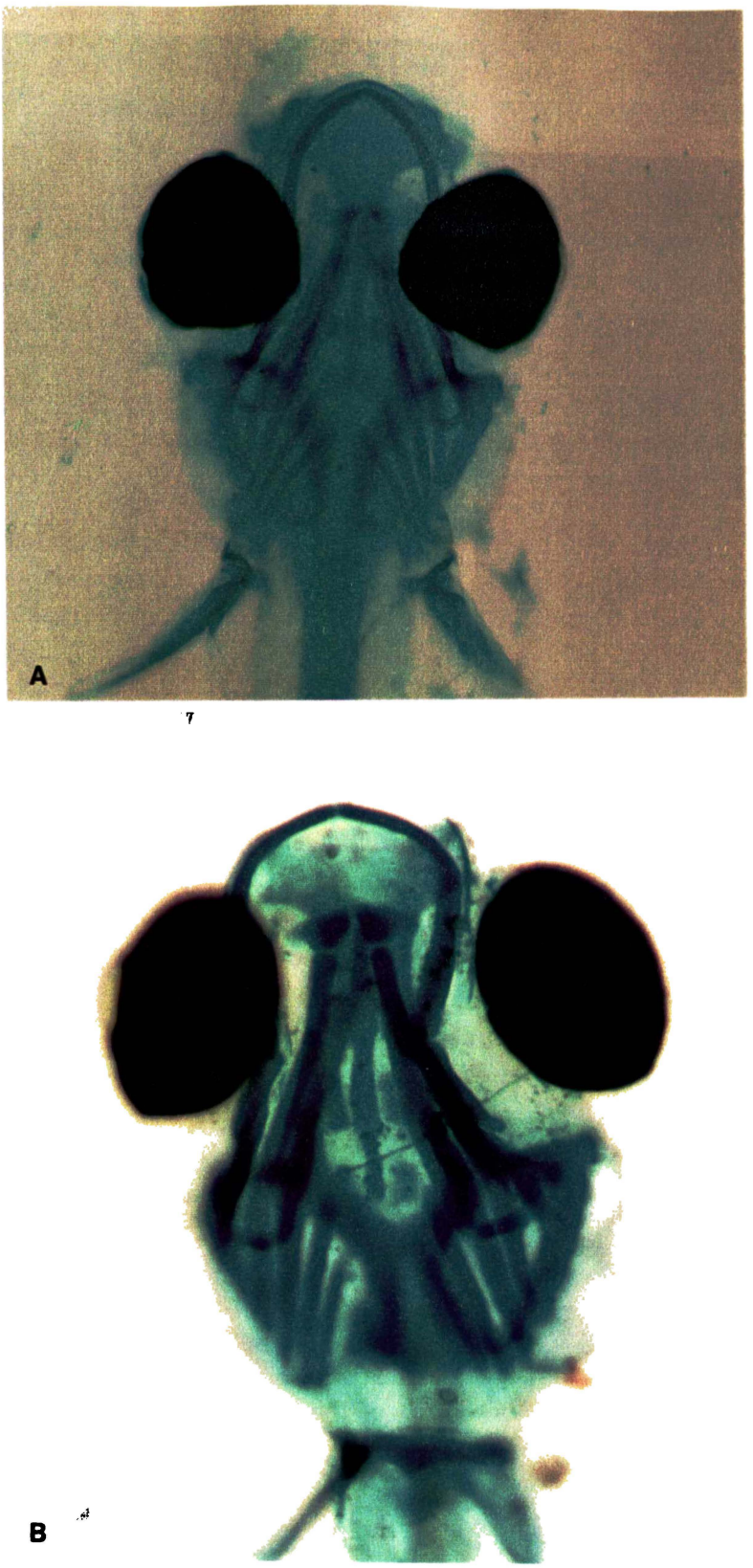


Figure 4.3 Normal crania and cranial malformations caused by exposure to PCP (A) normal smelt (B) normal inanga.

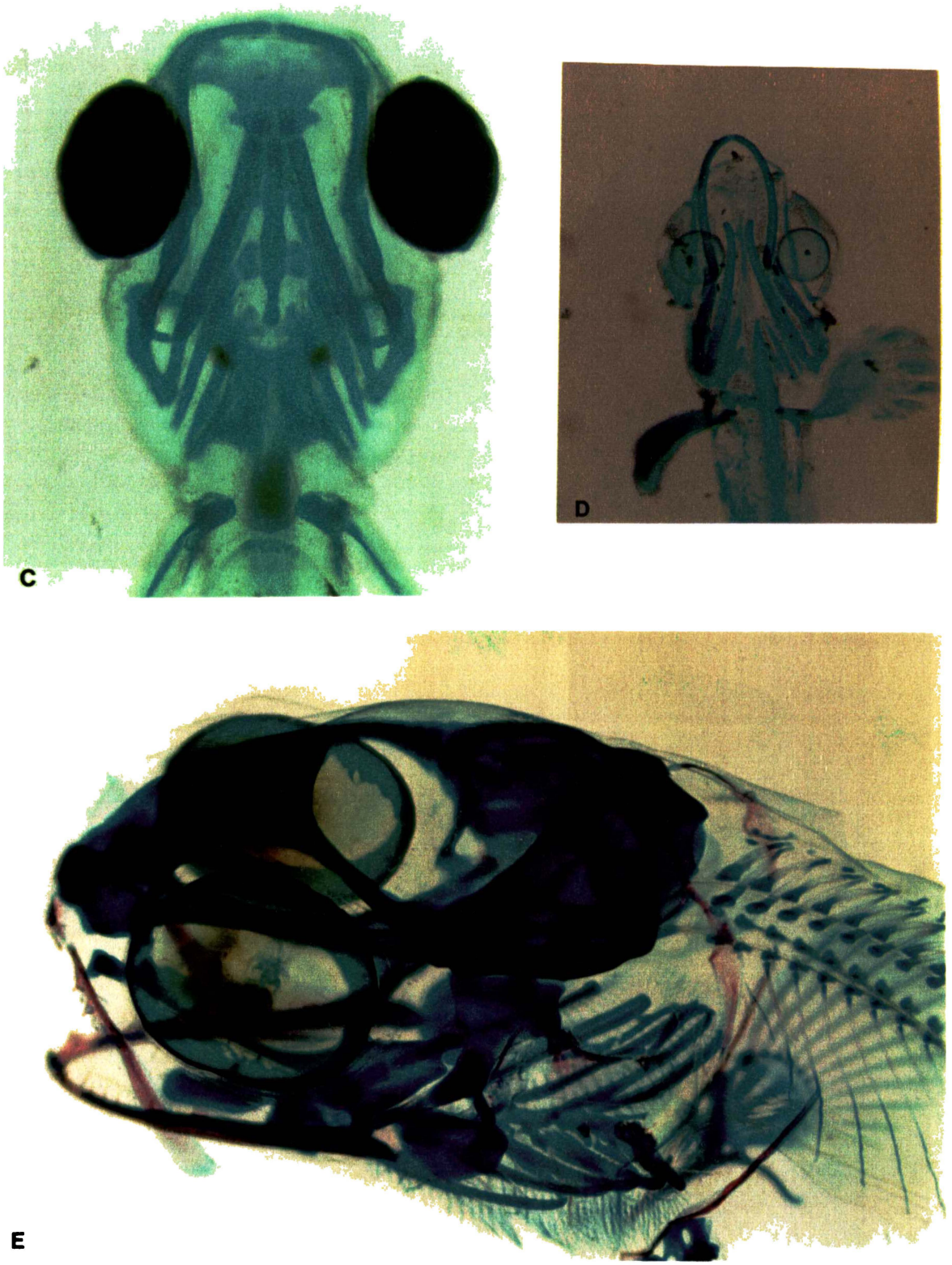


Figure 4.3 (continued) Normal crania and cranial malformations caused by exposure to PCP (C) normal koaro (D) normal common bully and (E) normal rainbow trout.

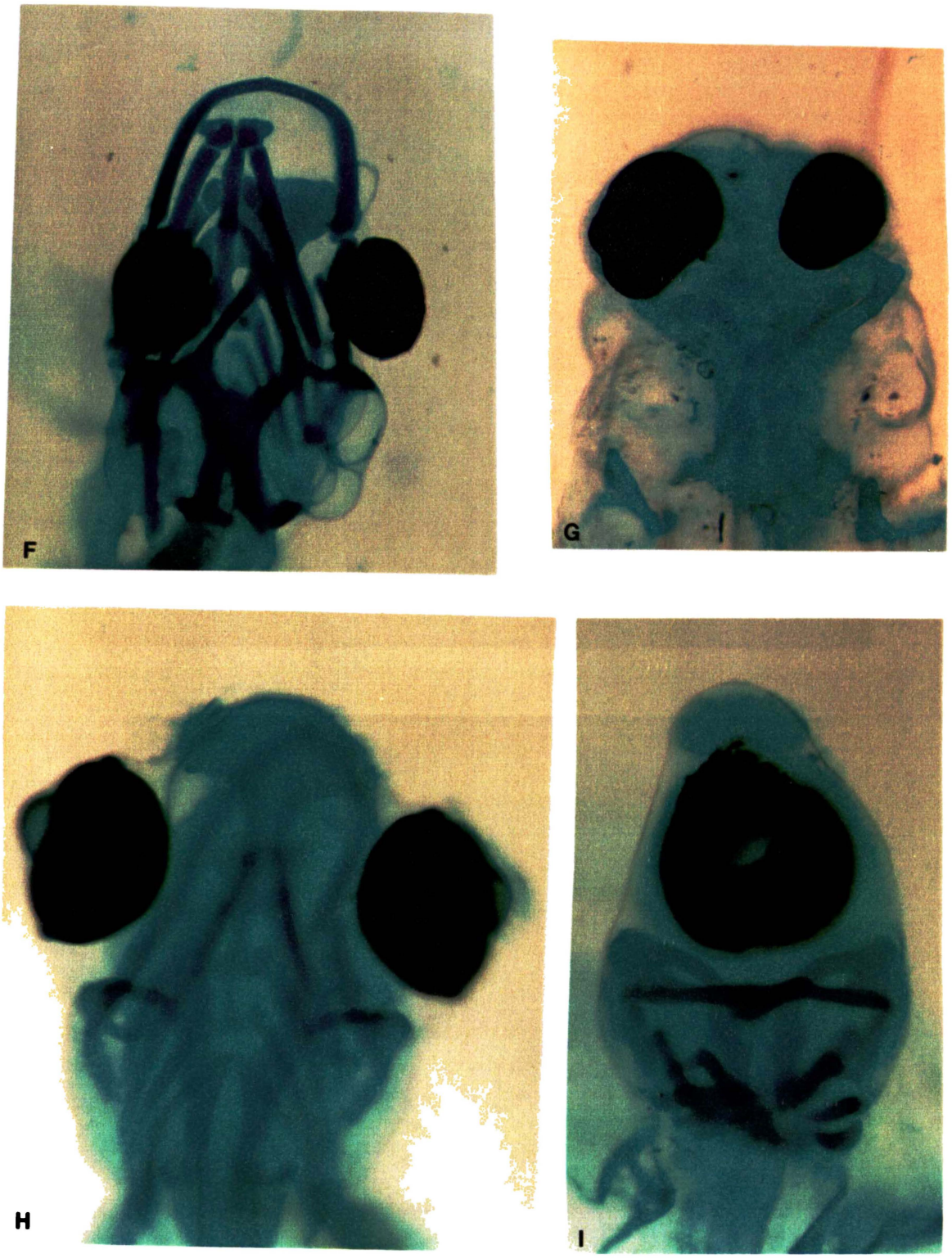
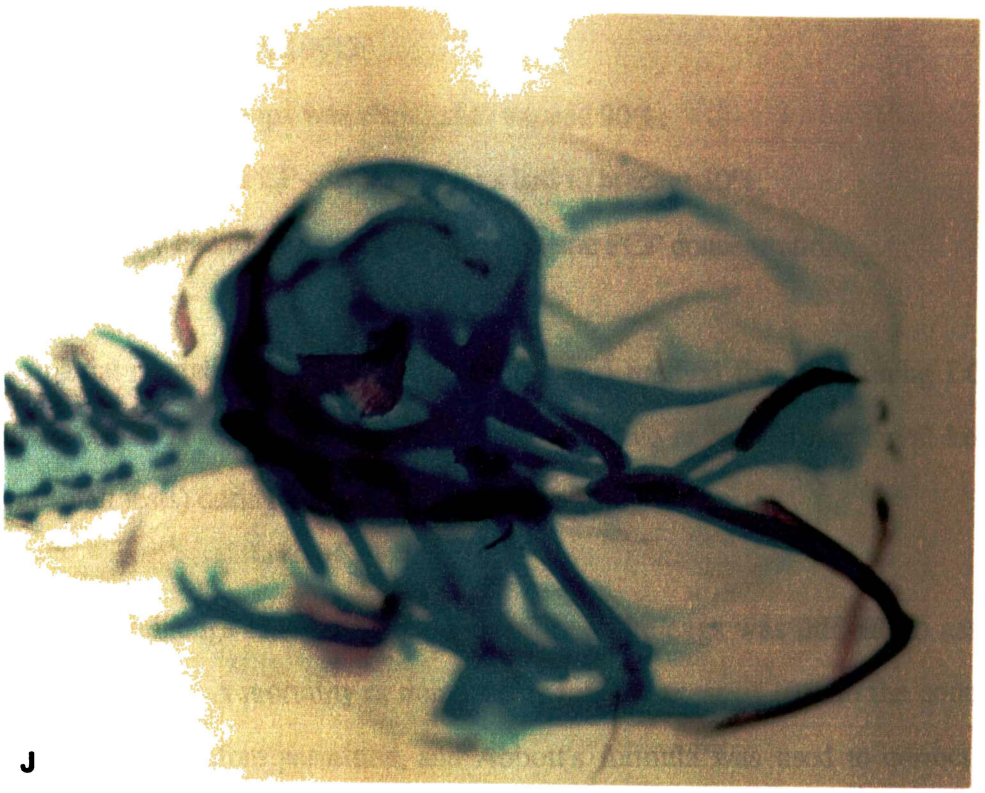


Figure 4.3 (continued) Normal crania and cranial malformations caused by exposure to PCP: (F), elongated Meckel's cartilage (smelt); (G), irregularly shaped and sized eyes, unable to identify cartilages (inanga); (H), Meckel's cartilage misshapen (smelt); and (I), cyclop (smelt).



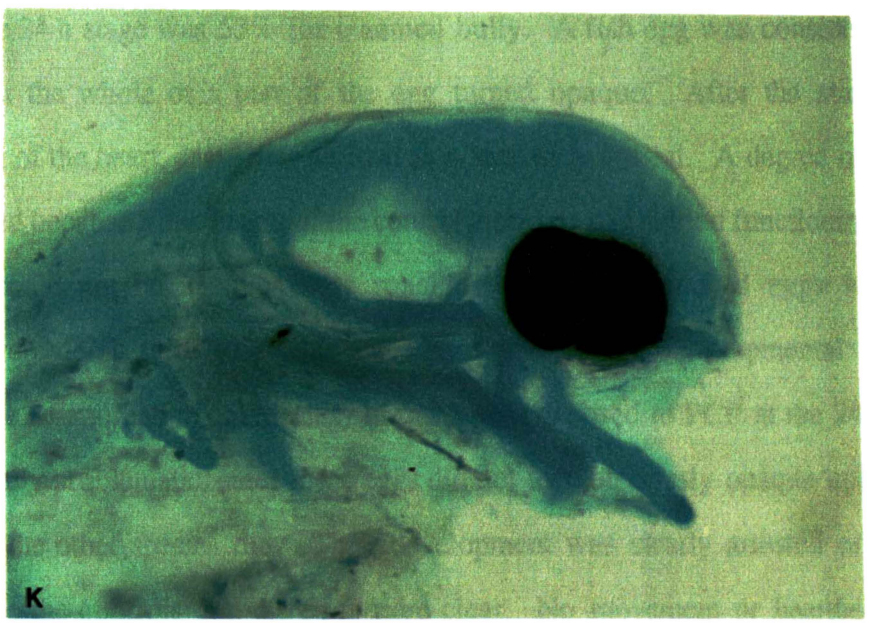


Figure 4.3 (continued) Normal crania and cranial malformations caused by exposure to PCP: (J) elongated and wide Meckel's cartilage, (rainbow trout); (K) small eyes, large otic capsules, Meckel's cartilage and copula point downwards (inanga).

4.8 Statistical analyses

For calculation of LC50 values, three conditions had to be fulfilled for a toxicity test to be considered valid (USEPA 1991):

1) Survival of control groups was required to exceed 90%;
2) The mortality rates in PCP-exposed groups had to bracket 50%;
3) Partial mortality had to be recorded for at least one PCP concentration.

When no partial mortalities were found, the graphical method for determining LC50 was utilised (USEPA 1991), which only occurred on two occasions (for larval koaro and larval smelt tests) during the definitive flow-through tests.

When testing 24 h inanga or bully eggs, the viability of the eggs was difficult to assess at the start of the tests. A mortality or nonviability rate in excess of 10% in the controls of these tests was therefore permitted, and Abbott's formula was used to correct for control mortality (Abbott 1925; USEPA 1991). The highest control mortality for eggs tested at the 24 h stage was 53% for common bully. A fish egg was considered dead when either the whole or a part of the egg turned opaque. After the start of the functioning of the heart, death was defined as a lack of heartbeat. A degree of caution was required because if the hearts of the control embryos had started functioning during a test, it could not be unequivocally concluded that PCP-exposed eggs lacking a heartbeat were dead, since PCP was capable of slowing the developmental rate. In addition, the determination of mortality in smelt eggs exposed to PCP at the 24 h stage was especially problematic, since these eggs did not turn obviously opaque upon death as eggs of the other species did. Their development was clearly arrested or slowed compared to the controls, but the eggs were clear. No movement or heartbeat could guide the decision of whether a smelt egg was alive at this early stage. No LC50 value for 24 h smelt eggs was, therefore, determined.

LC50 values were calculated using either the probit or Spearman-Kärber methods, according to the guidelines of USEPA (1991). The probit method was used when there were two or more partial mortalities (Finney 1971; USEPA 1991). The Spearman-Kärber method was used if there was only one partial mortality. A trim was used if there was neither 0% mortality in the lowest concentration nor 100% mortality in the highest concentration (Hamilton et al. 1977; USEPA 1991). The probit and Spearman-Kärber computer programmes for calculating the LC50 values were written by Dr Nicholas Ling, the University of Waikato.

Synthetic water and ethanol controls were compared using a *t*-test or a nonparametric Mann-Whitney test. The ethanol controls were compared to the treatment groups using one-way ANOVA and Dunnett's post-test using GraphPad InStat Mac software. If the conditions of ANOVA were not satisfied (i.e. a significant Bartlett's test) then the values were either log-transformed prior to conducting the ANOVA test or the nonparametric Kruskal-Wallis test was used (with Dunn's post-test). In some cases regression analysis was used. Fishers exact test was used for data involving counts.

CHAPTER 5

RESULTS FOR EARLY LIFE STAGE TOXICITY TESTS

5.1 Availability and quality of native fish eggs

For the purpose of toxicity testing, it is important to have a reliable source of material to work with. The availability of native fish eggs to use in toxicity testing in this study was, however, somewhat limited. The seasonality of the spawning activities of fish is, of course, a natural limitation to the collection of usable eggs. Prior to artificial or natural fertilisation of fish eggs, the fish have to be mature, even when gonadotropin is used to induce final maturation. Mature smelt were available in April-July, inanga in July-September, common bully in October-December, and koaro in March-April, at the sites where these fish were captured for this study.

In addition to natural limitations on the availability of eggs, there were also constraints imposed by the success rate of artificial fertilisation of eggs obtained either through hormone injections, or by using ripe fish captured in the field. The survival of fertilised eggs during the first couple of days was highly variable, due to both unfertilised and nonviable eggs. This is best illustrated by the survival rates of four groups of smelt eggs after two days post-fertilisation: One group of pooled eggs from many different females and males had a survival rate of 13%, and three groups from one female and one male smelt had survival rates of 61, 8 and 0%, respectively. The highest proportions of fertilised and viable eggs was seen amongst the koaro eggs. The common bully eggs collected from Lake Rotoiti were of good quality, but the combination of the 1 h transport time to the laboratory, and the detachment of the eggs from the substrates, may have caused some stress, and occasionally large proportions of these eggs died soon after the return to the laboratory.

Eggs of all species, except rainbow trout, were easily infected by fungi if left unattended. Therefore, frequent changes of water, large volumes of incubation water and daily removal of diseased or dead eggs were necessary to maintain the eggs in a disease-free state.

Keeping eggs in beakers in an incubation cupboard seemed to encourage fungal growth, and this method of incubation was avoided. In the few cases where zinc-free malachite green was used to prevent disease, it was not successful, and it was also avoided because of fear of additional toxicity from this treatment. Low concentrations of PCP also seemed to effectively control fungal growth, but the concentrations of PCP used in this study were too high to be of therapeutic value.

5.2 Survival of control eggs during toxicity tests and the subsequent incubations in clean water

The control mortality during toxicity tests is an indication of the suitability of the conditions during the tests. The mortalities reported here are for both the static and flow-through tests.

Control smelt eggs had a mortality rate of ≤1.9% in the toxicity tests that were started at the early and late eyed stages, and at the newly hatched larval stage. As mentioned in Section 4.8, the tests that started at the 24 h stage were particularly problematic in that neither viability or survival of these early eggs could be accurately determined.

Control inanga eggs had a mortality rate of ≤1.0% when tested at the early and late eyed stages, and at the newly hatched larval stage. Control inanga eggs that were tested at the 24 h stage had an average % mortality rate (± SD) of 15.3 ± 9.4% (n= 7 tests), probably reflecting the number of eggs that were nonviable at the start of the test rather than true mortality.

Control koaro eggs had a mortality rate of ≤0.9% in all tests except for one range finding test at the late eyed stage where the mortality rates were 10.5 and 4.8% for the synthetic water and solvent controls, respectively.

Control bully eggs had a mortality rate of ≤3.1% when tested at the early and late eyed stages, and at the newly hatched larval stage. The average % mortality (± SD) for control bully eggs tested at the 24 h stage was 28.8 ± 24.7% (n= 5 tests)

Control rainbow trout eggs tested at all stages had a mortality rate of ≤1.9%.

There were no significant differences in mortality during toxicity tests between solvent and synthetic water controls in any of the fish species tested (Fisher's exact test, applied only when there was at least one death in either group).

Little information on the optimal incubation conditions for eggs from the four native fish species is available from the literature, with the exception of the temperature optima for smelt and inanga eggs (Carlberg 1993; Mora and Boubée 1993). The conditions chosen in this study were, therefore, a result of what has been successful in the past, and what the conditions would have been for eggs naturally spawned in the field, especially with respect to the temperature.

It is, therefore, important to make an assessment of the suitability of the incubation conditions that were provided in this study for control eggs and thus were never exposed to PCP. As mentioned in the previous section, the yield of normal and fertilised eggs was occasionally small, but this was not taken to be a result of suboptimal incubation conditions, but rather as a result of the presence of unfertilised or nonviable eggs. The best estimate provided by this study of the suitability of the incubation conditions can be derived from an examination of the numbers of control survivors from toxicity tests at the 24 h stages. The estimates are, therefore, based on the period between completion of the flow-through toxicity tests (i.e. PCP exposure), at which point the eggs were three days old, to the stage when all eggs had either hatched or died, and the experiments completed. Table 5.1 lists the percent successful hatch of control eggs of the five tested species, while kept in the conditions as detailed in Sections 4.2 - 4.6. Overall, the survival rate was ≥ 70% for all synthetic water and solvent control eggs, with half of these having over 90% survival.

Table 5.1 Percentage hatch of control eggs that underwent a toxicity test starting at the 24 h stage. The percentage is based on the number of control eggs that were left at the end of the toxicity tests (number shown in the parentheses), at which stage they were three days old, until the time-of-hatch.

Species	% survival in synthetic water control (n)	% survival in solvent control (n)
Smelt	93 (91)	85 (106)
Inanga	100 (94)	94 (96)
Koaro	92 (101)	96 (106)
(two batches)	97 (106)	94 (109)
Common bully	83 (31)	94 (31)
(two batches)	89 (46)	76 (47)
Rainbow trout	72 (98)	70 (100)

5.3 The developmental rates of control eggs

The developmental rates until hatch at the chosen incubation temperatures for the five tested species can be seen in Table 5.2. The rates of development varied between batches of eggs. The time to 50% hatch varied significantly within a species, especially for inanga and koaro. This was, however, probably not necessarily a reflection of rates of development, but rather varying reactions to external stimuli such as pipetting and light. For instance, one trigger for hatching in inanga may be low oxygen tension (Mitchell 1989), but this was not provided for them. In some cases hatching seemed to be triggered by the daily pipetting of eggs, especially among smelt eggs.

Since common bully eggs were collected from the field, the exact time of their fertilisation was not known with absolute certainty, but it was deduced for one batch of eggs that was found on a substrate that had been checked and found to have been empty 24 hours earlier.

Table 5.2 Developmental rates of control eggs of the five tested species at the chosen incubation temperatures (also indicated in the table). 'Stage at 24 h' indicates the stage that the eggs were at when the first set of toxicity tests started. The age (in days) at the early eyed and late eyed stages indicates how old the eggs were when these tests were started. Days to 50% hatch is the measured range among all the tested control groups.

Developmental stage	**Smelt** 18°C	**Inanga** 18°C	**Koaro** 13°C	**Bully** 18°C	**Trout** 13°C
Stage at 24 h	epiboly (50%)	epiboly (50%)	blastula	epiboly (50-75%)	blastula
Early eyed (days old)	4	3-4	8	3	12
Late eyed (days old)	7	7-10	14-16	5	21
50% hatch (days old)	10-11	15-26	24-37	7-10	25-29
Degree days to 50% hatch	180-198	270-468	312-481	126-180	325-377

Selected photos showing the normal development of the four native fish species are presented in Figures 5.1 - 5.4.

5.4 Acute toxicity of PCP

The 48 h LC50 values with 95% confidence intervals for PCP exposure for the five tested species at four different early life stages derived from flow-through tests are listed in Table 5.3. The method for calculating each individual LC50 value is also indicated. The results from static range finding tests are not shown. When sufficient eggs were available, then more than one test for a certain life stage was performed. The number of eggs per concentration (divided evenly over 2-10 replicate test beakers for each concentration) and the origin of the eggs are also listed in Table 5.3. The 48 h LC50 for 24 h smelt eggs was not reliably determinable because dead eggs did not turn opaque unlike the other species tested.

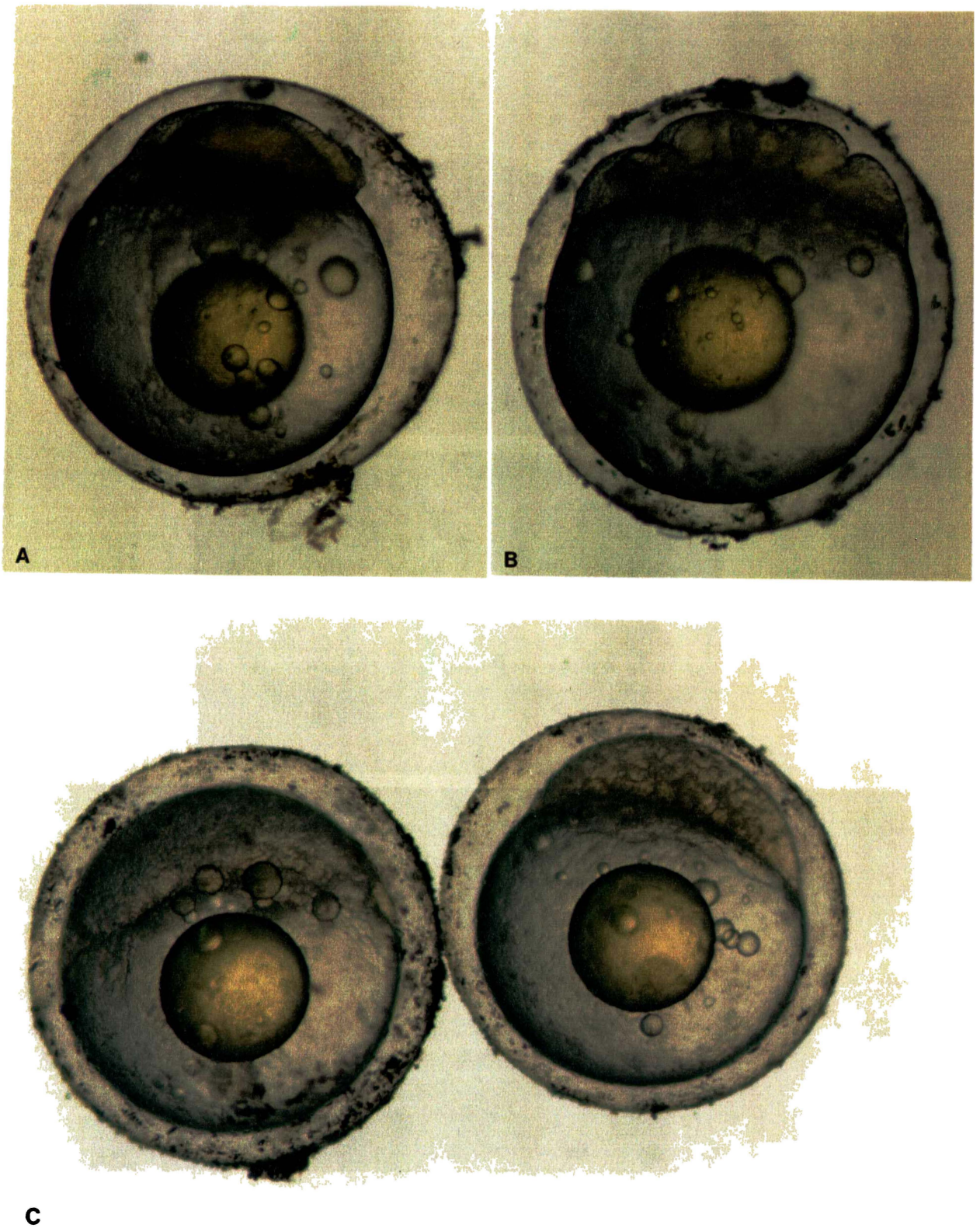


Figure 5.1 The normal development of smelt at 18°C until hatching. (A) 2 cells (B) 4 cells and (C) 10-12 hours. The diameters of the eggs are between 2.3 and 2.6 mm.

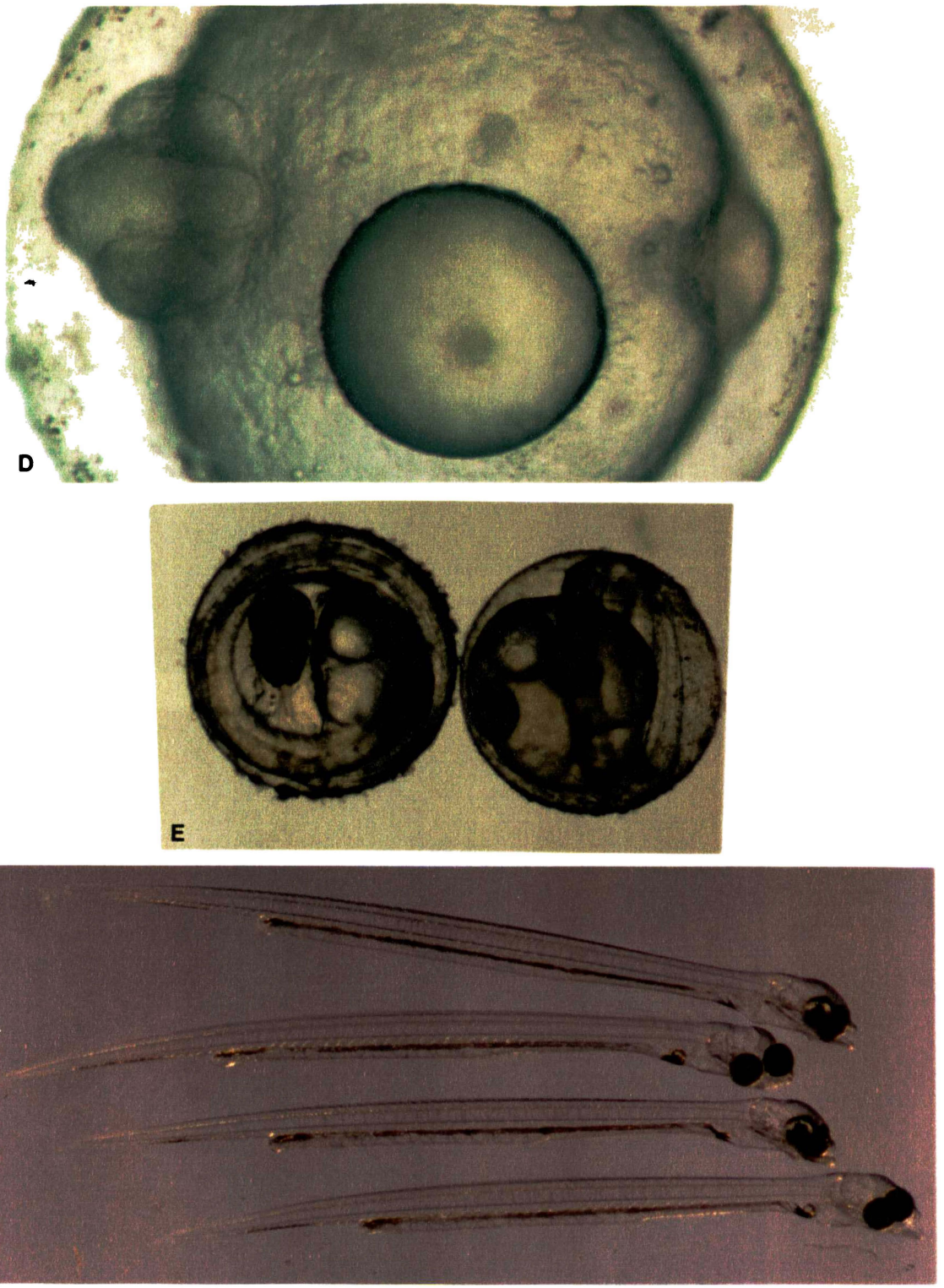


Figure 5.1 (continued) The normal development of smelt at 18°C until hatching. (D) early eyed (E) 10 days and (F) hatched larvae. The diameters of the eggs are between 2.3 and 2.6 mm, and the hatched larvae are 4.7 - 5.2 mm.

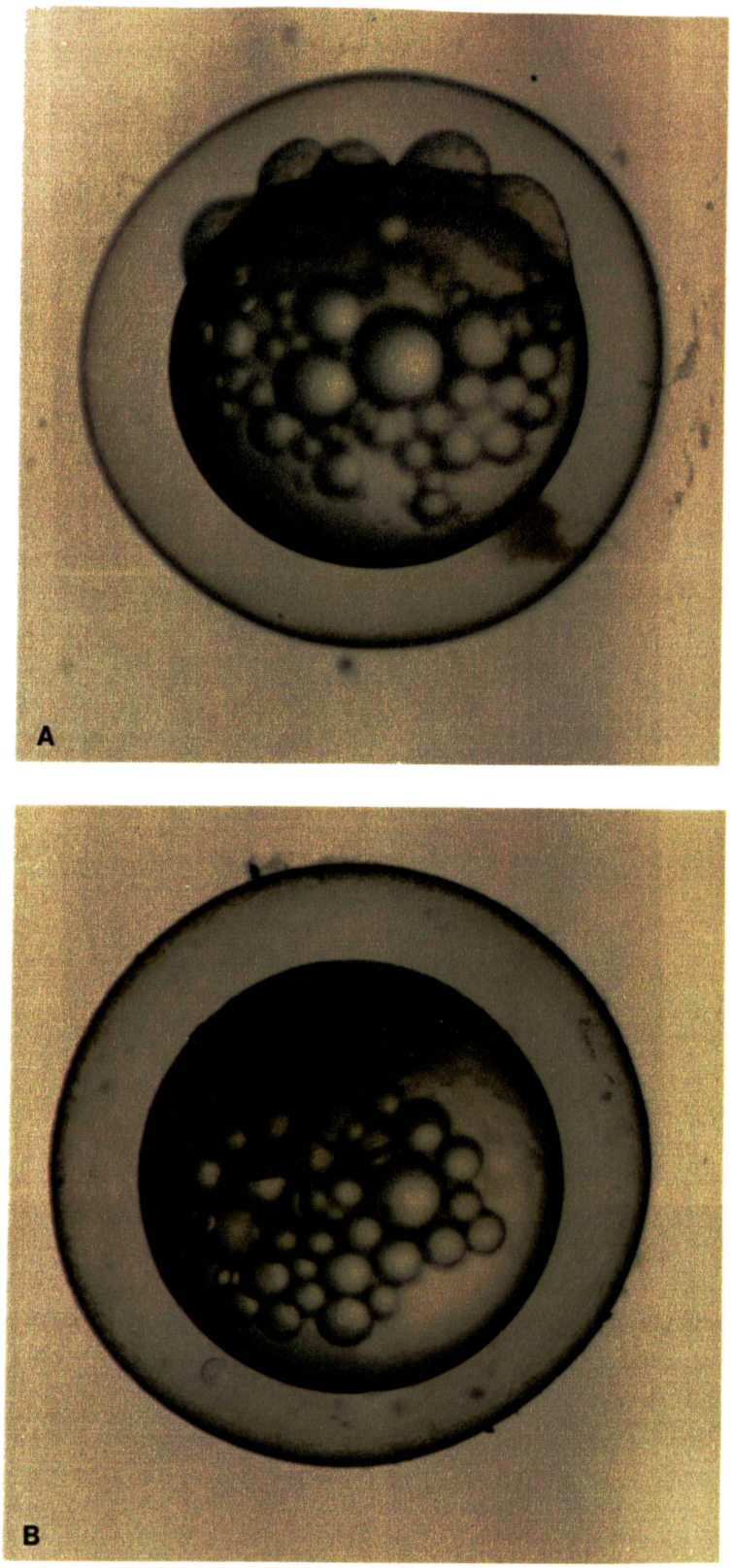


Figure 5.2 The normal development of inanga at 18°C until hatching. (A) 8 cells and (B) 1 day. The diameters of the eggs are between 2.9 and 3.4 mm.

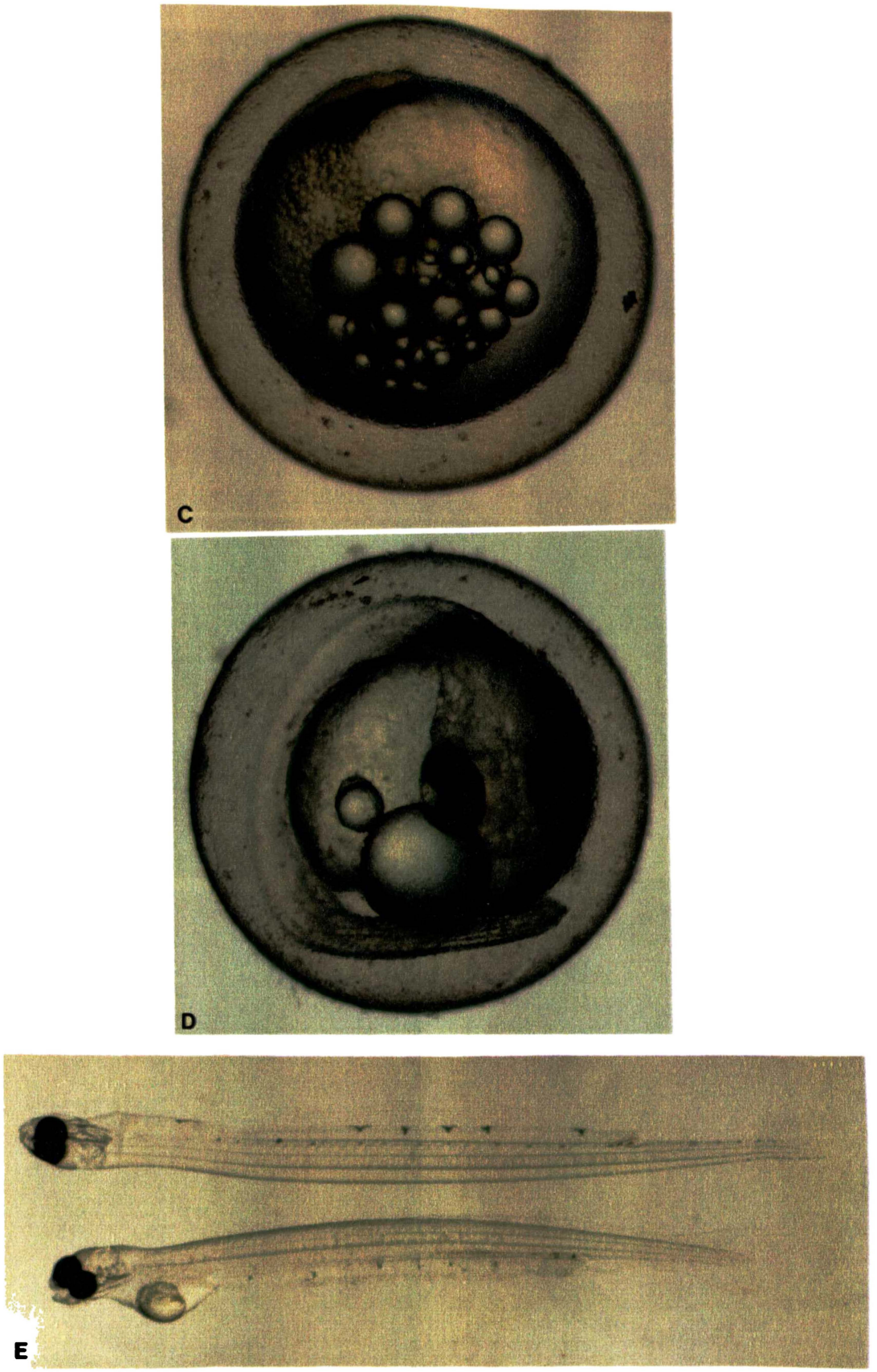


Figure 5.2 (continued) The normal development of inanga at 18°C until hatching. (C) 2 days (D) 7 days and (E) hatched larvae. The diameters of the eggs are between 2.9 and 3.4 mm, and the two larvae are 6.60 and 6.74 mm long (standard length).

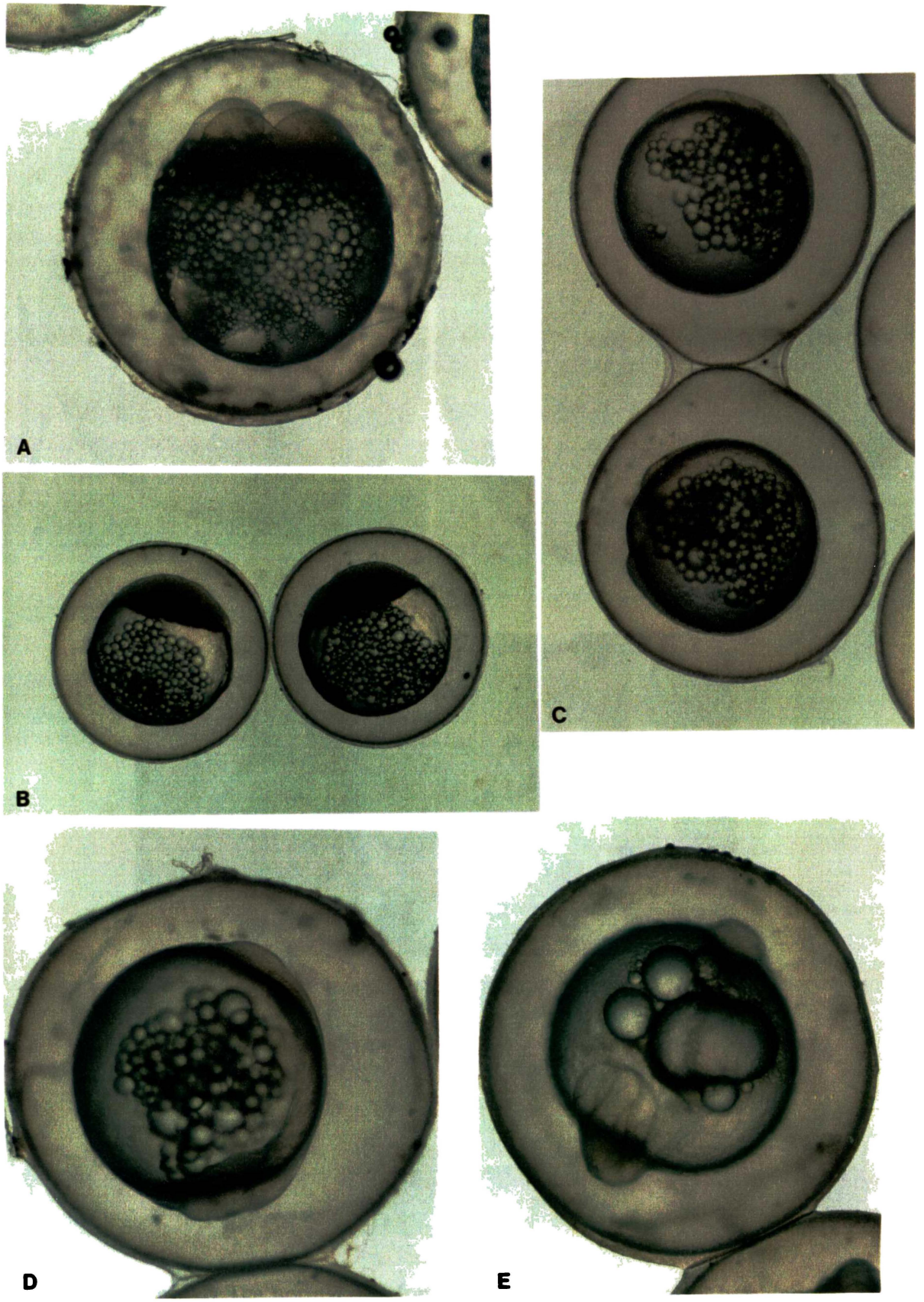


Figure 5.3 The normal development of koaro at 13°C until hatching. (A) 5.5 h (B) 29 h (C) 54 h (D) 3 days and (E) 5 days. The diameters of the eggs are between 2.0 and 3.0 mm.

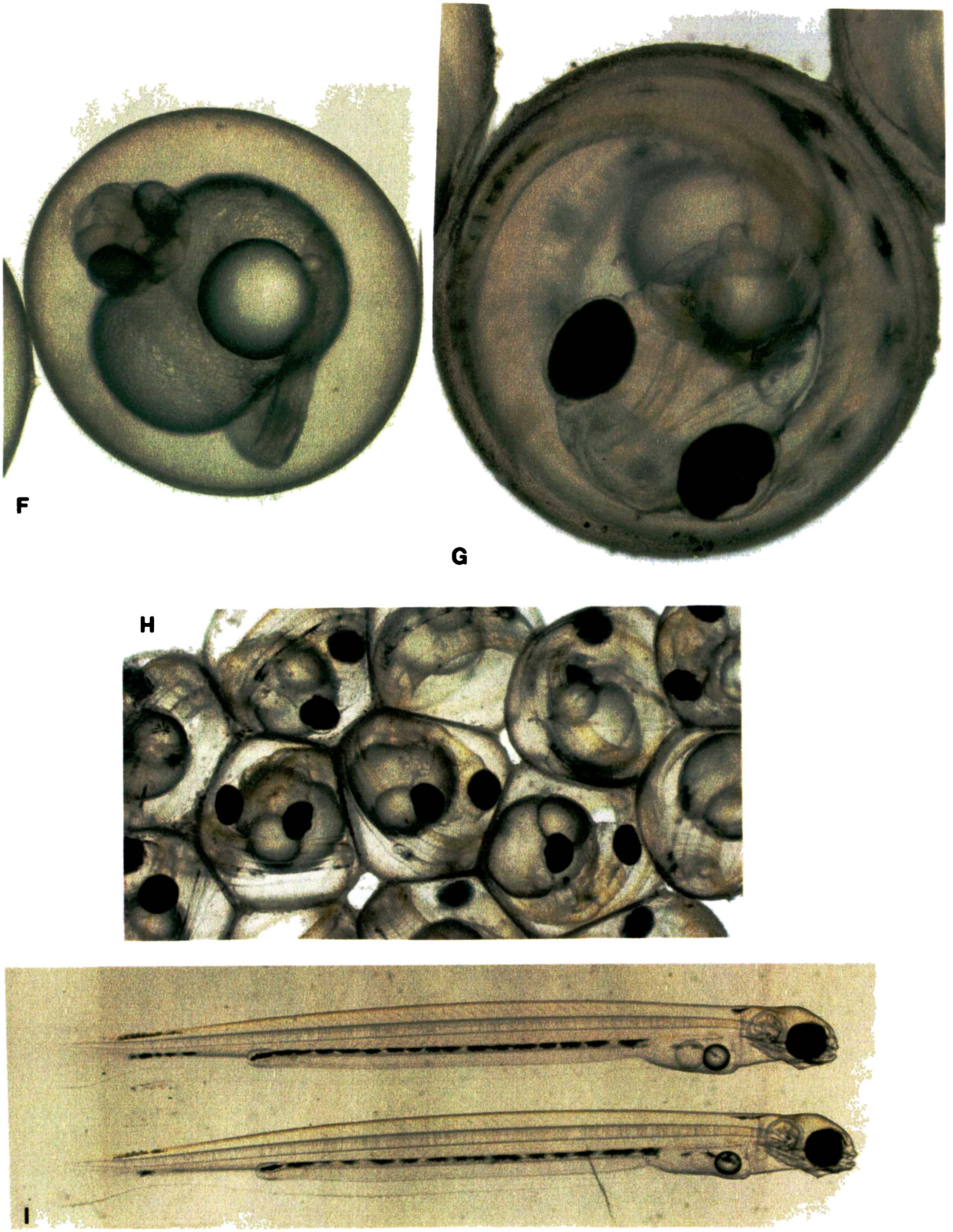


Figure 5.3 (continued) The normal development of koaro at 13°C until hatching. (F) 8 days (G) 20 days (H) 21 days and (I) hatched larvae. The diameters of the eggs are between 2.0 and 3.0 mm and the larvae are 8.1 and 8.3 mm long.

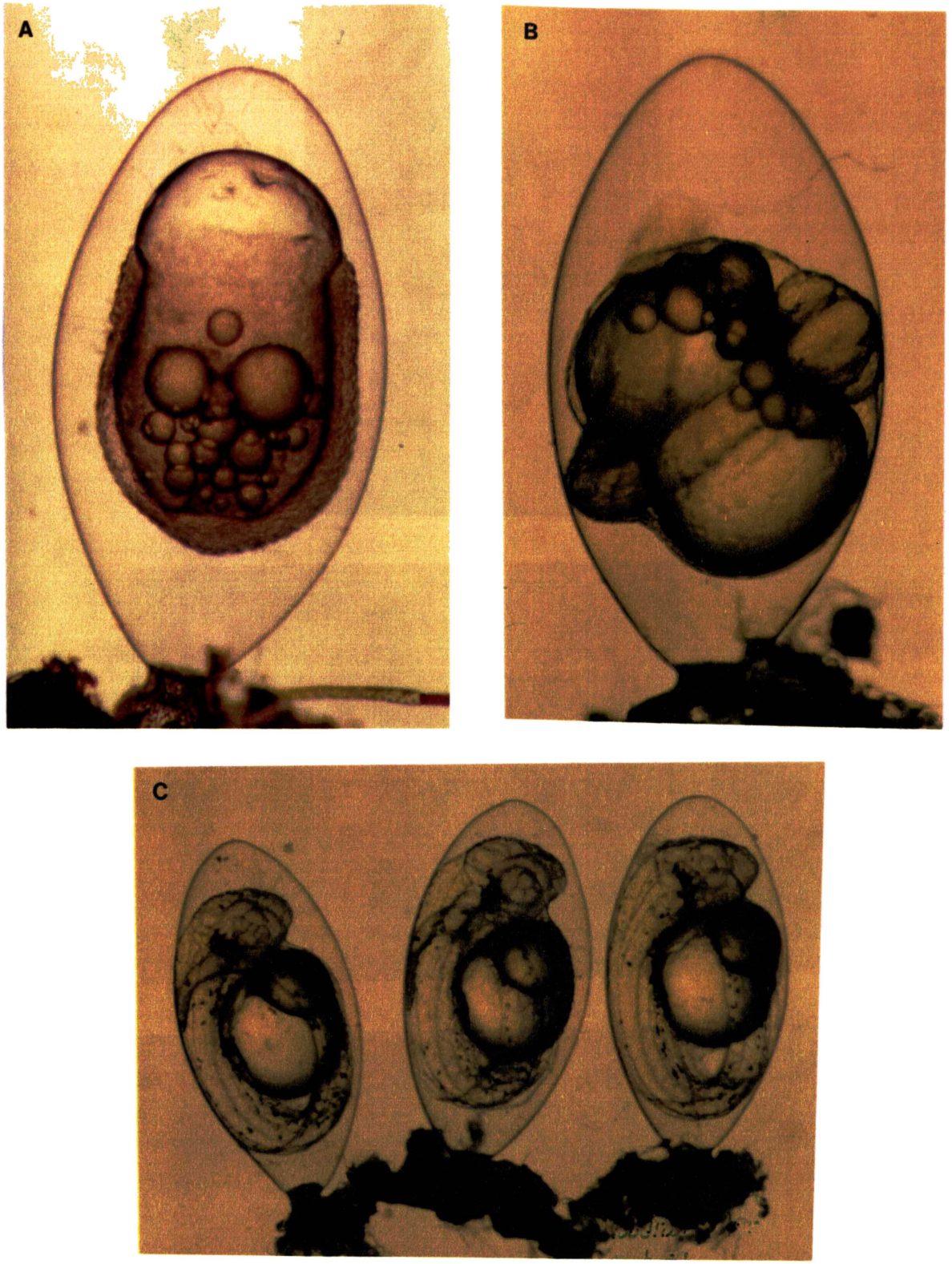


Figure 5.4 The normal development of common bully at 18°C until hatching. (A) epiboly (B) free tailbud and (C) early eyed stage. The eggs are 1.5 - 1.7 mm from base to top.

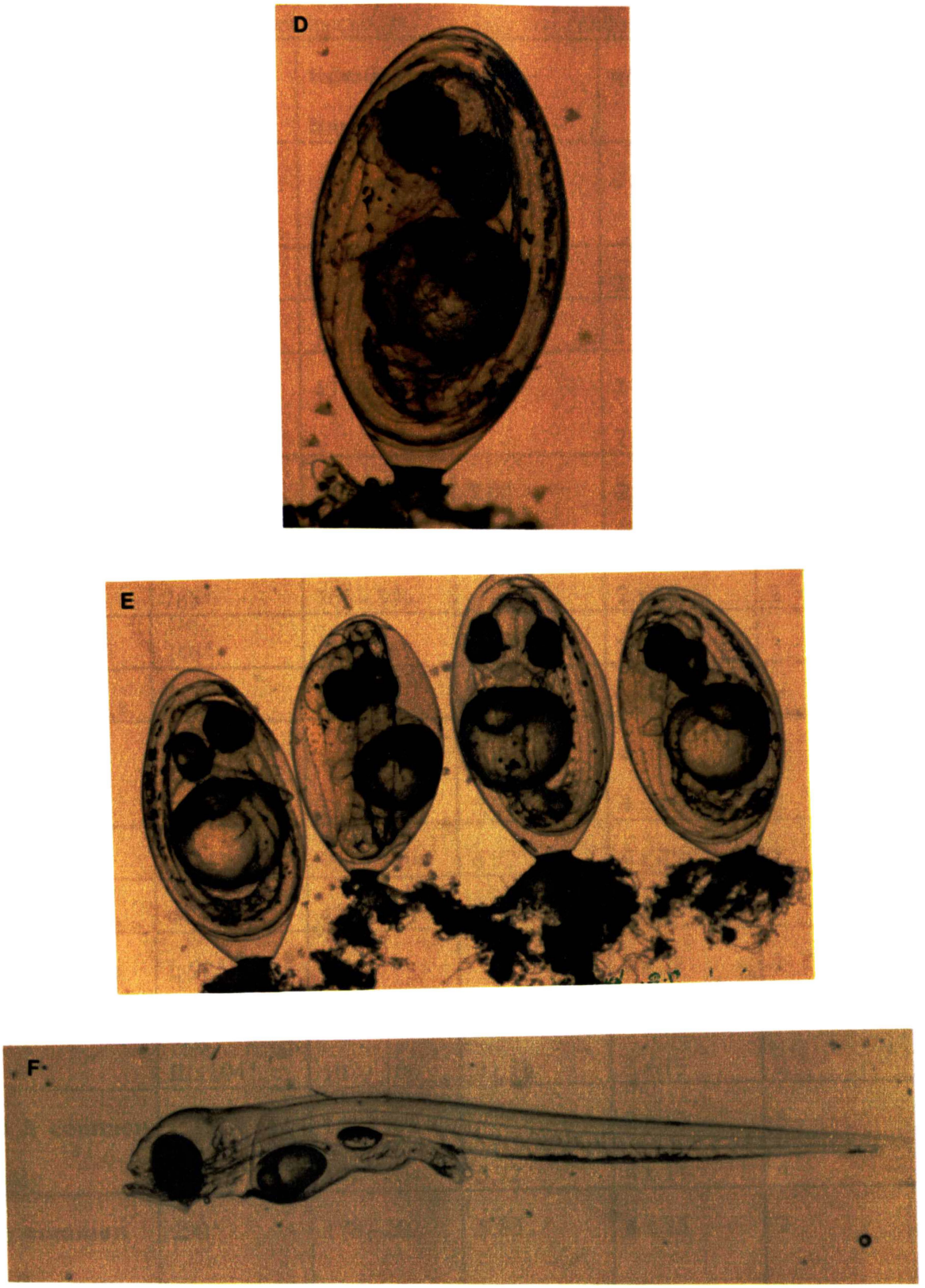


Figure 5.4 (continued) The normal development of common bully at 18°C until hatching. (D) and (E) late eyed stage and (F) newly hatched larva. The eggs are 1.5 - 1.7 mm from base to top, and the larva is 3.1 mm long (standard length).

Table 5.3 48 h LC50 (µg/L), 95% fiducial limits and slopes of the probit regression lines for the five tested species at the four different life stages. Also shown are the number of eggs or larvae tested per concentration and the origin of the eggs. I, II and III denote different tests when more than one test per species and life stage was conducted.

Species/stage	LC50	fiducial limits	slope	n/conc.	batch
24 h smelt	not determinable			2 x 50	1
e.e. smelt	389[a]	371 - 407	7.24	2 x 50	3
l.e. smelt	147[a]	135 - 159	8.14	2 x 31	3
larval smelt	I: 49[a]	45 - 53	7.77	2 x 21	1
	II: 31[c]			2 x 17	2
24 h inanga	I: 1034[a]	981 - 1090	7.64	2 x 48	3
	II: 918[a]	894 - 942	25.14	2 x 15	4
e.e. inanga	785[b]	765 - 806		2 x 50	2
l.e. inanga	246[a]	234 - 257	10.99	2 x 60	3
larval inanga	30[b]	28 - 32		2 x 10	4
24 h koaro	I: 155[a]	145 - 166	6.86	4 x 25	1
	II: 142[a]	131 - 153	5.37	4 x 26	2
e.e. koaro	1043[a]	994 - 1094	6.72	4 x 25	2
l.e. koaro	442[a]	423 - 463	6.67	4 x 25	2
larval koaro	I: 92[c]			2 x 22	1
	II:78[a]	76 - 79	25.61	2 x 25	1
	III: 104[a]	102 - 106	41.18	2 x 15	2
24 h common bully	I: 167[b]	145 - 192		2 x 15	3
	II: 191[a]	179 - 204	5.72	4 x 14	4
e.e.common bully	190[a]	178 - 202	5.83	4 x 25	2

Table 5.3 continued 48 h LC50 (µg/L), 95% fiducial limits and slopes of the probit regression lines for the five tested species at the four different life stages. Also shown are the number of eggs or larvae tested per concentration and the origin of the eggs. I, II and III denote different tests when more than one test per species and life stage was conducted.

l.e. common bully	180[a]	170 - 190	9.14	4 x 25	1
larval common bully	92[b]	87 - 98		2 x 25	1
24 h rainbow trout	663[b]	642 686		10 x 10	1
e.e. rainbow trout	910[a]	849 - 975	6.86	10 x 10	1
l.e. rainbow trout	423[a]	388 - 462	4.97	10 x 10	1
larval rainbow trout	77[a]	71 - 83	6.08	10 x 8	1

a = LC50 determined by the probit method; b = Spearman-Kärber method; c = graphical method
e.e. = early eyed; l.e. = late eyed

The common bully and the smelt had, on average, lower LC50 values than the other three species, but the differences were not statistically significant. The variability between species (coefficient of variation (CV) = (SD*100)/$\bar{x}$) of the LC50 values was 81% for the 24 h stages, 54% for the early eyed stages, 48% of the late eyed stages, and 44% for the larval stages. Within-species variation between the four life stages was slightly higher: 93% for smelt, 87% for inanga, 101% for koaro, 29% for common bully and 68% for rainbow trout. The LC50 values for the four life stages of common bully did not vary as much as they did for the other species. The larval stages had the lowest LC50 values for all tested species. The lowest overall acute LC50 was 30 ppb PCP for inanga larvae, and the highest was 1043 ppb for early eyed koaro eggs. For larval koaro, three flow-through toxicity tests were performed, producing an average LC50 value of 91.33 ± 13.01 ppb, and the coefficient of variation was 14% for these tests. When duplicating tests for 24 h inanga, koaro and

common bully and larval smelt, the results were close to each other, with two 95% confidence intervals overlapping (24 h koaro and 24 h common bully).

5.5 Sublethal effects

The survivors of the toxicity tests (PCP exposures) were incubated in filtered, aerated and dechlorinated tap water until hatch or death in order to study the sublethal and lethal effects of the previous exposures to PCP. The sublethal effects that were measured included hatching and survival rates, growth measured as length at hatch, average day of hatch, swimming performance (of common bully and koaro), heart rates (of smelt) and occurrence of terata including cranial malformations.

5.5.1 Hatching and survival rates

The survival and hatching rates of all five tested species during the follow-up after each flow-through toxicity test are shown in Figures 5.5 - 5.10. Day zero in the figures denotes the day the acute toxicity tests were terminated and when incubation in clean water initiated. The number of eggs surviving the acute test is set at 100% for day zero.

During the first few days after PCP exposure there was, in general, substantial mortality of eggs that had been exposed to PCP concentrations close to their respective LC50 values. Even though hatching is considered a sensitive stage in fish development, no unusual mortality was seen during this period, except for rainbow trout eggs tested at the 24 h and early eyed stages. Visual inspection of the hatching curves suggests that the time of hatching may have been affected in some exposed batches of eggs. This will be discussed in detail in Section 5.5.3.

Table 5.4 lists the final LC50 for all species, where the final mortality includes the total number of deaths that occurred during both the acute toxicity tests and in the subsequent follow-up incubation periods. The final LC50 is also compared to the post-test LC50 (from

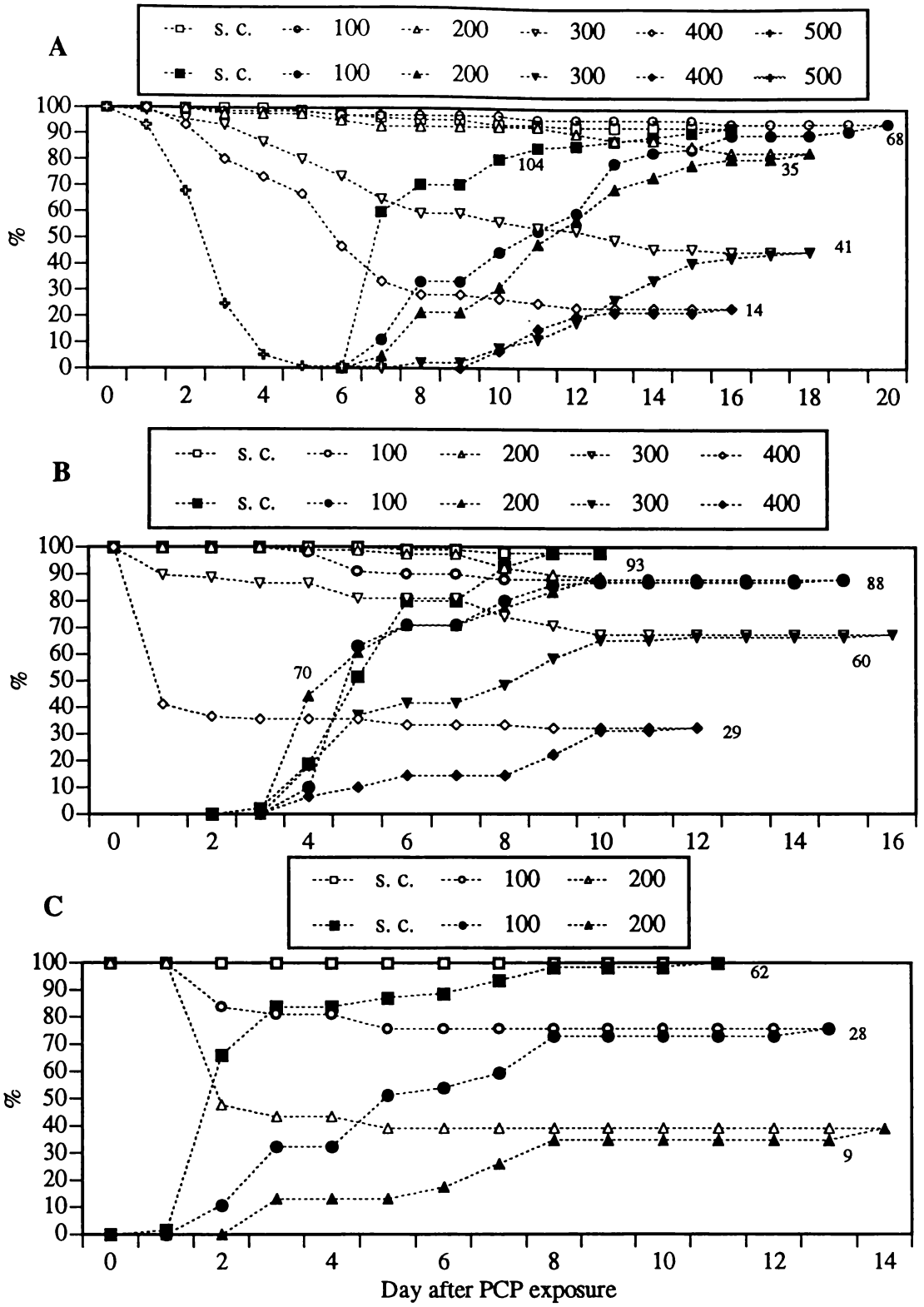


Figure 5.5 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of smelt eggs during the days following PCP exposure at the (A) 24 h, (B) early eyed and (C) late eyed stages at the different PCP concentrations (ppb) and the solvent controls (s.c) as shown in the legend. The total number of eggs surviving the exposures is set at 100% for day 0, which was the day of completion of the acute toxicity tests. The numbers of survivors at hatch are also shown in the graphs.

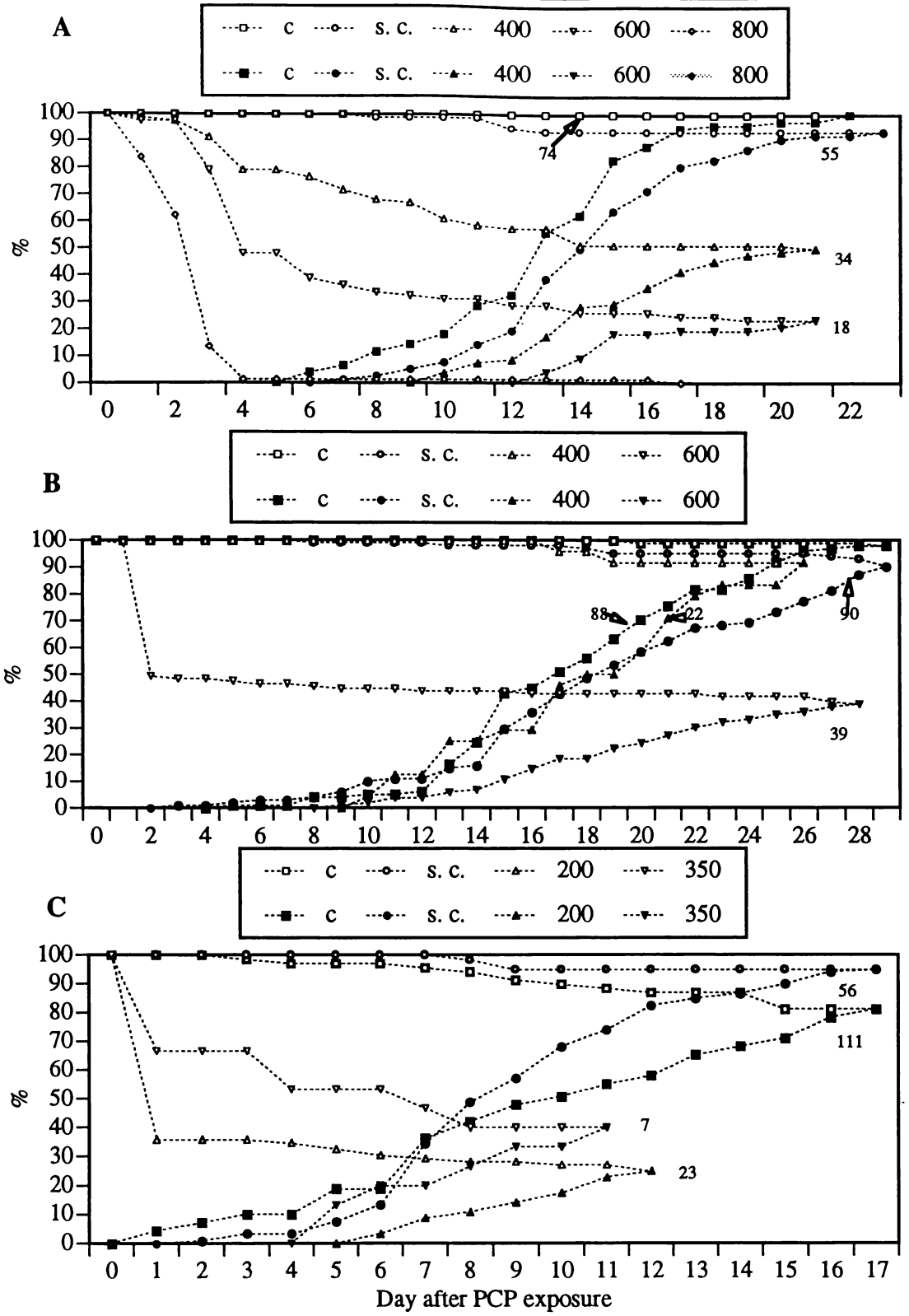


Figure 5.6 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of inanga eggs during the days following PCP exposure at the (A) 24 h, (B) early eyed and (C) late eyed stages at the different PCP concentrations (ppb) and the two controls (c = synthetic water control, s.c. = solvent control) as shown in the legend. The total number of eggs surviving the PCP exposures is set at 100% for day 0, which was the day of completion of the tests. The numbers of survivors at hatch are also shown in the graphs.

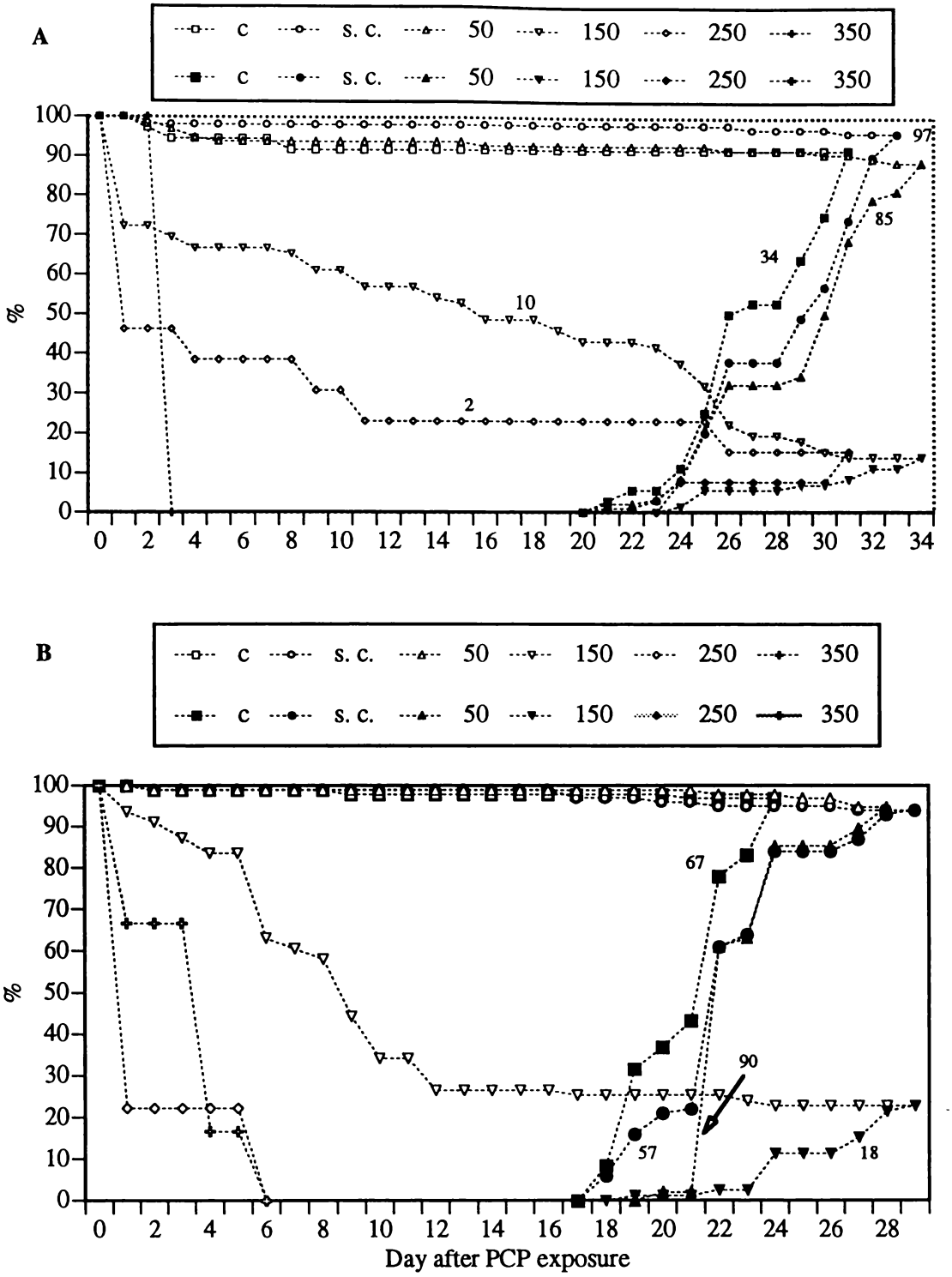


Figure 5.7 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of koaro eggs during the days following the completion of two tests at the 24 h stages (both A and B) at the PCP concentrations (ppb) and controls (c = synthetic water control and s.c. = solvent control) as shown in the legends. The total number of eggs surviving PCP exposure is set at 100% for day 0, which is the day of the completion of the acute toxicity tests. The numbers of survivors at hatch are also shown in the graphs.

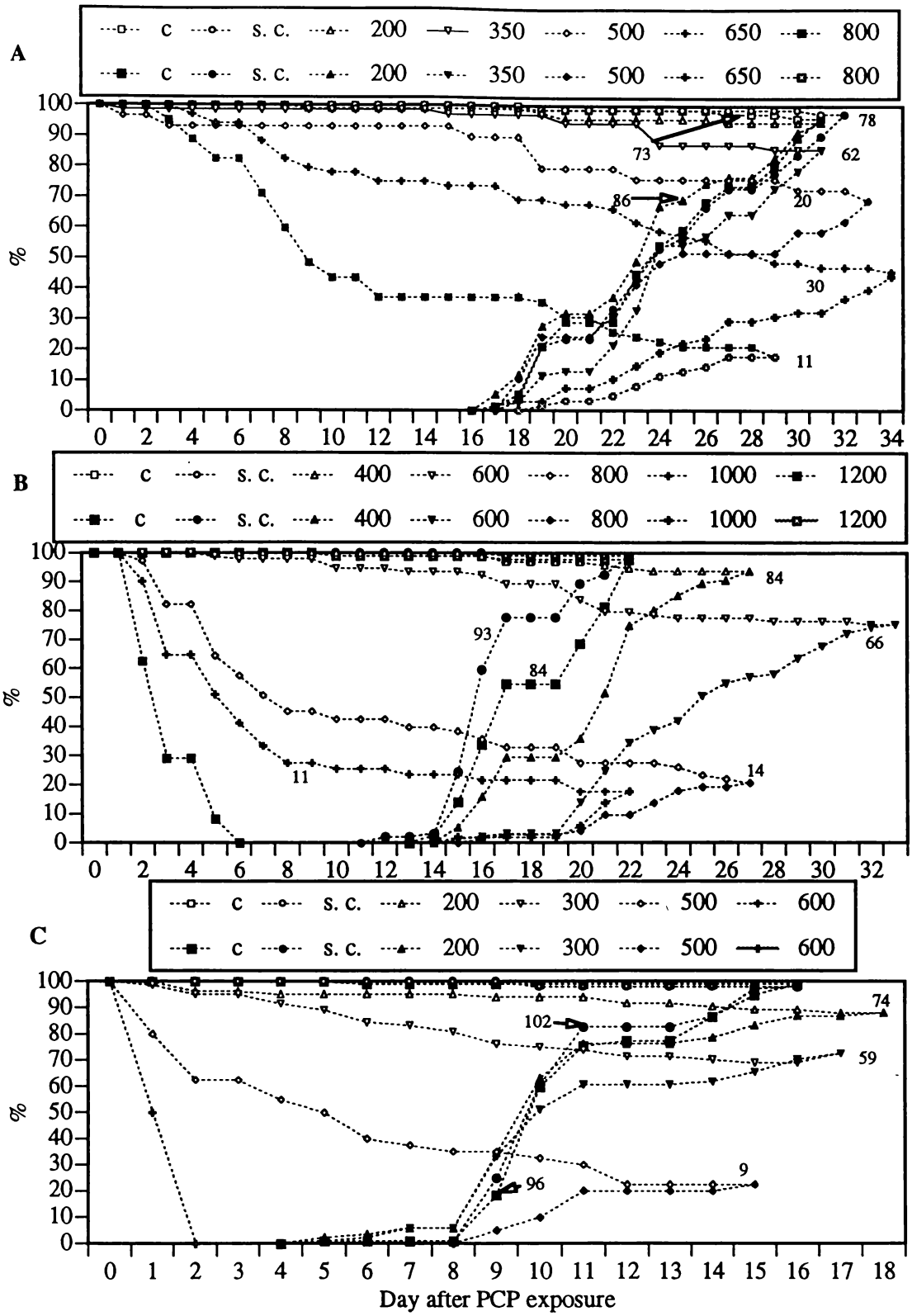


Figure 5.8 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of koaro eggs after completion of toxicity tests at the early eyed stages (A and B) and at the late eyed stage (C) at the PCP concentrations (ppb) and controls (c = synthetic water control and s.c. = solvent control) as shown in the legends. The number of survivors after PCP exposures is set at 100% for day 0, which was the day of the completion of the acute toxicity tests. The number of survivors at time of hatch is also shown in the graphs.

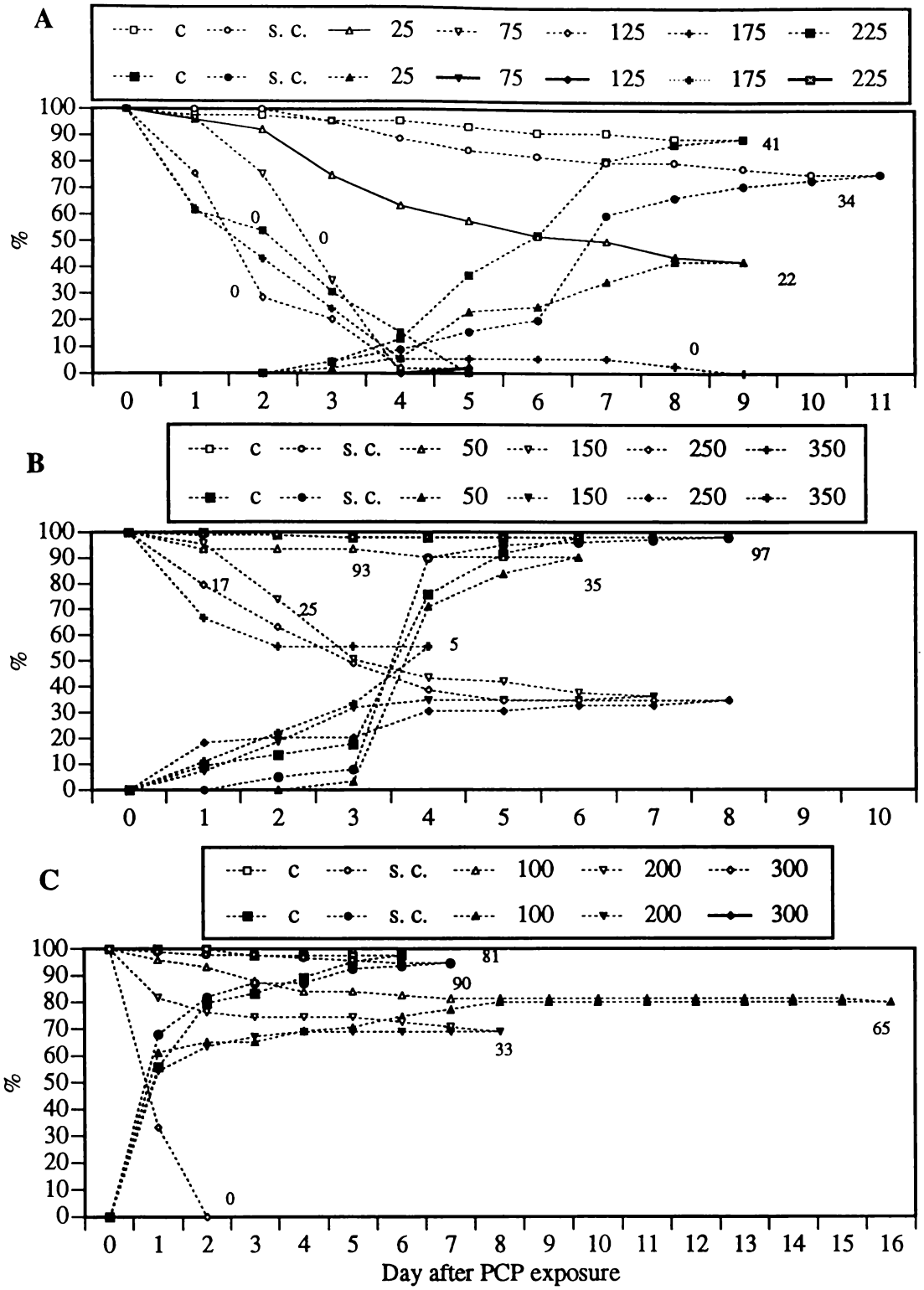


Figure 5.9 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of common bully eggs after completion of PCP exposure at the (A) 24 h, (B) early eyed and (C) late eyed stages at the PCP concentrations (ppb) and the controls (c = synthetic water control and s.c. = solvent control) as shown in the legends. The total number of eggs surviving PCP exposure is set at 100% for day 0, which is the day of completion of acute toxicity tests. The number of survivors at hatch is also shown in the graphs.

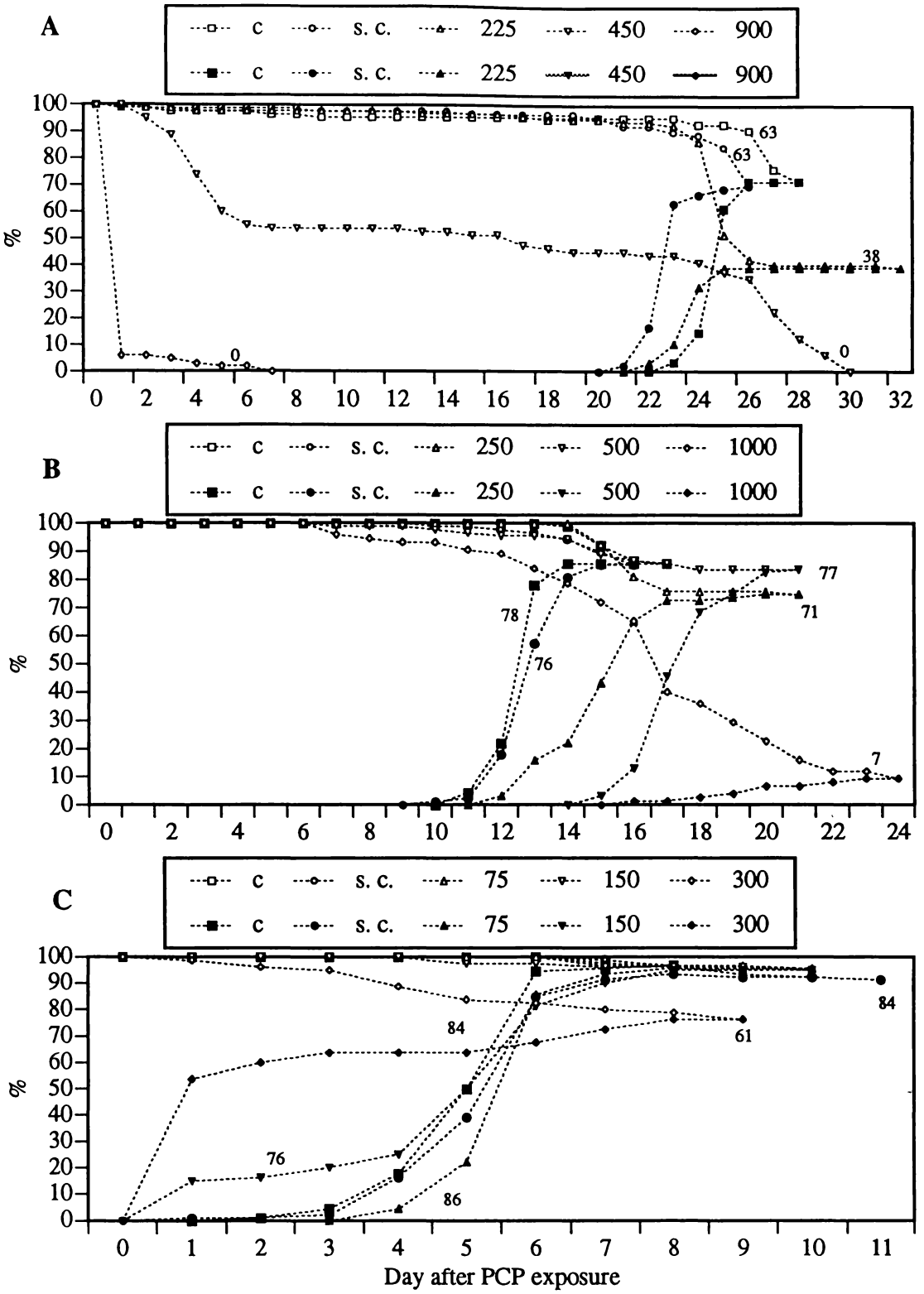


Figure 5.10 Percent survival (open symbols) and percent cumulative hatch (closed symbols) of rainbow trout eggs during the days following a toxicity test at the (A) 24 h, (B) early eyed and (C) late eyed stages at the different PCP concentrations (ppb) and the controls (c = synthetic water control and s.c. = solvent control) as shown in the legends. The total number of eggs surviving the PCP exposures is set at 100% for day 0, which is the day of completion of the tests. The number of survivors at time of hatch is also shown in the graphs.

Table 5.3) to give an indication of the delayed effect of PCP on survival. Common bully tested at the 24 h stage was the most severely affected with a post-test LC50/final LC50 of 11.6. Most of the ratios were, however, less than two.

Table 5.4 The final LC50 (µg/L), 95% fiducial limits and slopes of the probit regression lines after hatch for the five tested species at three life stages. The final LC50 values are also compared to the acute LC50 values obtained after the tests (post-test LC50/final LC50).

Species / stage	Final LC50	fiducial limits	slope	post-test LC50 /Final LC50
24 h smelt	139	131 - 148	5.76	-
e.e. smelt	233	220 - 248	5.73	1.7
l.e. smelt	122	113 - 133	8.35	1.2
24 h inanga	412	394 - 431	11.14	2.5
e.e. inanga	510	493 - 529	16.53	1.5
l.e. inanga	162	151 - 175	6.84	1.5
24 h koaro	80 86	73 - 89 78 - 94	6.76 6.96	1.9 1.6
e.e. koaro	622	598 - 647	9.99	1.7
l.e. koaro	292	278 - 307	8.96	1.5
24 h common bully	16	13 - 20	3.56	11.6
e.e. common bully	112	102 -123	4.47	1.7
l.e. common bully	138	130 - 147	8.32	1.3
24 h rainbow trout	161	144 - 179	5.74	4.1
e.e. rainbow trout	477	440 - 518	5.10	1.9
l.e. rainbow trout	215	199 - 232	5.82	2.0

e.e = early eyed; l.e. = late eyed

5.5.2 Lengths of hatched larvae

After exposing the five different species to PCP at the four early life stages, the lengths of the hatched larvae of all five tested species decreased in proportion to the toxicant concentration compared to the solvent controls with few exceptions (Figures 5.11 - 5.12).

The consistency of the response between batches of eggs tested at the same stage can also be compared for some tests. Two batches of bully tested at the completed epiboly stage exhibited a fairly similar response to PCP in their growth (Figure 5.12A). Two batches of inanga eggs tested at the early eyed stages were, however, quite different in their growth rates (Figure 5.11B), as well as two batches of koaro tested at the 24 h and early eyed stages (Figure 5.11C).

Rainbow trout that had been tested at the late eyed stage showed a decrease in length at 75 ppb, but not at 150 and 300 ppb where they were of similar size to those in the control group (Figure 5.12B). Smelt larvae were essentially unaffected by PCP concentrations up to 75 ppb (Figure 5.11A), but trout larvae were affected at 50 and 75 ppb PCP compared to the solvent control (Figure 5.12B).

5.5.3 Average and median time-to-hatch

The average and median time-to-hatch (± SE) is shown in Figures 5.13 - 5.14, for each species and at each PCP concentration. The control values in the figures are for only the ethanol control eggs.

Smelt eggs hatched significantly later than controls when exposed to PCP at the 24 h and late eyed stages, whereas the response was mixed for the early eyed stage, with a significant decrease in time-to-hatch at 200 ppb and a significant increase in time-to-hatch at 400 ppb (Figure 5.13A).

Inanga eggs did not respond to PCP with respect to their time-to-hatch. It is, however, interesting to note that eggs that had undergone an acute toxicity test at the 24 h stage hatched

significantly earlier than when they were exposed at the early and late eyed stages. The eggs tested at the 24 h and the late eyed stages were from the same egg batch (Figure 5.13B)

One batch of koaro eggs exposed at the 24 h and the early eyed stages showed a significant delay in their time-to-hatch. In contrast to these results, another batch exposed to similar PCP concentrations showed no response at any of the embryonic stages (Figure 5.13C)

Common bully had a tendency to hatch earlier when exposed to PCP at all embryonic stages, and the difference was significant at 150 and 250 ppb (p <0.01) for the early eyed stage, and at 100 and 200 ppb (p <0.001) at the late eyed stage (Figure 5.14A).

Rainbow trout eggs had a significantly delayed time-to-hatch when exposed to 225 ppb at the 24 h stage, and to 250, 500 and 1000 ppb at the early eyed stage. When exposed at the late eyed stage the time-to-hatch was significantly earlier at 300 ppb compared to the ethanol control (Figure 5.14B).

The synthetic water control was significantly different from the solvent control for a 24 h koaro test and a 24 h inanga test (significantly delayed hatching in the solvent control), and a 24 h rainbow trout test (significantly earlier than the solvent control). In all other tests the two controls were statistically the same.

It is also interesting to note that hatching took place for rainbow trout eggs within a more narrow time span than for the other species. The average coefficient of variation of time-to-hatch (CV = (SD*100)/$\bar{x}$) was 3.1% for rainbow trout, 15.7% for common bully, 16.4% for smelt, 18.8% for inanga and 9.1% for koaro in the solvent and synthetic water controls.

The CV for time-to-hatch was not significantly altered in PCP-exposed eggs compared to solvent control eggs, except for a significant increase in CV for rainbow trout eggs tested at the late eyed stage (p = 0.026) and when koaro had been tested at the 24 h stage (p = 0.002), as determined by regression analysis for both species.

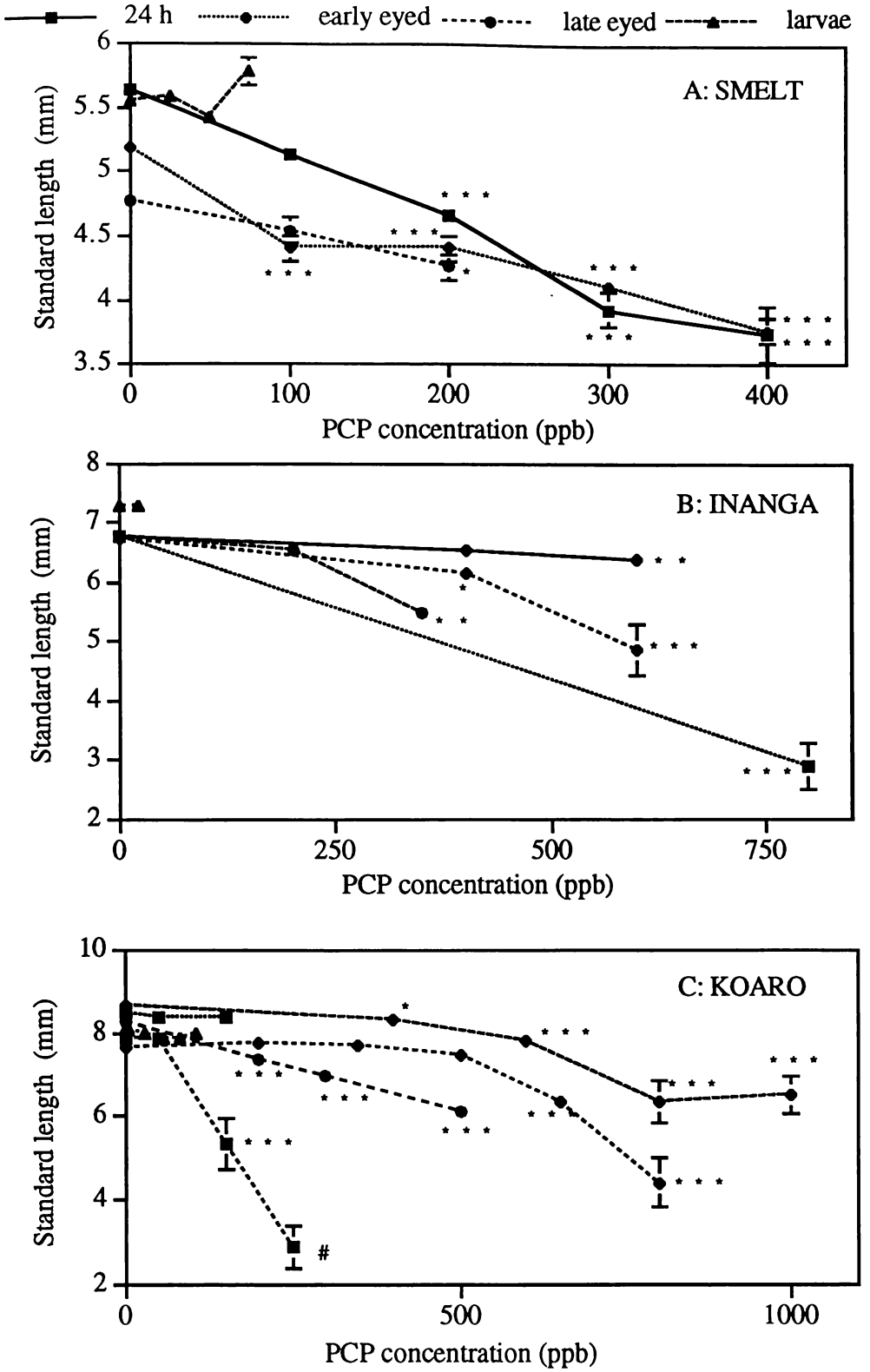


Figure 5.11 Standard lengths of post-hatch individuals (average ± SE) of (A) smelt, (B) inanga and (C) koaro when exposed to PCP at the concentrations shown on the x-axes at the 24 h, early eyed and late eyed stages as shown in the legend. For larval tests, the lengths were measured 2 days post-test. Note that some tests were duplicated. Significant differences are indicated as ***(p <0.001), **(p <0.01) and *(p <0.05). # n=2

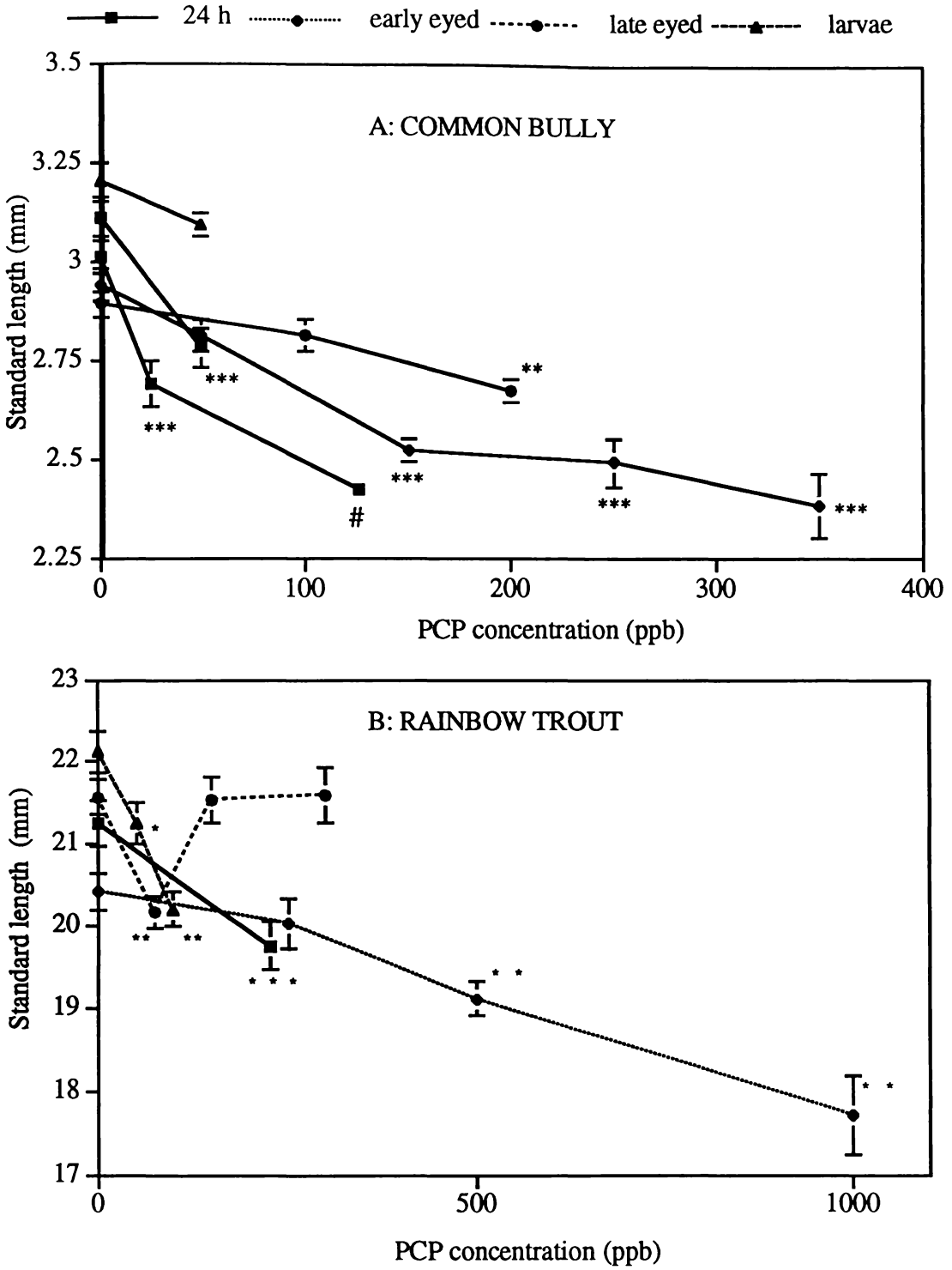


Figure 5.12 Standard lengths (average mm ± SE) of (A) common bully and (B) rainbow trout when exposed to PCP at the concentrations shown on the x-axes at the 24 h, early eyed and late eyed stages as shown in the legend. For larval tests, the lengths were measured 2 days post-test. Note that some tests were duplicated. Significant differences are indicated as ***(p <0.001), **(p <0.01) and *(p <0.05). # n=1

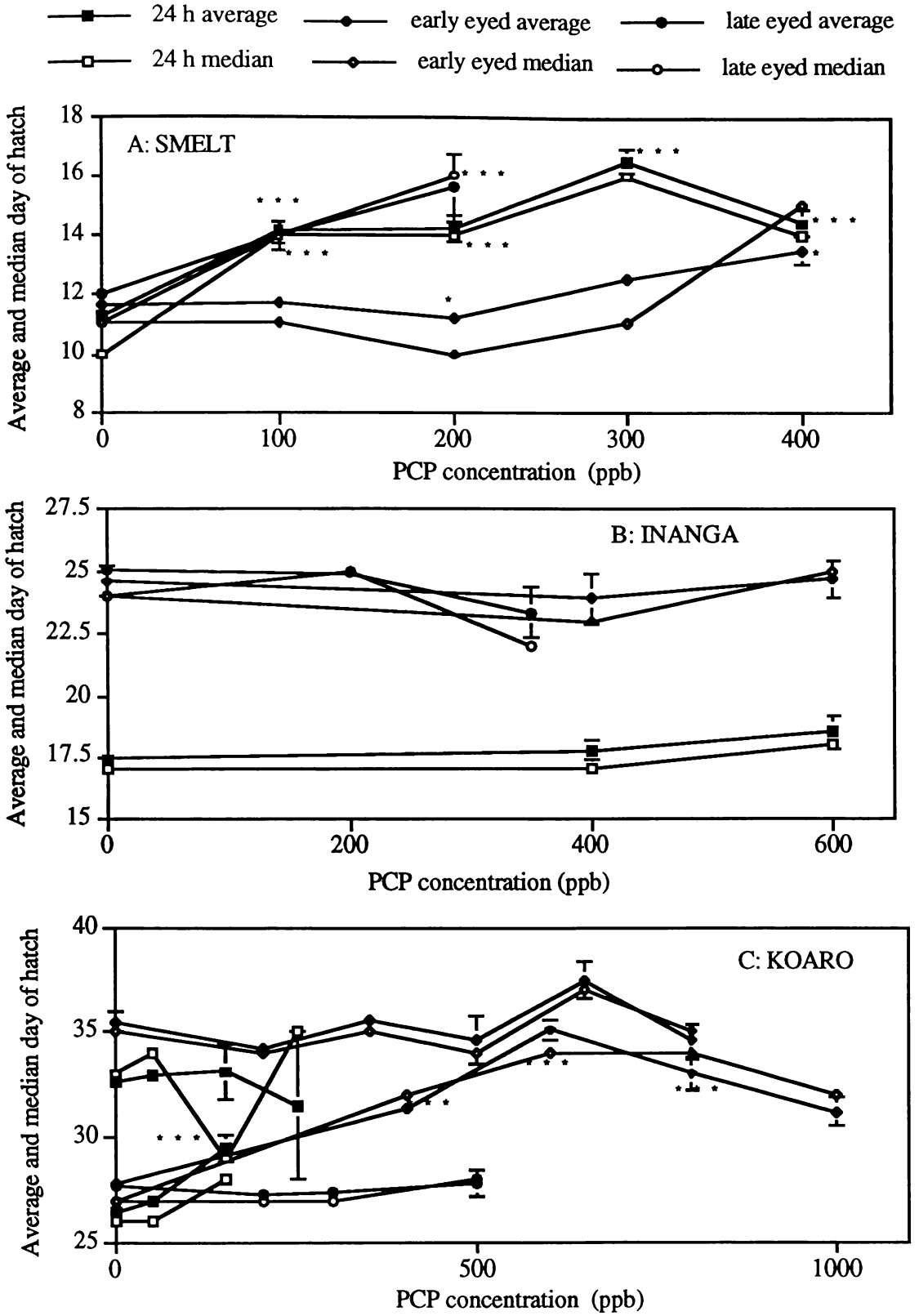


Figure 5.13 Average (filled symbols) and median day (open symbols) of hatch for (A) smelt, (B) inanga and (C) koaro when tested at the 24 h, early eyed and late eyed stages as shown in the legend above the graph. Standard errors are given for average but not for median day of hatch. Significant differences in comparison to the solvent controls are indicated in the graphs as ***(p <0.001) and *(p <0.05), for the average values only.

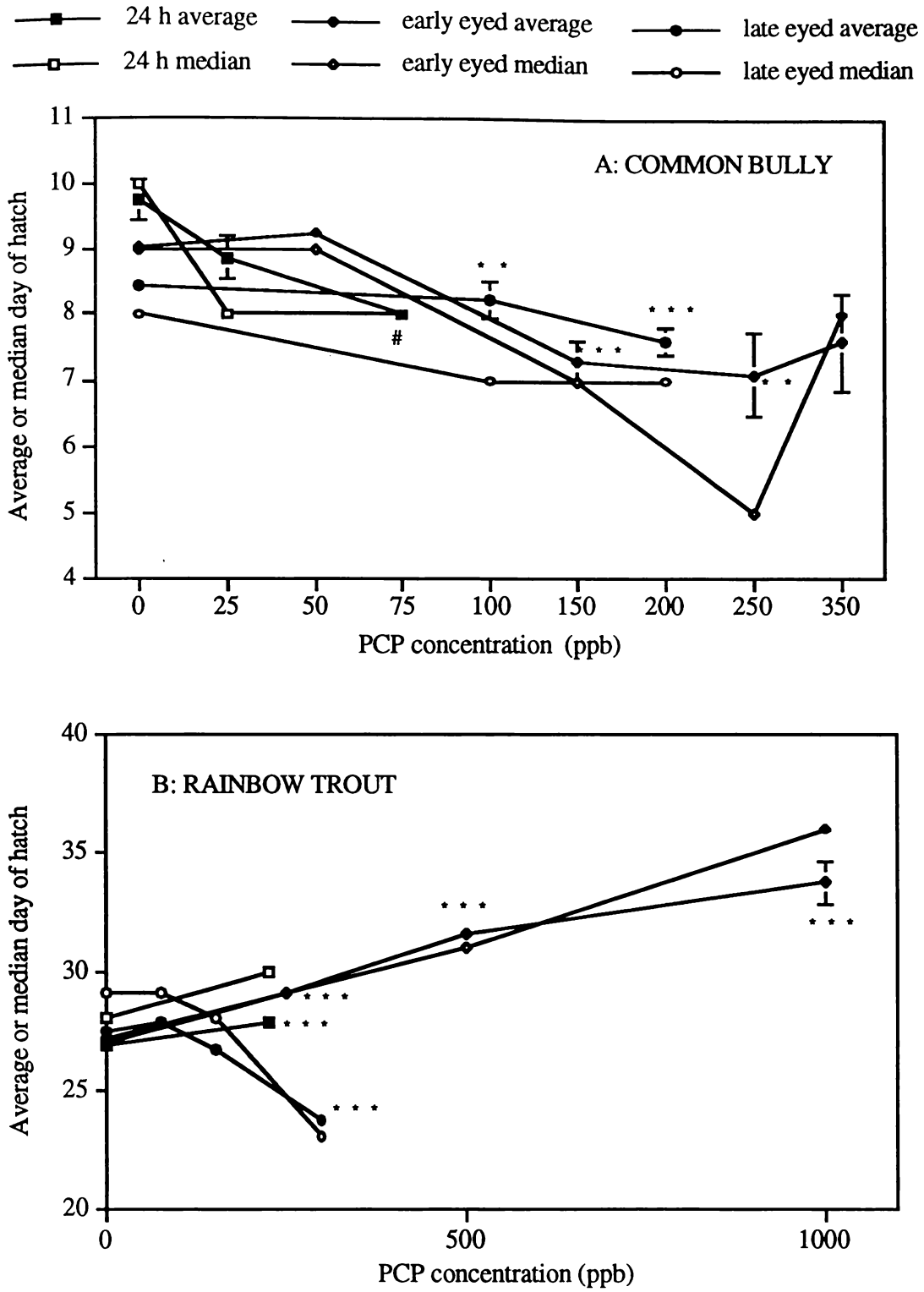


Figure 5.14 Average (filled symbols) and median day (open symbols) of hatch for (A) common bully and (B) rainbow trout when tested at the 24 h, early eyed and late eyed stages as shown in the legend above the graph. Standard errors are given for average but not for median day of hatch. Significant differences are indicated in the graphs as ***(p <0.001) and **(p <0.01), for average values only. # n=1

5.5.4 Teratology associated with PCP exposure

The percentage of hatched larvae that were abnormal can be seen in Figures 5.15 - 5.20 for the five tested species. The numbers of abnormalities are shown in Appendix 2. The prevalence of abnormal larvae increased in proportion to the PCP concentration with few exceptions and for all life stages tested. One of the most common deformities was axial abnormalities. This type of abnormality made up the majority of the total number of terata for late eyed rainbow trout, late eyed bully, and for all stages of smelt.

Generally the terata were more numerous, diverse and severe when eggs were exposed to PCP at the 24 h stages. For instance, cranial and eye deformities were largely absent when testing eggs at the late eyed stages.

It is interesting to note the difference in response for two batches of koaro eggs exposed to PCP at the 24 h stage (Figure 5.17A and B). Eighty percent of hatched larvae from batch 1 were malformed when exposed to 150 ppb PCP, whereas the same concentration produced no malformations in batch 2 eggs. The same two batches tested at the early eyed stage were fairly similar in their responses (Figure 5.18A and B).

Some of the final malformation data was amenable to calculation of effective concentration (EC50) values, and from this analysis a teratogenicity index (TI) could be calculated (TI = LC50/EC50). The TI has been developed for use in conjunction with the Frog Embryo Teratogenesis Assay - Xenopus, where both acute mortality and teratogenicity are assessed at the end of a 96 h testing period (Bantle et al. 1989). Here the approach for calculation of the TI was different. For the final LC50 used for the calculation of the TI, the total number of eggs dying during the tests and during the following incubation period until hatch was used. The EC50 values and the TI values are listed in Table 5.5. The highest TI was 1.39 for common bully tested at the early eyed stage, and the lowest TI was 0.53 for inanga tested at the early eyed stage. The correlation between EC50 and LC50 was significant (r = 0.8674, p= 0.0003).

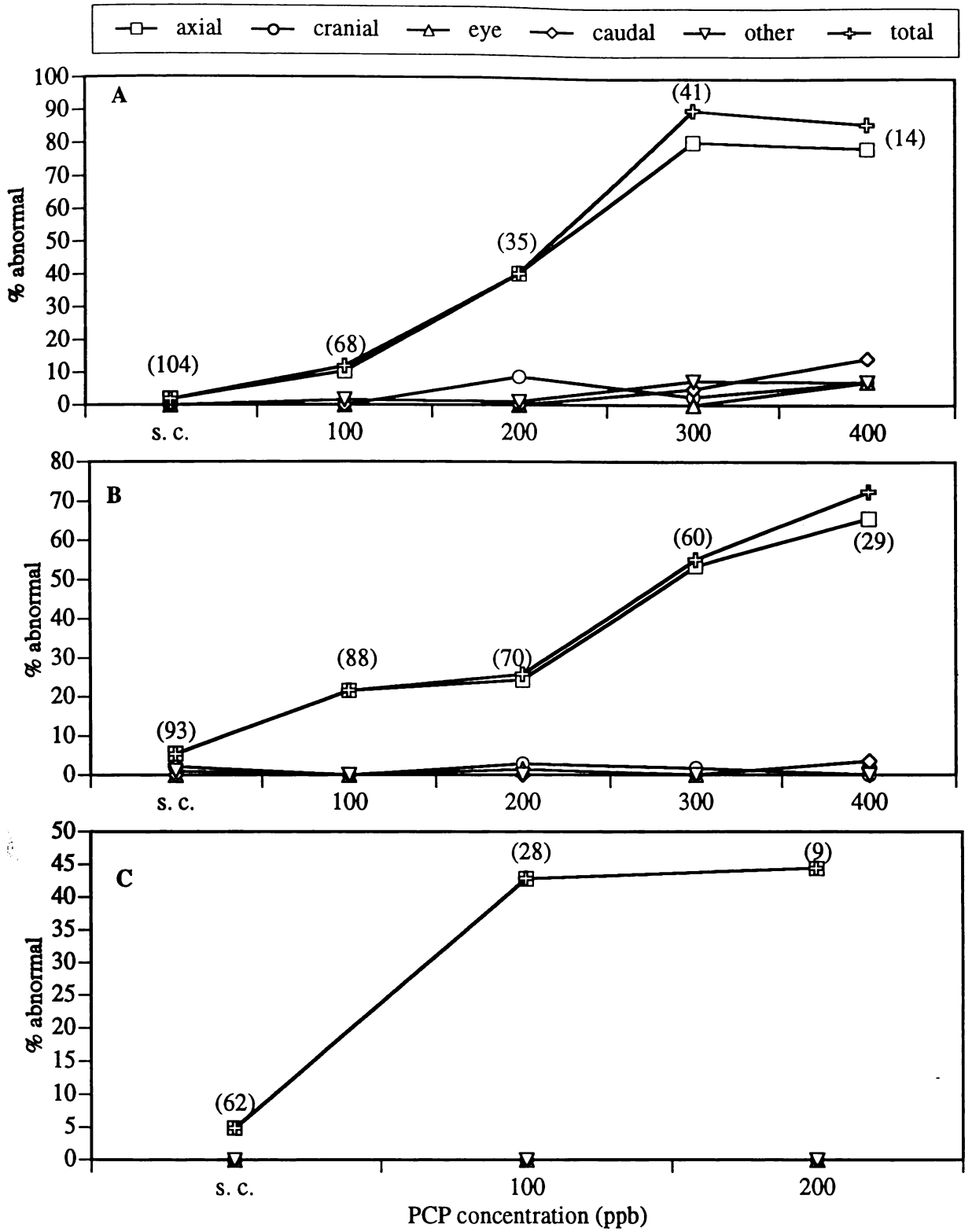


Figure 5.15 Percent abnormal smelt larvae when the eggs were exposed to PCP (ppb) at the 24 h (A), early eyed (B) and late eyed (C) stages compared to the solvent control. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. s.c. = solvent control.

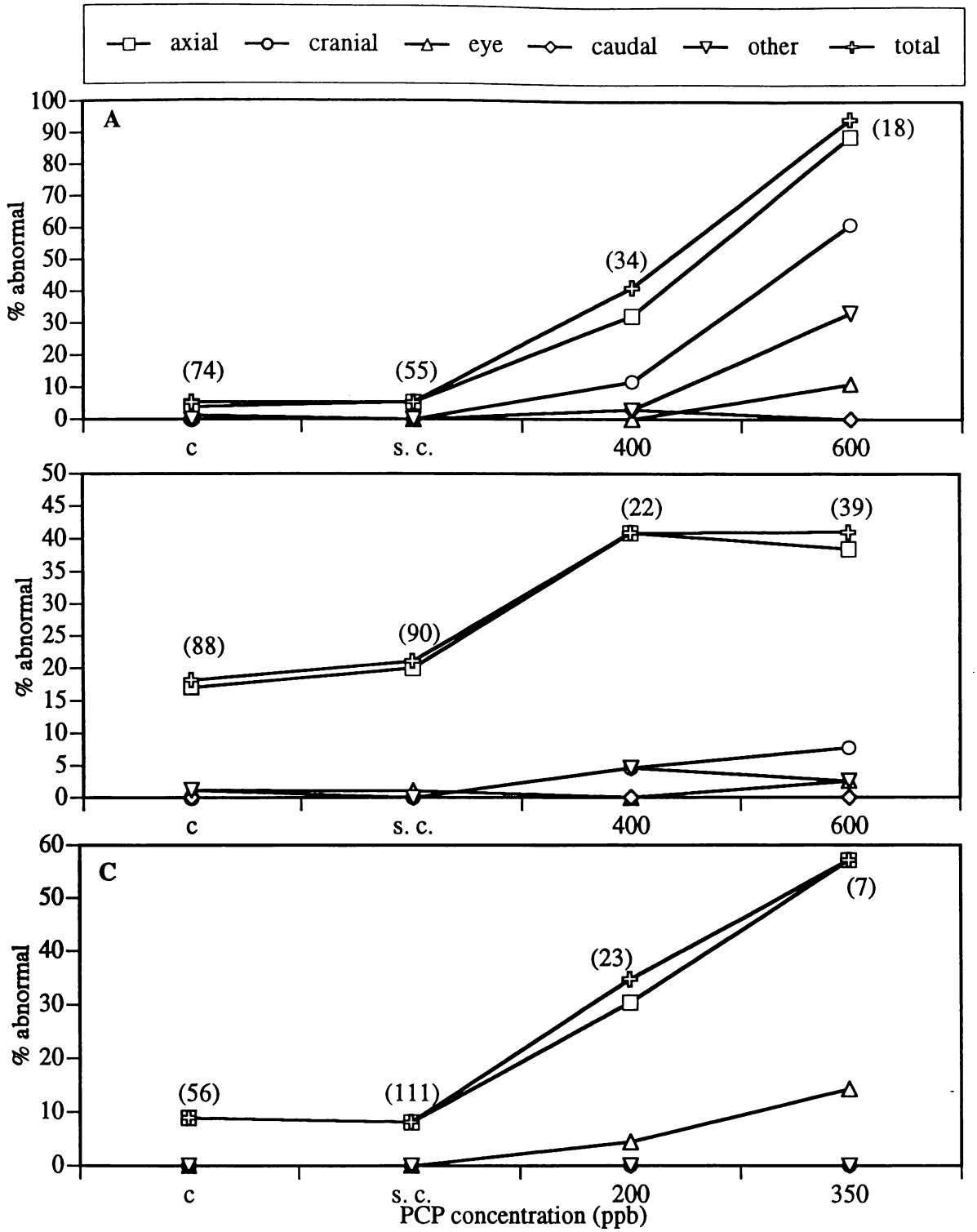


Figure 5.16 Percent abnormal inanga larvae when the eggs were exposed to PCP (ppb) at the 24 h (A), early eyed (B) and late eyed (C) stages compared to the controls. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. c = synthetic water control; s.c. = solvent control.

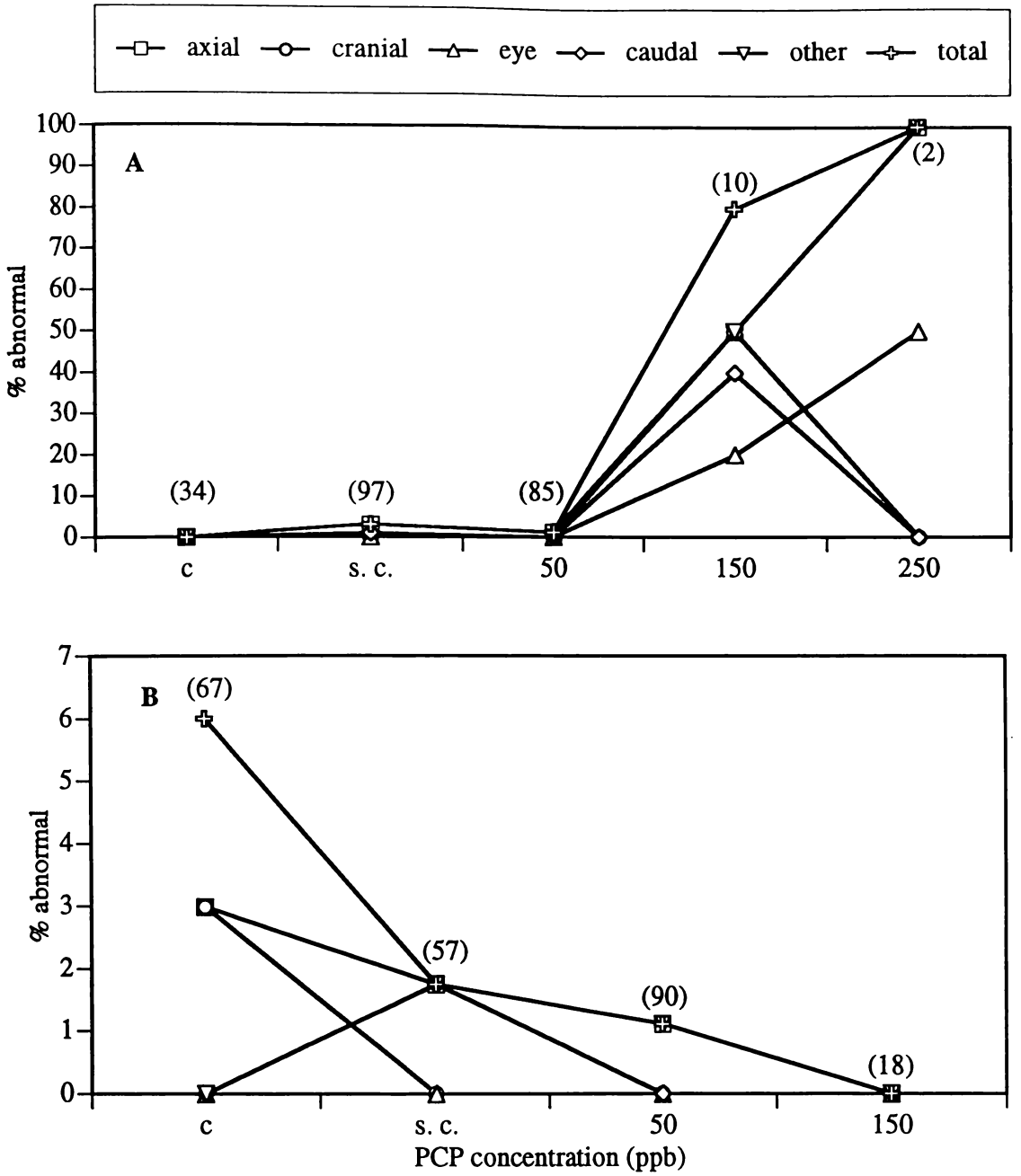


Figure 5.17 Percent abnormal koaro larvae when the eggs were exposed to PCP (ppb) at the 24 h stage (A and B) compared to the controls. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. c = synthetic water control; s.c. = solvent control.

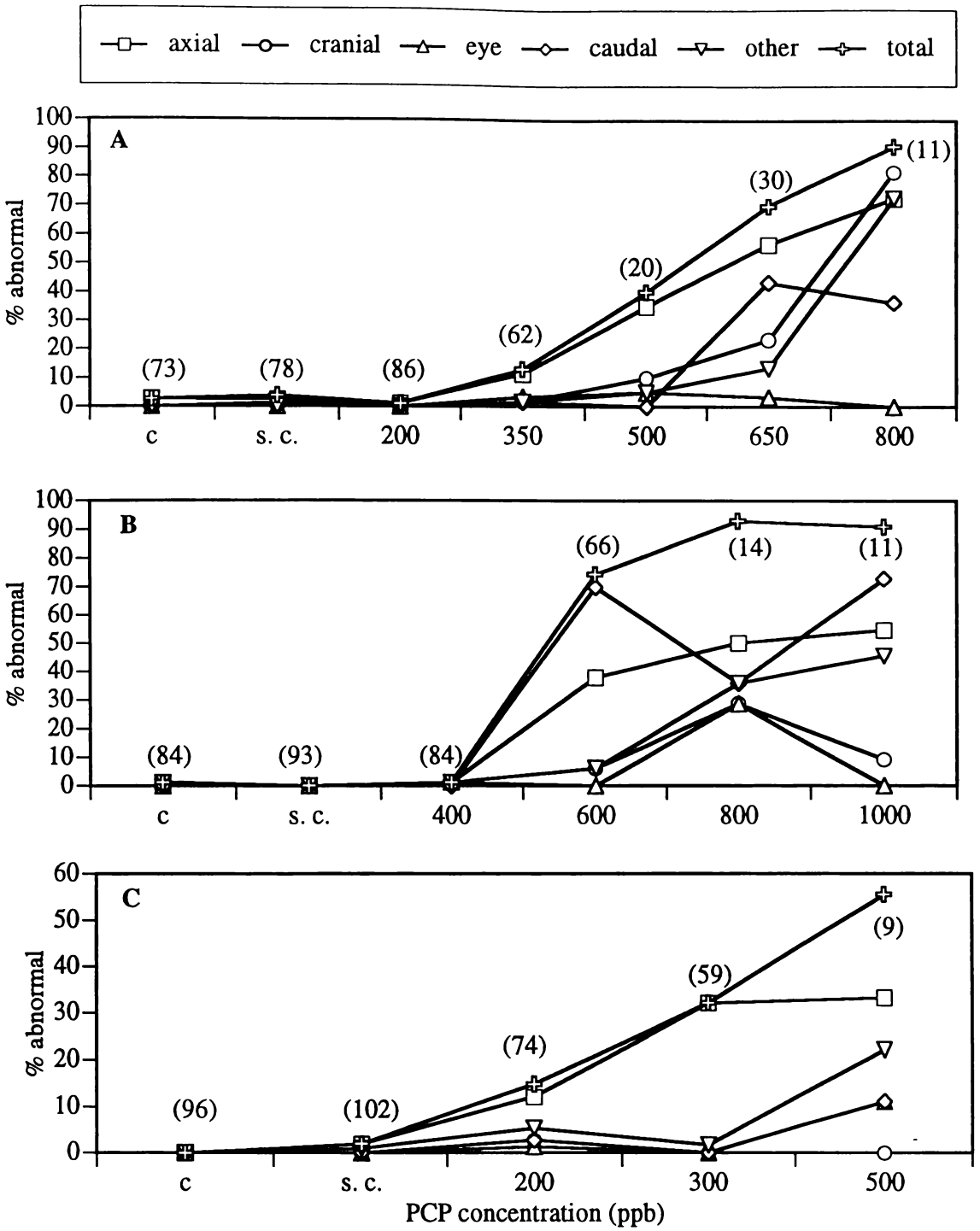


Figure 5.18 Percent abnormal koaro larvae when the eggs were exposed to PCP (ppb) at the early eyed (A and B) and late eyed (C) stages compared to the controls. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. c = synthetic water control; s.c. = solvent control.

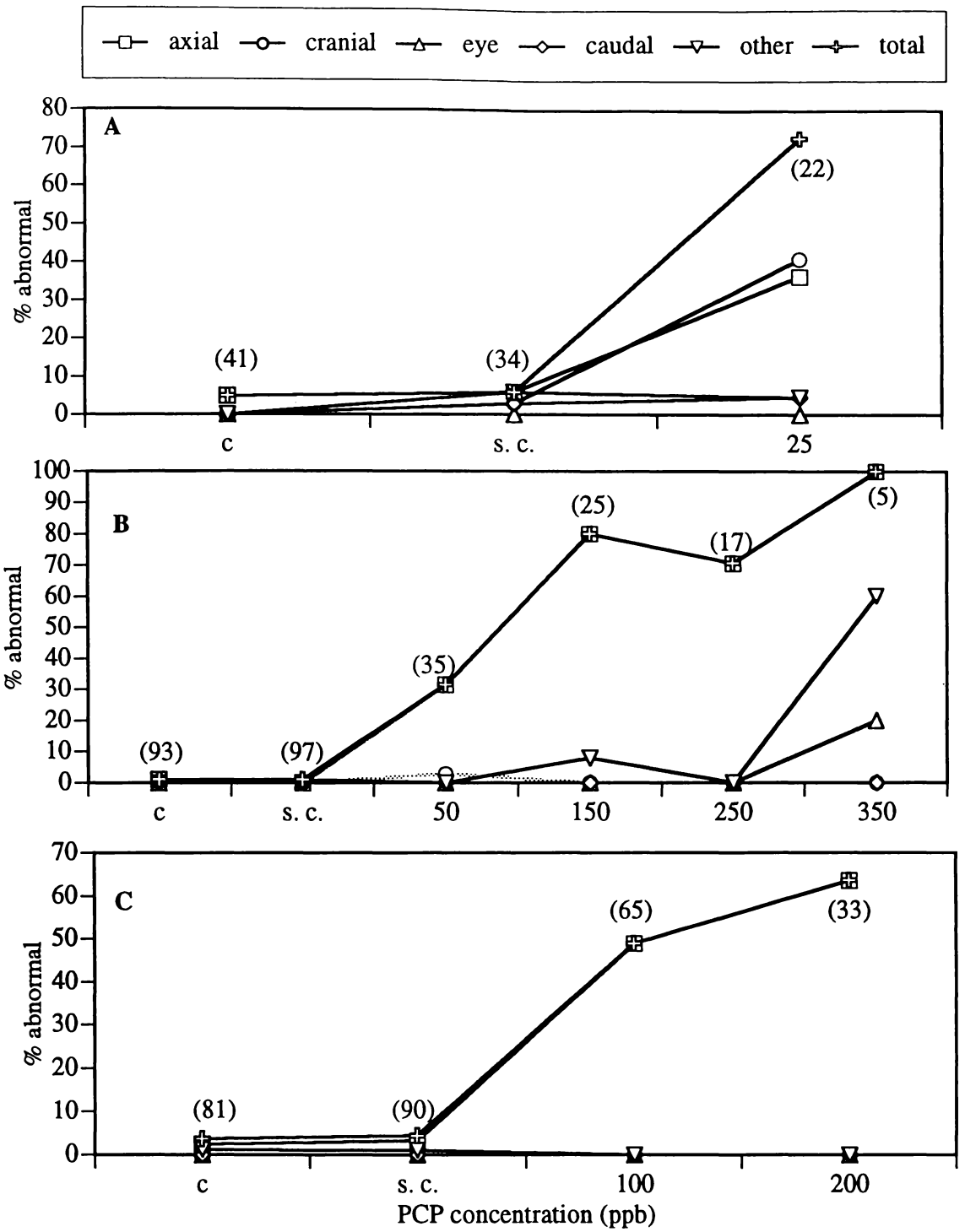


Figure 5.19 Percent abnormal common bully larvae when the eggs were exposed to PCP (ppb) at the 24 h (A), early eyed (B) and late eyed (C) stages compared to the controls. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. c = synthetic water control; s.c. = solvent control.

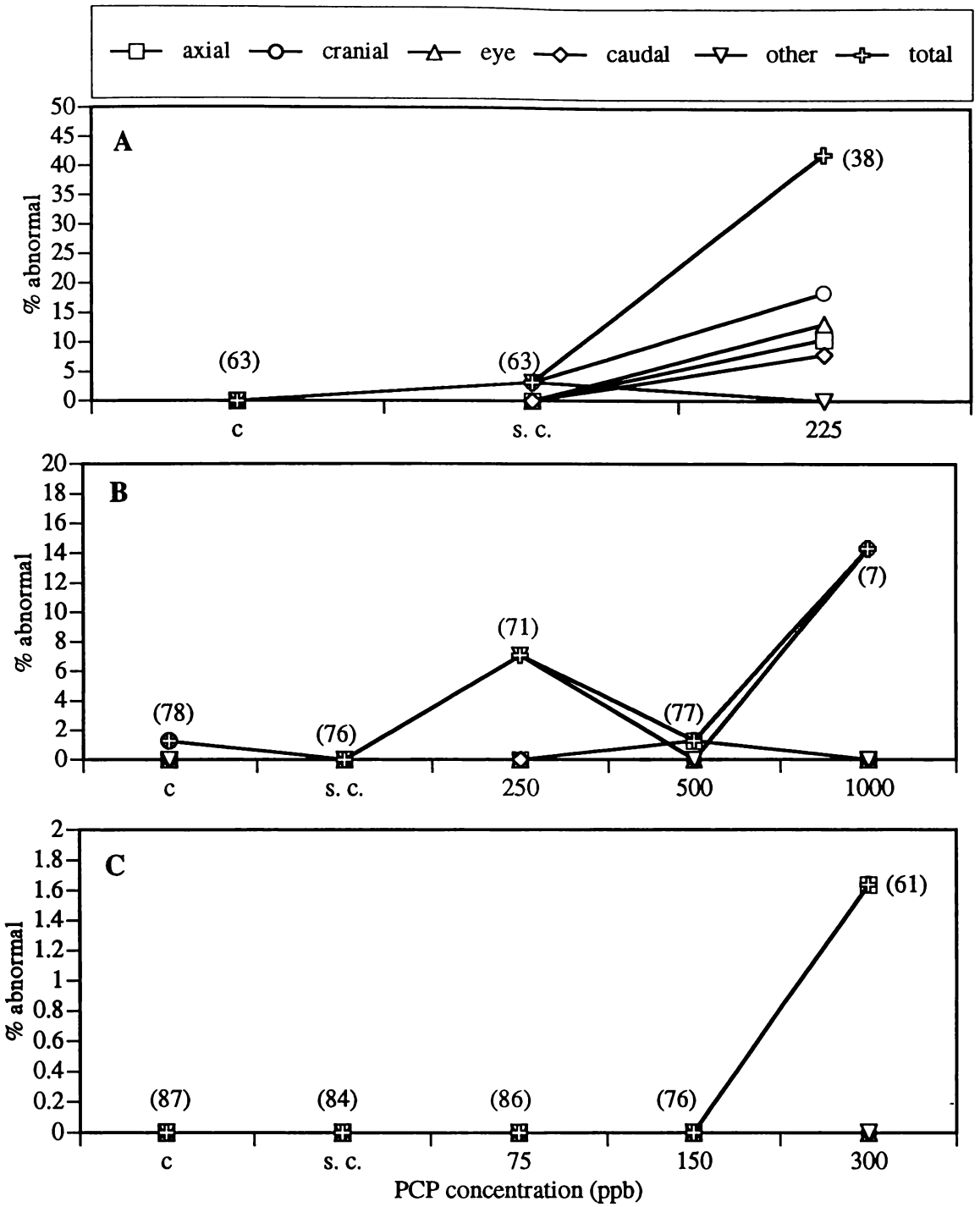


Figure 5.20 Percent abnormal rainbow trout larvae when the eggs were exposed to PCP (ppb) at the 24 h (A), early eyed (B) and late eyed (C) stages compared to the controls. "Total" denotes the total percent abnormal larvae and the other curves show the percent of larvae with axial, cranial, eye or caudal deformities. Note that one individual could have multiple abnormalities. Also shown are the total number of hatched larvae (n). Note the different scales of the x- and y-axes. c = synthetic water control; s.c. = solvent control.

Table 5.5 EC50 values and teratogenicity indices (final LC50/EC50(abnormality) for the four native fish species.

SPECIES	24 h stage		Early eyed stage		Late eyed stage	
	EC50	TI	EC50	TI	EC50	TI
Smelt	195	0.71	265	0.88	no EC50	
Inanga	310	1.33	960	0.53	250	0.65
Koaro	102	0.79	615	1.01	442	0.66
Common bully	17	0.97	81	1.39	100	1.38
Rainbow trout	No tests produced a greater than 50% abnormalities (no EC50)					

The abnormality rates of controls were below 10% for rainbow trout, common bully, koaro, and smelt at all stages and inanga tested at the 24 h and late eyed stages. The abnormality rates of controls for the inanga tested at the early eyed stage were, however, elevated; 18 and 22% for the synthetic water and solvent controls, respectively (Figure 5.16B).

For quantifying the number and type of cranial malformations certain classification procedures need to be mentioned. For example, if one cranium had many cartilages affected in the same way, only one count per malformation category was counted. However, one cranium could have malformations belonging to all four categories and, therefore, the different malformation categories are not additive in Figures 5.21 - 5.26. In addition, the success rate of cartilage staining was not 100%, with some crania either not staining properly, or being damaged in the process of staining. The number of successful stains for each PCP concentration and each species is also shown in Figures 5.21-5.26. The actual numbers of larvae with various types of malformations are shown in Appendix 3.

Smelt eggs responded to PCP exposure with deformed crania in a concentration-dependent manner. The highest percentage of abnormal crania was 30.6% when 24 h eggs had been exposed to 300 ppb PCP. The controls tested at the three other embryonic stages had ≤6% malformed crania. The most common type of abnormality observed in smelt for all stages was eye malformations, followed by abnormal sizes or shapes of cartilages, and asymmetry

and disorganisation. The least common type was missing cartilages (based on total counts from all life stage tests; Figure 5.21).

The crania of inanga had an abnormality rate of 90% when 24 h eggs were exposed to 800 ppb, but for all other stages and PCP concentrations the rates were ≤31%. It is evident that the malformations were more numerous and severe for the 24 h stage than for later stages. Control inanga tested at all embryonic stages had less than 6% malformed crania. The most common malformation was abnormal size or shape of the cartilages and the least common was missing cartilages (Figure 5.22).

Koaro eggs showed malformations in proportion to PCP dose except for one batch of eggs tested at the 24 h stage. The controls had a low malformation rate of ≤1.4%. Two batches of koaro exposed at the 24 h stages responded differently with 50% malformed crania at 150 ppb for batch 1, but 0% for batch 2 at the same PCP concentration (150 ppb) (Figure 5.23A and B). The most common type of malformation was asymmetry and disorganisation, whereas the least common types were missing cartilages and eye malformations for all stages (Figures 5.23 - 5.24).

Common bully eggs had generally very high rates of abnormalities in their crania, and the response was proportional to the PCP concentrations (Figure 5.25). The controls showed some abnormalities with between 1.7 and 11.8% of the eggs having at least 1 abnormality. The highest abnormality rates were found in eggs exposed at the early eyed stages. The most common type of cranial abnormality was missing cartilages, followed by abnormal sizes or shapes, and asymmetry and disorganisation. The least common type of abnormality was eye malformations.

Rainbow trout crania were 100% abnormal when early eyed trout eggs were exposed to 1000 ppb PCP, and 30.8% when 24 h eggs were exposed to 225 ppb. Rainbow trout crania exposed at other stages and other concentrations were, in general, not very responsive to PCP, as indicated by the low abnormality rate which was ≤4.8% (Figure 5.26). The most

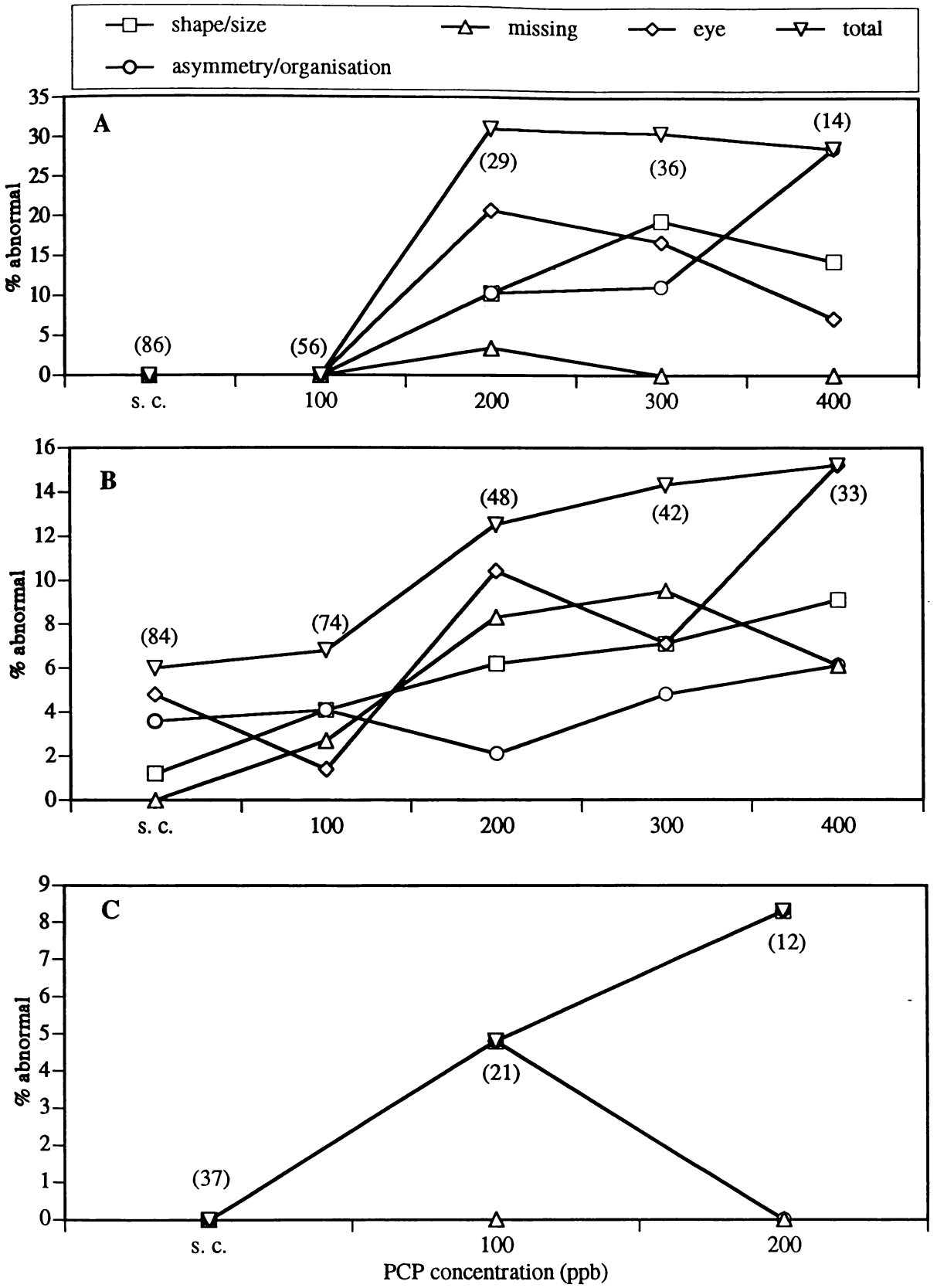


Figure 5.21 Percent smelt larvae that had abnormal crania when exposed to PCP at the (A) 24 h, (B) early eyed and (C) late eyed stages. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. s.c. = solvent control.

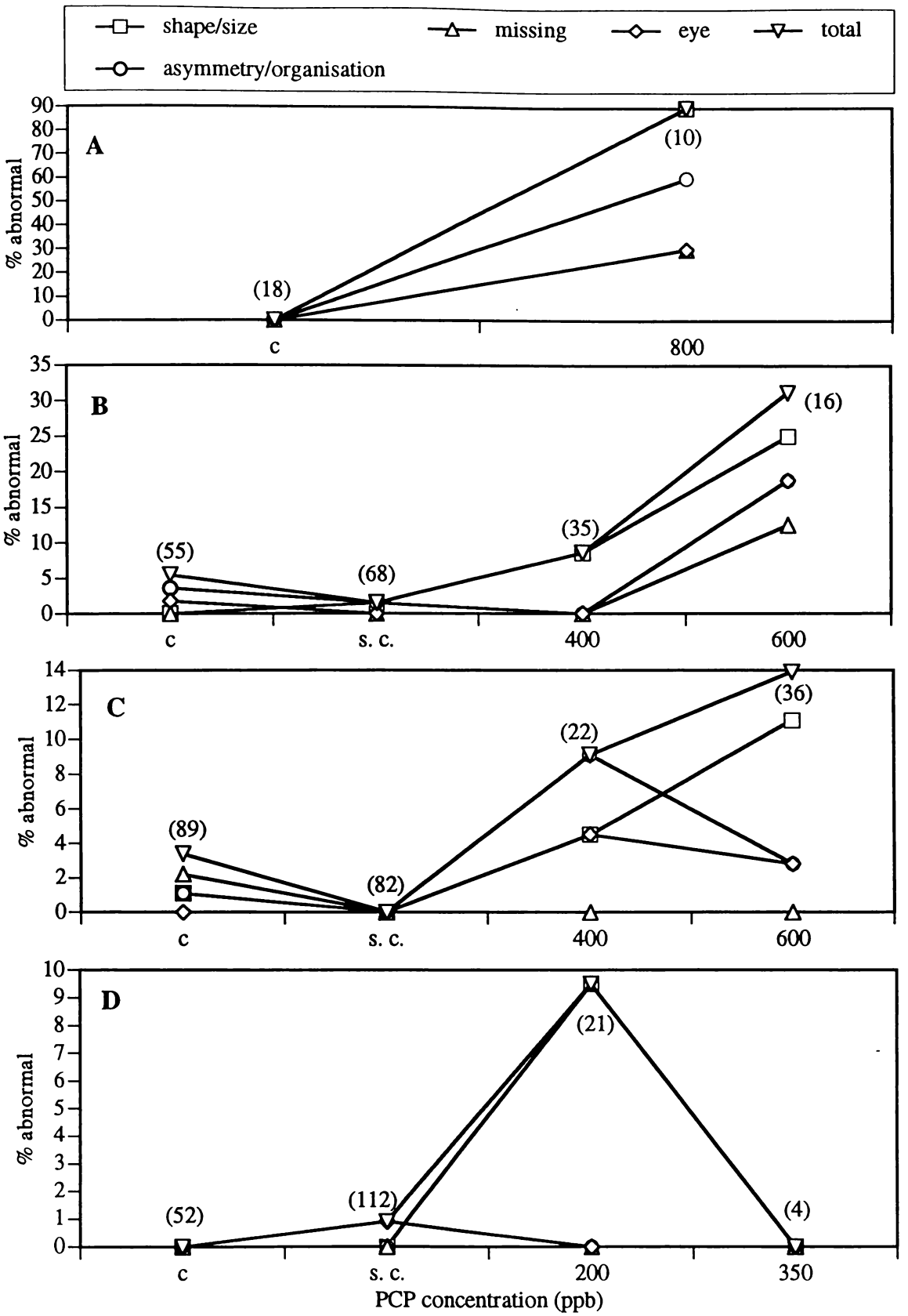


Figure 5.22 Percent inanga larvae that had abnormal crania when exposed to PCP at the (A and B) 24 h, (C) early eyed and (D) late eyed stages. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. c = synthetic water control; s.c. = solvent control.

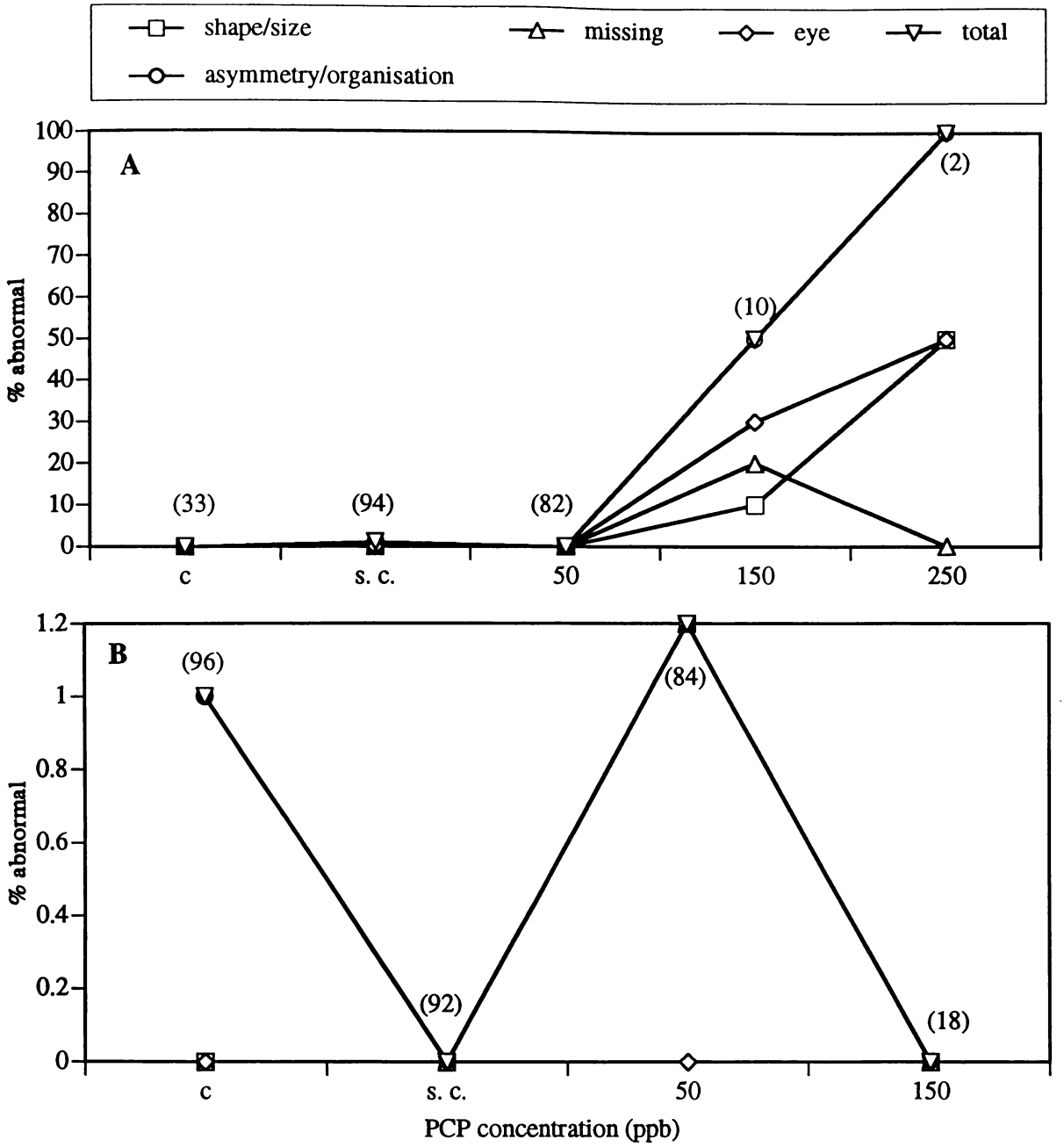


Figure 5.23 Percent koaro larvae that had abnormal crania when exposed to PCP at the (A and B) 24 h stage. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. c = synthetic water control; s.c. = solvent control.

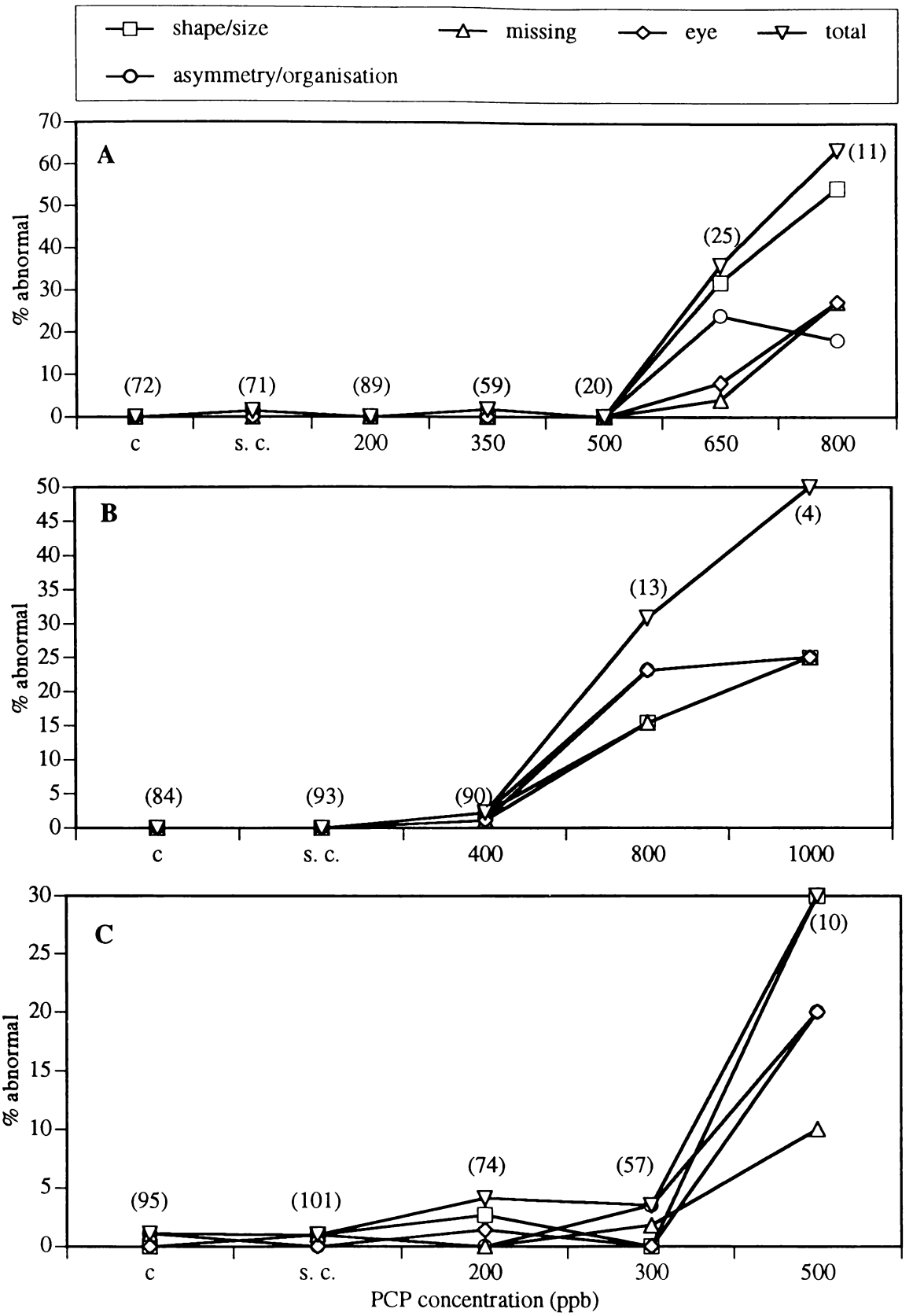


Figure 5.24 Percent koaro larvae that had abnormal crania when exposed to PCP at the (A and B) early eyed and (C) late eyed stages. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. c = synthetic water control; s.c. = solvent control.

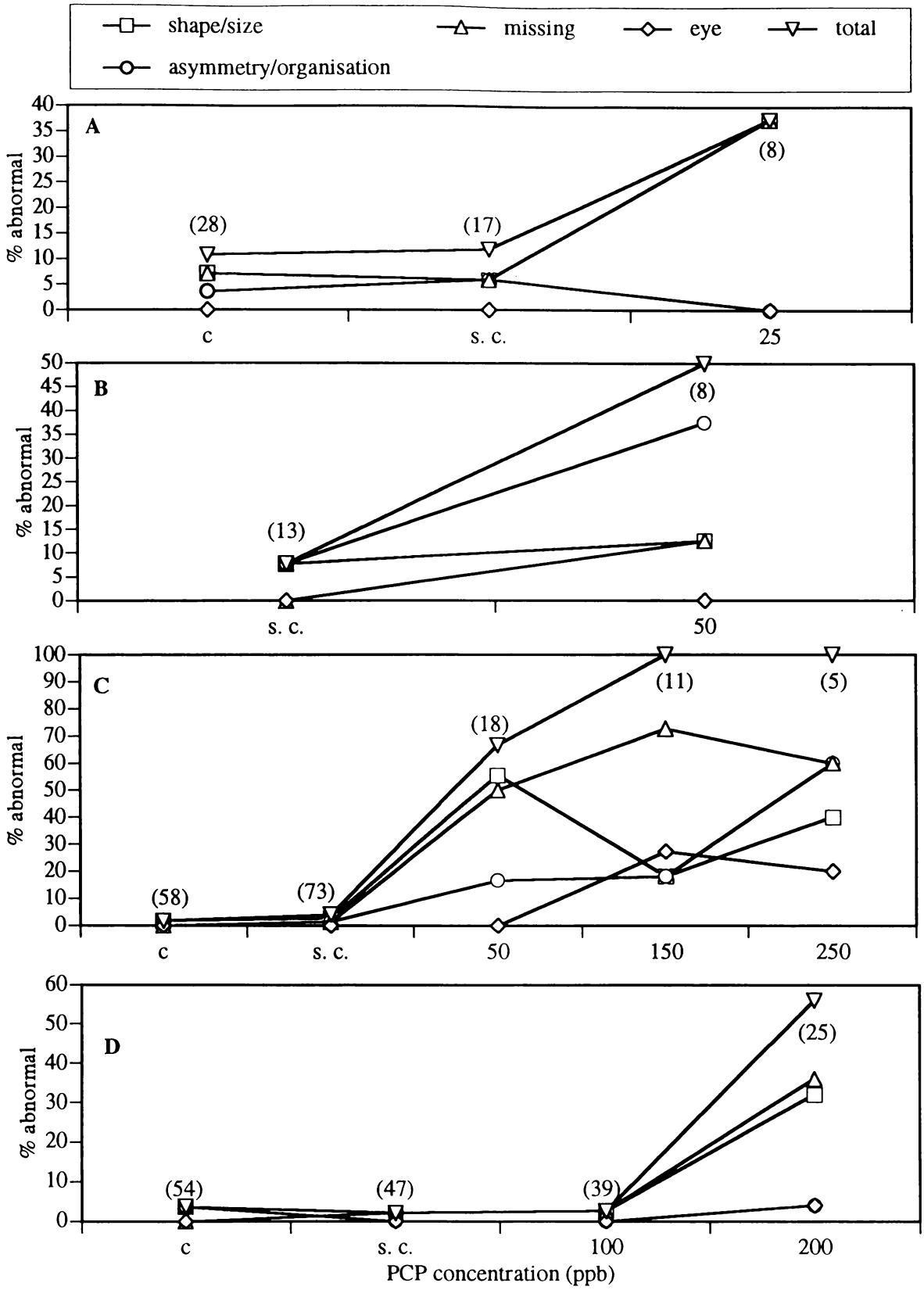


Figure 5.25 Percent common bully larvae that had abnormal crania when exposed to PCP at the (A and B) 24 h, (C) early eyed and (D) late eyed stages. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. c = synthetic water control; s.c. = solvent control.

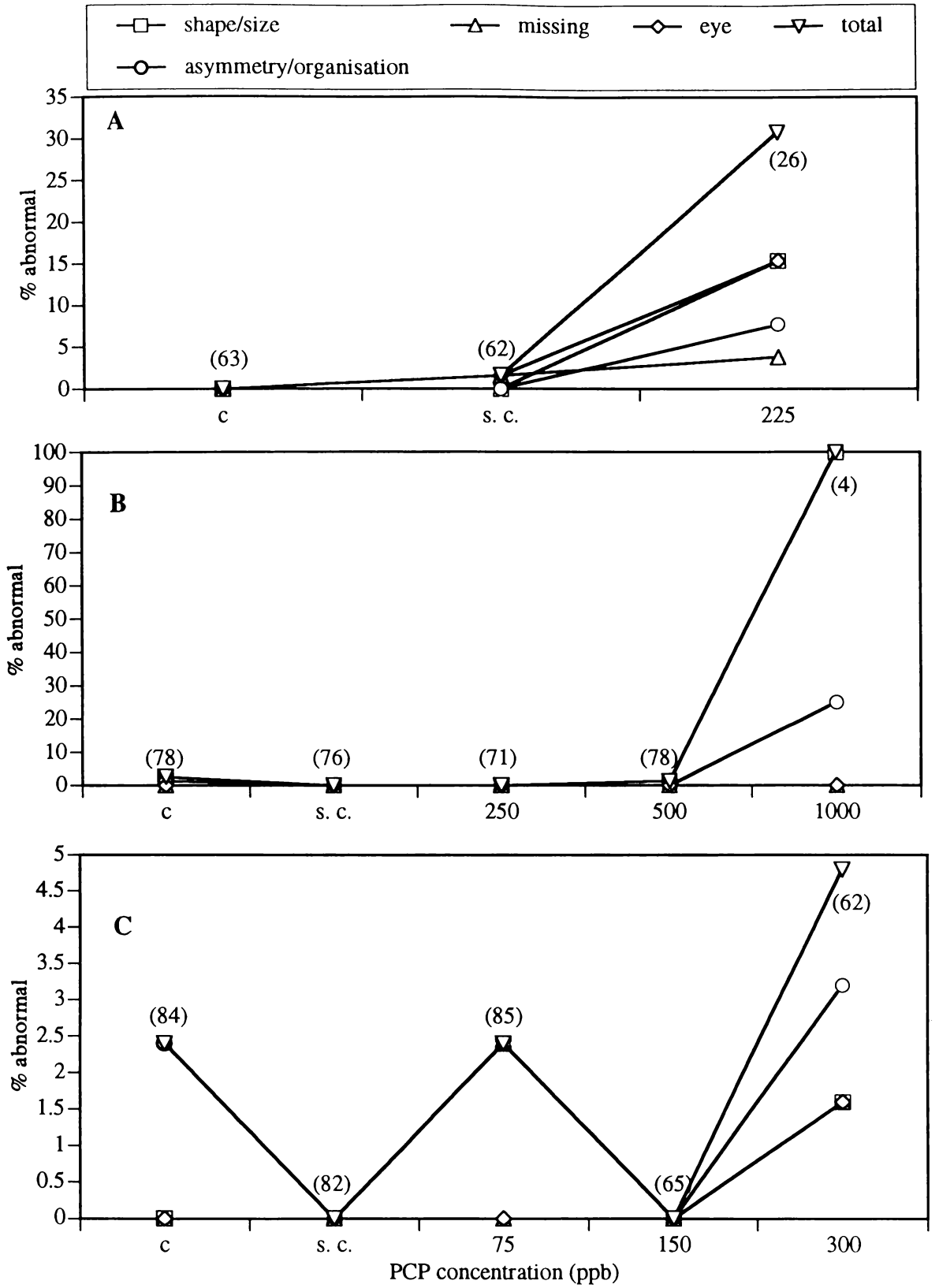


Figure 5.26 Percent rainbow trout larvae that had abnormal crania when exposed to PCP at the (A) 24 h, (B) early eyed and (C) late eyed stages. The number of successfully stained larvae is also shown in parentheses. Note that the x- and y-axes have different scales. c = synthetic water control; s.c. = solvent control.

common type of malformation was the shape or the size of the cartilages, and the least common was missing cartilages.

5.5.6 Swimming performance

The percentages of koaro and common bully larvae that swam normally after hatching are shown in Figures 5.27 A and B, respectively. Normal swimming generally declined in proportion to the PCP concentration, although some exceptions occurred. Two batches of koaro larvae tested at the 24 h stages reacted quite differently in response to PCP, with one batch being insensitive to up to 150 ppb PCP. Interestingly, one group of common bully larvae tested at the late eyed stage had 100% normally swimming larvae at 125 ppb PCP, but showed a decrease in swimming performance at 25 ppb.

5.5.7 Heart rates of smelt

When smelt eggs were exposed to PCP at the 24 h stage, their heart rates were decreased significantly (p <0.01) compared to the ethanol control 3 days after the completion of the toxicity test at 200 - 400 ppb (Figure 5.28A), but this effect had disappeared 3 days later and at the time-of-hatch. When they were exposed at the early eyed stage (Figure 5.28 B) the heart rates were significantly depressed (p <0.001) at 100 - 400 ppb one day after the completion of the acute toxicity test, but not 4 days later for 100 - 300 ppb. The heart rates of smelt were not significantly different when the eggs were exposed to PCP at either the late eyed or newly hatched stages immediately and one day after the tests were completed (Figure 5.28 C and D, respectively). The concentrations that were used for late eyed eggs and newly hatched larvae were lower than for the 24 h and early eyed eggs, but were comparable to the acute LC50 values for each stage.

It was also found that, in general, the heart rates of the control smelt embryos and of the larvae increased with further development. This was observed when smelt eggs were exposed to PCP at the early eyed stage (Figure 5.28 B). The controls and eggs that had been exposed to up to 300 ppb PCP had significantly increased heart rates at 4 days after the test, when compared to the heart rates of the same treatment group one day after the test.

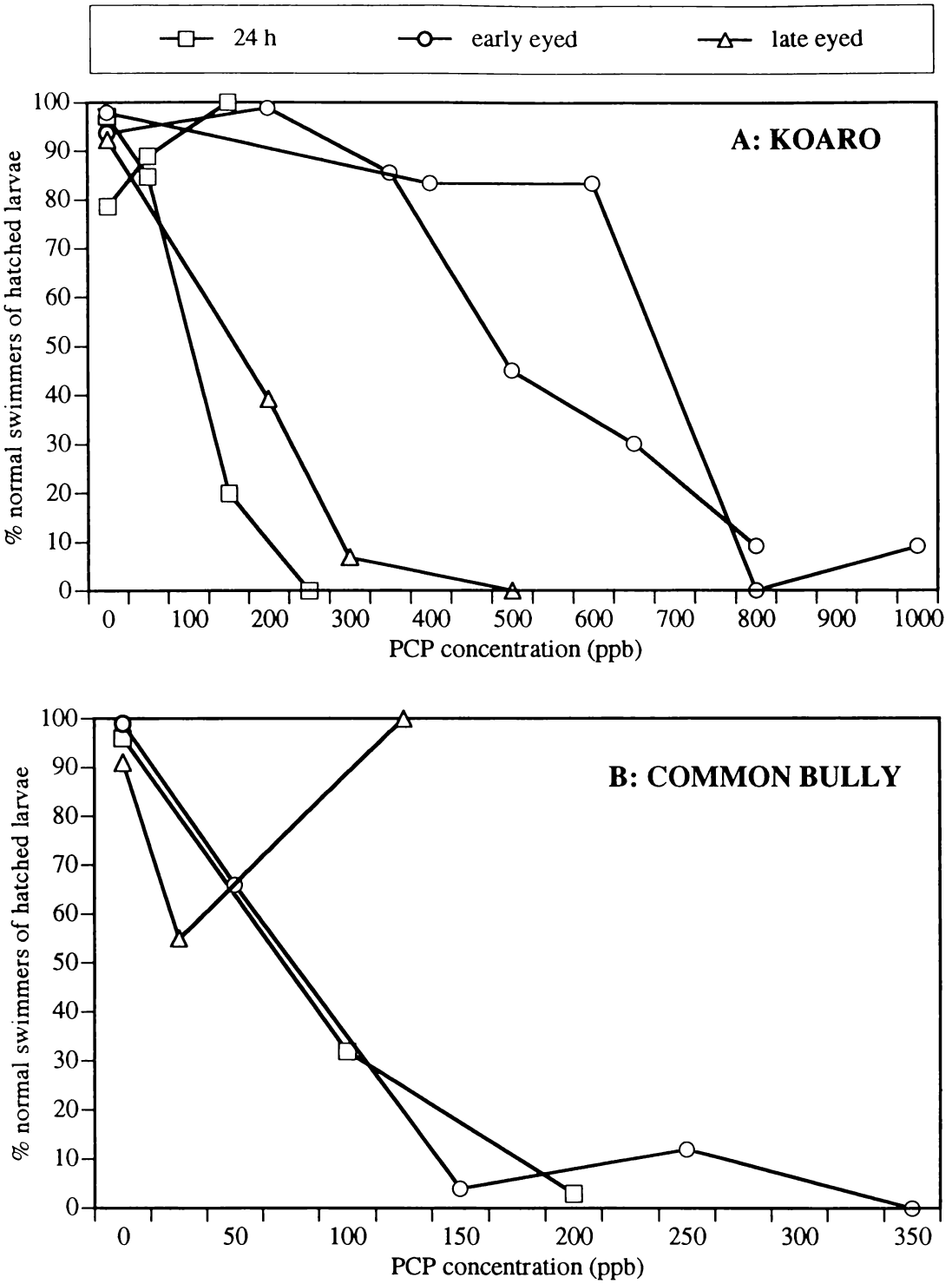


Figure 5.27 Percentage of hatched koaro larvae and common bully larvae that swam normally following exposure at 24 h, early eyed or late eyed stages (as shown in the legend) to the PCP concentrations shown on the x-axes. Note that two tests were done for koaro at the 24 h and early eyed stages. The remainder of the hatched larvae either did not swim at all, or they swam in an uncoordinated fashion.

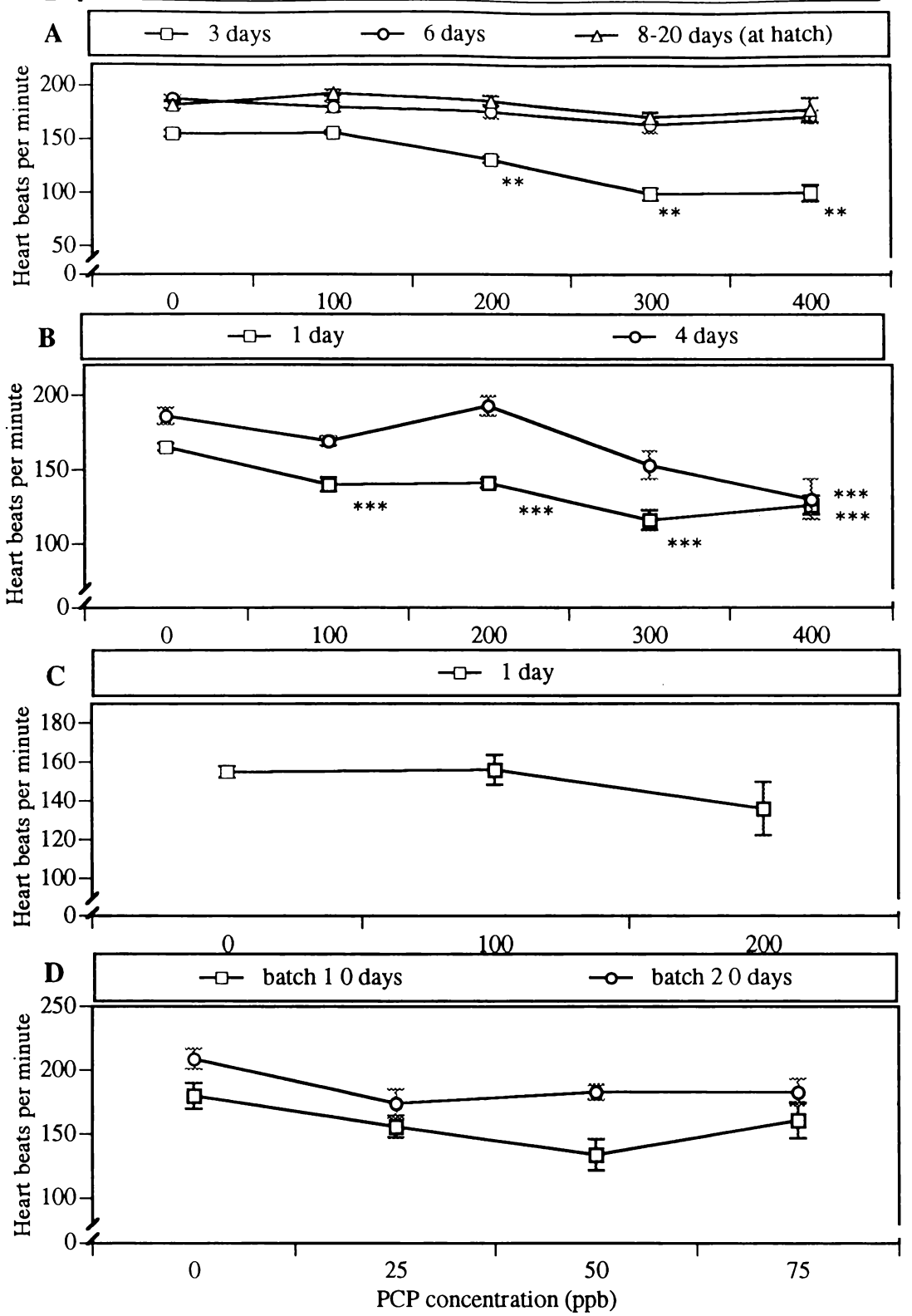


Figure 5.28 Smelt heart rate (mean ± SE) following tests at the (A) 24 h, (B) early eyed, (C) late eyed and (D) newly hatched larval stages. Graphs A and B show the change in heart rate at three and two occasions after the tests, respectively, shown as number of days after completion of acute toxicity tests in the legends. Graph C and D show the heart rate on one occasion, but graph D shows it for two different batches of eggs. Significant differences to the solvent control are shown as ** (p <0.05) and *** (p <0.001).

This was, however, not the case for eggs exposed to 400 ppb; these had apparently not recovered 4 days after the test, and the heart rates were statistically the same as those on the first day post-test. When smelt eggs were exposed to PCP at the 24 h stage (Figure 5.28 A), all treatment groups recovered within 6 days from the test. This is in contrast to what was found 3 days after the test, where the heart rates in all treatment groups were significantly increased.

5.6 Summary of results for early life stage toxicity tests

When eggs of the five tested species were exposed to PCP for 48 h at the 24 h, early eyed and late eyed stages, they showed a variety of sublethal responses that were relatively similar between the different species. The responses of the 24 h stages were usually more severe than those of the later stages. For comparison between the different sublethal responses that were measured in this study, a table of the lowest LOEC (lowest observed effect concentration, calculated using *t*-tests or Fisher's exact tests) for each species and each embryonic stage was compiled, together with the category of responses observed at that PCP concentration (Table 5.6). It can be seen that abnormalities of the hatched larvae and percentage of successful hatch (calculated from all survivors during toxicity tests and follow-ups to time-of-hatch) were the most sensitive responses. Cranial malformations, time-to-hatch and growth (measured as lengths) were generally the least responsive to PCP exposure. For the two species where swimming performance was assessed (common bully and koaro), this parameter was affected at the lowest LOEC five times out of six tests. Finally, heart rates reacted at the lowest LOEC once out of three possible exposure groups. Overall, if the LOEC values are compared to the LC50 values (Table 5.3) it becomes apparent that the sublethal responses of embryonic stages were often less sensitive than lethality of larval stages.

Table 5.6 The lowest LOEC values for each species and each embryonic life stage, together with the sublethal parameters that responded at these LOECs.

Species	Life stage	Lowest LOEC	Response
Smelt	24 h	100 ppb*	abnormalities, length, time-to-hatch
	early eyed	100 ppb*	abnormalities, successful hatch, heartbeat, length
	late eyed	100 ppb*	abnormalities, successful hatch, time-to-hatch
Inanga	24 h	400 ppb*	abnormalities, successful hatch, length
	early eyed	400 ppb*	cranial abnormalities
	late eyed	200 ppb*	abnormalities, successful hatch
Koaro	24 h	50 ppb*	swimming performance
	early eyed	350 ppb*	abnormalities, successful hatch
	late eyed	200 ppb*	swimming performance, abnormalities, successful hatch, length
Common bully	24 h	25 ppb*	swimming performance, abnormalities, successful hatch, length
	early eyed	50 ppb*	swimming performance, abnormalities, cranial abnormalities, successful hatch
	late eyed	100 ppb*	swimming performance, abnormalities, successful hatch, time-to-hatch
Rainbow trout	24 h	225 ppb*	abnormalities, cranial abnormalities, successful hatch, length, time-to-hatch
	early eyed	250 ppb*	abnormalities, time-to-hatch
	late eyed	300 ppb	successful hatch

* lowest concentration tested

CHAPTER 6

MATERIALS AND METHODS FOR ADULT FISH

6.1 Capture and maintenance of inanga and common bully

Inanga were caught by beach seining or white bait netting in the Parawai stream at Raglan (37°50'S, 174°58'E) and returned to the laboratory in buckets filled with aerated stream water. The fishing took place in December, 1995 (for toxicity testing), January, 1996 (for toxicity testing), and in October, 1996 (for sublethal exposure). The fish were transferred to 10% artificial sea water (3 ppt NaCl solution) at 18°C as a preventive measure against white spot disease. The artificial sea water was gradually replaced by aerated, dechlorinated tap water of the same temperature during the weeks following each fish collection. The volume of the aquarium was 280 L, and the stocking density was less than 0.7 g/L. For the heat shock experiments the control temperature was 20°C, and in all other experiments the fish were maintained at 18°C. The temperature was maintained by a Julabo FT200 cooler, and the water was continuously filtered with a Fluval 303 charcoal filter. Debris and dead fish were removed daily and the fish were fed daily, *ad libitum*, with frozen chironomid larvae until the day before exposure to PCP. The fish were acclimatised for at least two weeks, and any batch of fish with a mortality in excess of 10% was not used for experiments.

Common bully were captured in the Waikato River in Hamilton by beach seining in November, 1996, for a range finding toxicity test, and the fish were maintained at the temperature of the river which was 18°C. In February, 1997, bullies were captured with Gee minnow traps (3 and 5 mm) in the University of Waikato ponds for heat-shock treatment and the controls were kept at the temperature of the ponds which was 21.5°C. In March, 1997, bullies were captured by beach seining in the Waikato River

in Hamilton for both definitive toxicity tests and sublethal exposures to PCP. These latter fish were kept at the temperature of the river which was 17°C. The bullies were fed, *ad libitum*, with frozen Chironomid larvae until the day just prior to the experimental procedures being initiated. No artificial sea water treatments were used for prevention of disease with any of the bully experiments. Similar to the maintenance procedures for adult inanga, the volume of the aquarium was 280 L, the stocking density was less than 0.7 g/L, and the water was filtered with a Fluval 303 charcoal filter.

6.2 Determination of the 96 h LD50 and subsequent sublethal exposures of inanga and common bully

The adult fish were exposed to the identical batch of PCP as was used for the early life stage tests, i.e. Aldrich, 99.54% purity, as measured by GC-MS (Section 4.7.1). Exposures to PCP were achieved by intraperitoneal injection. This circumvented the logistical difficulty of exposures in multiple temperature-controlled glass aquaria, and the disposal of large amounts of PCP-contaminated water (Hodson et al. 1984). The protocol used for the exposure of adult fish to PCP had been previously approved by the Animal Ethics Committee at Waikato University.

To prepare the PCP for injections, PCP was dissolved directly in commercially available coconut oil which was heated to 25°C to maintain it in liquid form. In addition, the injection syringe was also heated to prevent the PCP mixture from solidifying during injections. After injection into the peritoneal cavity of the fish, the PCP mixture solidified quickly and leakage was not encountered. The fish were anaesthetised with 33 mg/L ethyl-*p*-aminobenzoate (Summerfelt and Smith 1990), which induced anaesthesia within about 1 min (as indicated by loss of equilibria) with subsequent recovery within 2 min for inanga. For bully, the same dose induced

anaesthesia within about 5 min and recovery occurred within 1-2 min. The fish were weighed while anaesthetised to the nearest 0.05 g, and the data recorded for later analysis. Intraperitoneal injections were performed with a 5, 10 or 25 µL Hamilton glass syringe at 5 mL/kg fish. The injection volumes were, therefore, kept constant in relation to the weights of the fish, and the toxicant dosages were varied by altering the concentrations of PCP in the oil.

Initial range finding tests were performed with both species with 5 fish per 3-4 doses of PCP. For determination of the definitive 96 h LD50, 6 or 7 randomly chosen groups of fish received PCP doses in an arithmetic series and a control group received coconut oil only. The number of fish per dose was 10 when performing definitive toxicity tests. Fish receiving different doses were maintained in the same tank and identified by fin clipping. The definitive LD50 (96 h) was determined twice for inanga, and once for common bully.

The concentrations chosen for sublethal exposures were 25, 50 and 75% of the definitive 96 h LD50. A control group of fish in each test was injected with coconut oil only. The intraperitoneal injection procedure was identical to that of the acute tests. The number of fish per treatment group was 30 for inanga and 20 for common bully, and the duration of the sublethal exposures was 96 h. The term sublethal is here used for the relatively high doses of PCP that the fish received, because the aim was to study sublethal physiological responses rather than acute mortality.

6.3 Blood and tissue sample collection

Four days following the sublethal injection, the fish were sampled in random order. Blood was sampled from the caudal vein of unanaesthetised fish with a 1 mL syringe

and microfine® III needle. The anticoagulant in each syringe was 0.15 mg EDTA-K salt, which had been allowed to dry inside the syringe. This minimised dilution of the minute volumes of blood obtained from the fish.

The appropriate volumes of blood were dispensed with the aid of Accu-fill 90™ Micropet 5 µL pipettes for the determination of the total white blood cell counts (2 µL), total red blood cell counts (2 µL), differential white blood cell count (1 µL), lactate or glucose (5 µL each) as described in Sections 6.4 - 6.8. Thus, the total volume of blood required for the haematological analysis of each fish required a total of 15 µL. Unfortunately, this was not achieved with some fish, particularly with some inanga individuals, for which only 5 - 10 µL or less was recovered. The allocation of blood samples to the different analyses was, therefore, partially determined by availability. An adult fish was deemed "mature" if the gonads had the appearance of maturity, i.e. mature or ripe stages, as described in Section 4.1, otherwise they were classified as "immature".

Following the sampling of blood, the fish were sacrificed by cervical translocation and the whole liver and gills were removed. These tissue samples were blotted dry and immediately frozen and stored in liquid N_2, for enzyme analyses of the liver, and heat shock protein analyses of the gills. Livers and gills were collected from every fish, with some pooling being required prior to analysis as detailed in Sections 6.9.2 and 6.10.1. Finally, the sex and maturity of the fish were determined by visual inspection. An adult fish was deemed 'mature' if the gonads had the appearance of maturing, mature or ripe stages as described in section 4.1, otherwise they were classified as 'immature'.

6.4 Whole blood glucose determinations

For the initial step in the determination of whole blood glucose levels, 5 µL of whole blood was deproteinated in 10 µL of 12% trichloroacetic acid (TCA), the samples vortexed, and then stored at -20°C until later analysis. Upon thawing, the samples were remixed and centrifuged at 13 000 *g* for 2 min with a Beckman Microfuge E™. Supernatant (9 µL) was added to the 261 µL of the hexokinase reagent and incubated at 37°C for 5 min. This represented a reduction in the volumes suggested in the instructions of the manufacturer of the test kit (Sigma diagnostics kit No. 16-10), and it was accounted for when calculating final concentrations. The absorbance at 340 nm was determined with a Shimadzu UV-250 dual beam spectrophotometer. The final glucose content was calculated using the millimolar absorption coefficient of NADH (6.22 at 340 nm), which was the product of the reaction.

Because of the small blood volumes obtained from the fish, a single sample was analysed per fish. One bully sample was large enough to enable four glucose determinations, so that an estimation of the precision of the method could be accomplished. As a reference, nonfasting human blood was also analysed in triplicate, concurrently with both the inanga and bully samples.

6.5 Whole blood lactate determinations

As for the analysis of blood glucose, 5 µL of whole blood was added to 10 µL of 12% TCA, the mixture vortexed, and the samples stored at -20°C. Upon thawing, the samples were remixed and centrifuged at 13 000 *g* for 2 min. The lactate concentrations were determined using Sigma diagnostics kit No. 826-A, according to the instructions of the manufacturer. The volumes of the supernatant and the NAD^+-reagent were reduced to 9 and 261 µL, respectively. The absorbance at 340 nm was determined with

a Shimadzu UV-250 spectrophotometer. A lactate standard-curve (0.55-4.44 mM) was also constructed and this curve was used for calculating the final lactate concentrations of the samples.

Similar to the situation with the determination of blood glucose levels, the blood sample volumes of the fish were small, and thus did not allow duplication of the lactate analyses. However, two bully samples were analysed in triplicate, and a single and a duplicate resting human blood sample were analysed concurrently with the inanga and bully samples, respectively.

6.6 Total red blood cell count

For determination of the total number of erythrocytes, 2 µL of blood was added to 1 mL of erythrocyte formalin-citrate counting fluid (Dacie and Lewis 1991). The samples were kept at 4°C until counting took place within two days after sampling. The samples were vortexed for 1 min immediately prior to adding them to the improved Neubauer haemocytometer, and at least 500 cells were counted.

6.7 Total white blood cell count

For counting the total number of leukocytes, 2 µL of blood was added to 98 µL of Shaw's solution (Klontz and Smith 1968), and stored at 4°C until counting took place within two days after sampling. The Shaw's solution used in this study was modified due to the relatively poor staining of the white blood cells (see Appendix 4). The samples were vortexed for 1 min immediately prior to adding them to the improved Neubauer haemocytometer, and at least 100 cells were counted.

6.8 Differential white blood cell count

Blood films were prepared using the standard "wedge" technique and allowed to air dry. They were fixed in methanol for 10 min, and after drying were stained using May-Grünwald (0.15%) and Giemsa dyes (0.1%) at pH 7.8 (Dacie and Lewis 1991).

At least 100 white blood cells were counted per sample and they were classified as either lymphocytes, thrombocytes or granulocytes. The criteria used for discriminating a lymphocyte were (Ellis 1977): the cells were of variable size and had a round nucleus with either no or only a small amount of surrounding cytoplasm. Thrombocytes had elongated nuclei, with cytoplasm stretching to either of both sides, but occasionally no cytoplasm was present. Inanga granulocytes had abundant and granular cytoplasms, were larger than erythrocytes, and the nuclei were only rarely lobular. Bully granulocytes were more often lobulated.

6.9 Enzyme analyses

6.9.1 Preparation of post-mitochondrial fractions

The microsomal fraction has traditionally been used for the analysis of detoxifying enzymes of the liver. Initial trials with inanga livers indicated that the microsomal fraction had higher ethoxyresorufin-*O*-deethylase (EROD) activities than the post-mitochondrial fraction (8.34 versus 2.06 pmol min^{-1} mg^{-1} protein, respectively). It was, however, decided that the minute sizes of the livers prevented the routine use of the microsomal fraction for enzyme analyses, and thus the post-mitochondrial fraction (PMF) was used for the determination of all liver enzyme activities and protein concentrations.

For preparation of PMFs, the livers were homogenised in 4 volumes of cold 0.25 M sucrose, 10 mM HEPES, pH 7.4, with a hand-held teflon pestle and a glass tube at 4°C. The homogenates were centrifuged at 13 000 *g* for 15 min with a Beckman Microfuge E™ at 4°C, and the resulting supernatants then used for enzyme and protein analyses. When preparing post-mitochondrial fractions for enzyme analysis, it was noticed that bully livers contained large amounts of lipids, which were not present in inanga livers. The lipid could be seen as a white layer on top of the supernatant after centrifugation at 13 000 *g* and was removed before proceeding with the enzyme analysis.

6.9.2 Determination of uridine 5'-diphosphoglucuronyl transferase activity

The uridine 5'-diphosphoglucuronyl transferase (UDPGT) activity was determined essentially as described by Castrén and Oikari (1983), with *p*-nitrophenol as the aglycone. The cofactor (uridine 5'-diphosphoglucuronic acid, Sigma) was excluded from the blank. The PMFs were diluted with 0.25 M sucrose, 10 mM HEPES, pH 7.4, for the protein contents to be in the linear range of the assay. The reactions were initiated by adding an aliquot of PMF, and the samples were incubated for 20 min at 25°C, and then the reactions were stopped by the addition of ice-cold 3% TCA. After centrifugation for 5 min (13 000 *g* at room temperature), 5 M NaOH was added to the supernatant. The disappearance of *p*-nitrophenol from the samples compared with the blanks was measured at 400 nm with a Shimadzu UV-250 spectrophotometer. Because of the minute sizes of the inanga livers (approximately 5-10 mg per liver), pooling of livers (from 2-4 fish) within groups was necessary, as well as scaling down the volumes used in the assay, so that the final reaction volume of each sample assay mixture was 580 µL. The proportions of the reagents in the triplicate assays were the same as those of Castrén and Oikari (1983). Pooling of livers only occurred between fish of the same sex and sexual maturity. Common bully livers usually weighed more

than 25 mg, and only 8 cases of the pooling of livers from two fish took place out of 80 fish. The detailed protocol used in this study for the analysis of UDPGT activity is given in Appendix 5.

6.9.3 Determination of ethoxyresorufin-*O*-deethylase activity

The EROD activity was determined according to the method of Burke and Mayer (1974) with a Perkin Elmer Luminescence spectrometer, model LS50B. The buffer consisted of 0.1 M HEPES, pH 7.4, 1.5 mg/mL bovine serum albumin (BSA) fraction V (Sigma) and 10 mM $MgSO_4$. The cofactor, NADPH (Sigma), was present at 245 µM in the assay. The reaction was started by the addition of ethoxyresorufin to a concentration of 0.245 µM. The reaction was monitored during 2 - 20 min, until the increase in fluorescence could be reliably measured. For each assay the linear portion of the graph was used to calculate the final activity, with the aid of an internal resorufin standard that was added at the end of each assay. The extinction coefficient of resorufin at 572 nm was 16.68 mM^{-1} cm^{-1} and the final volume in the assay was 390 µL. The excitation wavelength was set at 530 nm and the emission at 585 nm, and the reaction temperature was 20°C. The detailed protocol for the analysis of EROD activity is described in Appendix 6.

6.9.4 Protein concentration determinations

The protein contents of the post-mitochondrial fractions were determined in duplicate using a variation of the Bradford method (Kruger 1994), with chicken ovalbumin (Sigma) as the standard. The microassay version of the assay was used to minimise the amount of sample required. The reaction was started by addition of the protein detection reagent, and after 2 - 60 min the absorbance at 595 nm was measured against blanks containing MILLI-Q® water and protein detection reagent. The unknown

samples produced absorbances in the range of the ovalbumin standards, i.e. between approximately 0.03 (for 10 μg/mL) and 0.4 (for 100 μg/mL). The detailed protocol for the analysis of protein concentration with the Bradford microassay method is summarised in Appendix 7.

6.9.5 The effect of ß-naphthoflavone on UDPGT and EROD activities

ß-naphthoflavone (ßNF) was used to investigate the extent of induction of EROD and UDPGT activities in inanga and common bully. Five fish of each species were injected with 100 mg/kg of ßNF in corn oil, at 5 μL/g fish, and the control groups were injected with corn oil alone. After 48 h at 17°C without feeding, the fish were sacrificed by cervical translocation and the livers were sampled, frozen in liquid N_2, and processed as detailed above (Sections 6.9.1 - 6.9.4). The inanga were all mature females, except for two immature males. The inanga weighed between 2.46 and 5.05 g, and no pooling of livers took place. The bullies were immature of unknown sex, and they weighed between 1.50 and 4.84 g. No pooling of bully livers took place.

6.10 Heat shock protein 70 (hsp70)

6.10.1 Hsp70 and protein determinations

For the determination of hsp70, the mouse monoclonal antibody (N27) originally isolated from HeLa cells was used. This antibody is specific for both the constitutive and inducible forms of hsp70. It was a generous gift from both Dr S. Ryan (formerly of The University of Auckland) and from the original source of the antibody, Professor W.J. Welch (The University of California, San Francisco). In addition, the monoclonal antibody C92 (obtained from W.J. Welch) which reacts only with the inducible form of hsp70, was tested for its efficacy in the identification of inanga hsp70.

Gill samples were pooled within their own experimental treatments in groups of three prior to homogenisation. For homogenising gill tissue, a mortar and pestle made of stainless steel was used. These were precooled in liquid N_2 before transferring the gills into the mortar and powdering the tissue with a hammer on the pestle. The tissue was then diluted with 15 volumes of hot solubilisation buffer (2% sodium dodecyl sulphate, 10% glycerol (v/v), 0.0625 M TrisHCl, pH 6.8), and extracted for 30 min at 100°C, followed by centrifugation at 13 000 *g* for 15 min at 4°C (summarised in Appendix 8).

The protein assay utilised was a modified method of Lowry (Lee and Dunbar 1994), with the same volume of solubilisation buffer being incorporated into the standards as was present in the samples. The standard was bovine serum albumin (fraction V, Sigma) (Appendix 9).

For preparation of the samples for loading onto SDS-PAGE gels, bromophenol blue and ß-mercaptoethanol were added to 0.025% (w/v) and to 5% (v/v), respectively. The samples were diluted such that the samples contained identical protein contents and volumes prior to loading onto SDS-PAGE gels. A set of molecular weight standards (Sigma SDS-7B with molecular weights between 32 and 195 kDa) and bovine serum albumin (BSA, 66 kDa) were also loaded in separate lanes to aid in determining the positions of detected bands in the gels or blots (Appendix 8).

The resolving gels were 7.5% gels (acrylamide-bisacrylamide 30:0.8) with 5% stacking gels (Hames 1990). The gels were run at 4°C in a Bio-Rad Mini-Protean® II system at 45 V until the dye front reached the resolving gel and then the voltage was increased to 80-120 V (Appendix 8). Wet electrophoretic transfer was performed using a transfer buffer containing 50 mM TrisHCl, 380 mM glycine, 20% methanol and 0.1% SDS

(Harlow and Lane 1988) at 40 V and 4°C for 14 - 16 h in a Bethesda Research Laboratories (BRL) Western blotting module. The blotting membranes used were 0.2 μm nitrocellulose (Pharmacia). To ascertain that transfer had taken place, the polyacrylamide gels were stained with Coomassie blue (0.25%) after Western blotting (Appendix 10).

The heat shock proteins were detected with an enhanced chemiluminescence kit according to the manufacturer's instructions (Amersham). Nonfat dried milk (5%) in phosphate buffered saline (PBS-T; 0.1% Tween-20) was used as a blocking agent. The primary antibody N27 was diluted 1:1000 and the secondary horseradish peroxidase-conjugated sheep anti-mouse Ig antibody 1:5000, with both antibodies being diluted in blocking agent (dilution of the antibodies in PBS-T was found to be associated with an increase in the background level). Hyperfilm-ECL (Amersham) was used to detect the luminescent bands. The exposure and development of the films took place in total darkness, since Hyperfilm-ECL was sensitive to red safelights (Appendix 10). The lengths of exposures depended on the strength of the signal, which was assessed after an initial 60 s exposure. Development of films was performed by standard methods and used Agfa developer for 5 min, a 2% acetic acid solution for 30 s for stopping the development, and a fixing solution (Agfa) for at least 5 min. Finally, the films were rinsed in water for at least 15 min.

The resulting films were scanned using a Molecular Dynamics scanner, subtracting the same background value from each integrated volume on the same film. Identical integration 'objects' were copied around each band. To enable comparisons of the amounts of hsp70 on different gels, the same sample was loaded in one lane on each gel, and all other bands were compared to this marker sample. The blots were stained with 0.1% India ink or 0.1% Amido Black (Harlow and Lane 1988) after ECL-

detection to ascertain the degree of transfer and the positions of detected bands (summarised in Appendix 10).

6.10.2 Positive controls for hsp70

In order to show that the hsp70 that was recognised by N27 was inducible, positive control samples consisting of heat-shocked fish were prepared. When choosing a suitable temperature for heat shock of the two species, critical thermal maxima (CTMax) that have been reported in the literature were used as a guide. The CTMax is determined by increasing the water temperature for fish at a constant rate and recording the temperature at which half of the fish become narcotised and fail to respond to stimuli (Becker and Genoway 1979; Paladino et al. 1980). Easton et al. (1987) used a heat shocking method where salamanders were exposed to 2-5°C below CTMax and then allowed to recover for 3 h, to study the synthesis of heat shock proteins. For inanga acclimated to 20°C, the CTMax has been determined to be 32.9°C (Simons 1986), and a heat shock temperature of 30°C was chosen (Easton et al. 1987). For bullies acclimated to 20°C the CTMax is 34°C (Simons 1986), and therefore, a heat shock temperature of 31°C was chosen. The fish were maintained in aerated and dechlorinated tap water at these elevated temperatures for one hour, and then returned to the control temperatures for three hours. The control fish were kept at the control temperatures, which were 20°C and 21.5°C for inanga and bully, respectively. The gills of the control and heat-shocked fish were processed as detailed in Section 6.10.1.

6.11 Statistical analyses

The data were analysed by ANOVA and Dunnett's post-test using GraphPad InStat Mac software. The program applies Kramer's extension to the Dunnett's test if the groups are of unequal size. If the Bartlett's test was significant (p <0.05), indicating

unequal variances between the groups or nonGaussian distributions, the data were log-transformed. If this produced a significant Bartlett's test result, the data were analysed using a nonparametric Kruskal-Wallis test and Dunn's post-test. The Kruskal-Wallis test was also employed when the sample size was too small to perform Bartlett's test. Some data were analysed using regression analysis. When two groups were compared, a Student's *t*-test was used, or a nonparametric Mann-Whitney test when the standard deviations were significantly different. Percentage data from the differential white blood cell counts were arcsin transformed prior to performing ANOVA.

CHAPTER 7

RESULTS FOR ADULT FISH

7.1 Mortality during lethal and sublethal exposures of inanga and common bully

One range-finding toxicity test with inanga indicated that the 96 h LD50 would be between 0.14 and 0.28 mmol/kg. Two definitive tests showed the LD50 to be 0.32 mmol/kg (95% confidence interval 0.30 - 0.36 mmol/kg, calculated by the trimmed Spearman-Kärber method) and 0.27 mmol/kg (95% confidence limits 0.24 - 0.30 mmol/kg, also calculated by the Spearman-Kärber method) (USEPA 1991). No unexposed control fish died during any of the acute tests.

For sublethal exposure of inanga, 25, 50 and 75% of 0.30 mmol/kg dosages were used, i.e. 0.075, 0.150 and 0.225 mmol/kg. Thirty to thirty-one fish per PCP concentration were exposed per treatment group. One inanga died during the sublethal exposures in both the control and the 0.150 mol/kg groups. Exposure of inanga to 0.225 mmol/kg for 96 h caused a mortality rate of 40%.

One range-finding toxicity test indicated that the 96 h LD50 for common bully would be approximately 0.23 mmol/kg PCP. The definitive test with 10 fish per treatment group indicated a 96 h LD50 of 0.234 mmol/kg (95% confidence limit 0.225 - 0.244, r^2=0.947, slope=0.031) as calculated by the probit method (Finney 1971). No deaths occurred in the unexposed controls during the acute exposures.

Sublethal exposures of common bully were performed at 25, 50 and 75% of the LD50, i.e. at 0.059, 0.117 and 0.176 mmol/kg. Twenty fish were exposed per group. Two deaths occurred among the fish exposed to 0.117 mmol/kg, and no mortality occurred in any of the other treatment groups.

The LD50 exposure groups of 25, 50 and 75% for both species will henceforth be referred to as the low, medium and high doses.

7.2 Weights and maturity of exposed fish

The majority of inanga in the sublethal test were immature, and none of the mature inangas were in spawning condition. Ten percent of the common bullies in the sublethal test were mature females, and the rest were immature and of unknown sex (Table 7.1).

Table 7.1 Weight (g ± SD) and maturity of inangas and common bullies exposed to sublethal doses of PCP. Note that the sex and maturity of the fish were determined at the end of the experiments and, therefore, "total" partially reflects the number of fish surviving the tests. The weights of the fish were determined at the start of the experiments and are based on 30 or 31 and 20 fish per treatment group for inanga and bully, respectively.

	DOSE			
	Control	**Low**	**Medium**	**High**
INANGA				
No. immature (sex unknown)	26	27	24	13
No. immature females	0	0	3	1
No. immature males	1	0	0	0
No. mature females	0	0	0	1
No. mature males	3	2	2	0
Total	30	29	29	15
Weight, (g ± SD)	1.20 ± 0.52	1.15 ± 0.52	1.37 ± 0.76	1.37 ± 0.65
COMMON BULLY				
No. immature (sex unknown)	1	0	0	0
No. immature females	10	10	8	15
No. immature males	7	10	9	2
No. mature females	2	1	2	3
No. mature males	0	0	0	0
Total	20	21	19	20
Weight, (g ± SD)	2.94 ± 1.99	2.62 ± 1.19	3.09 ± 1.12	2.93 ± 1.25

The weights for the inangas and common bullies did not differ significantly between their individual treatment groups. Because Bartlett's test for homogeneity of variances was significant with the data of the bullies a log transformation was done prior to performing ANOVA (Table 7.1.).

7.3 Sublethal effects of PCP exposure

7.3.1 Blood glucose

The blood glucose concentration of inanga decreased significantly (p <0.05) at the low and medium doses of PCP (Figure 7.1). Because the blood volumes were small and the mortality rate high there was no sample, unfortunately, for the high dose. The blood glucose concentration of common bully were unaffected by PCP, i.e. statistically insignificant, at all doses examined (Figure 7.2).

The quadruplicate bully blood samples from a single individual had a mean (± SD) glucose concentration of 2.75 ± 0.31 mmol/L, with a coefficient of variation of 11%. Human blood was also analysed in triplicate in the same manner, and the results (4.72 - 7.44 mM) were close to the expected ranges for human blood glucose (3.88 - 6.39 mM). The mean glucose concentration for human blood was 5.21 ± 0.47 (9% coefficient of variation) and 6.33 ± 0.94 mM (15% coefficient of variation), when the samples were analysed concurrently with the inanga and bully blood samples, respectively.

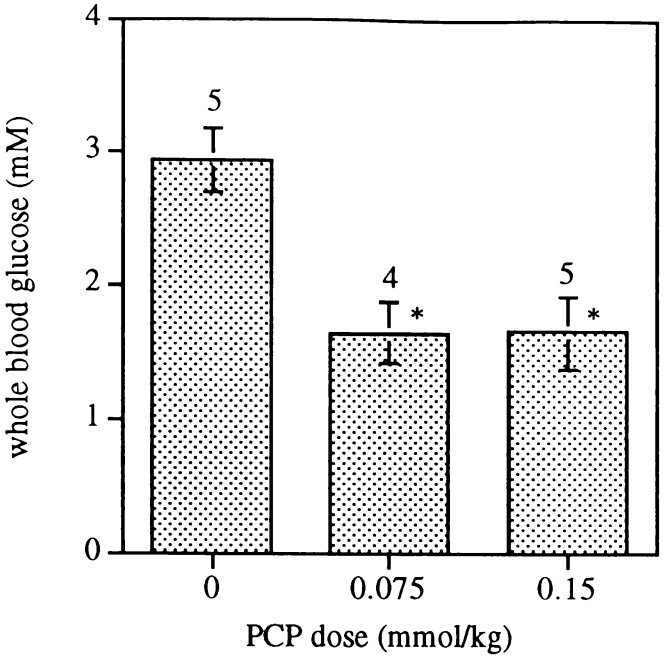


Figure 7.1 Blood glucose concentration (mean ± SE) of inanga when exposed to 0.075 and 0.15 mmol/kg PCP. The number of analysed samples is shown above the error bars. Significant differences to the control are marked as *(p <0.05) (Dunn's test).

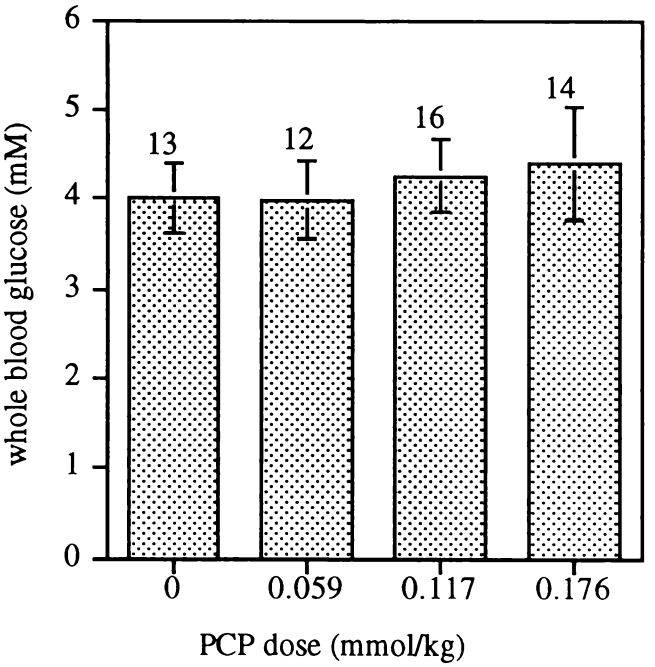


Figure 7.2 The effect of increasing PCP dose on the blood glucose concentration (mean ± SE) of common bully. The differences are not statistically significant. Also shown are the number of analyses per group above the error bars.

7.3.2 Blood lactate

The blood lactate concentration of inanga were unaffected significantly by exposure to PCP (Figure 7.3). There was no sample for the high dose due to small sample sizes and a low number of surviving inanga in this group. The blood lactate concentration of common bully showed a mixed response, with a decrease in lactate at the low and medium doses, and an increase at the highest dose (Figure 7.4). None of the differences were, however, significant.

Two bully blood samples were analysed in triplicate for lactate and the results were 0.29 ± 0.22, and 1.36 ± 1.01 mM, with coefficients of variation of 76 and 74%, respectively. Human blood was also analysed as controls, and the resulting values (0.90 - 1.49 mM) overlapped with normal resting values (0.33 - 1.33 mM, Sigma). The single human blood sample analysed with the inanga samples had a lactate concentration of 1.49 mM, and the duplicate human blood sample analysed concurrently with the bully samples had blood lactate concentration of 0.90 and 1.00 mM.

7.3.3 Total red blood cell count

The total red blood cell number for inanga was unaffected significantly by PCP exposure (Figure 7.5). The red blood cell count of common bully was also unaffected significantly when the fish were exposed to PCP (Figure 7.6).

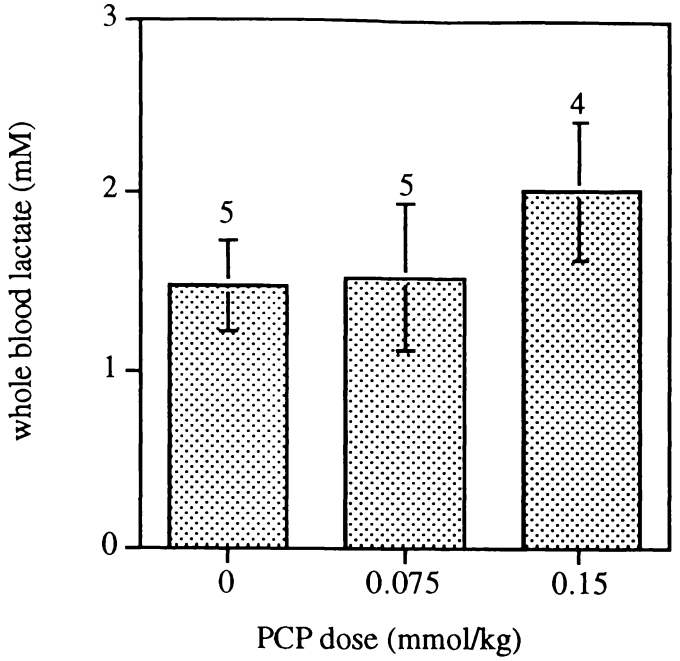


Figure 7.3 The effect of increasing doses of PCP on the blood lactate concentration (mean ± SE) of inanga. The increase is not significant. Also shown above the error bars is the number of analyses per group.

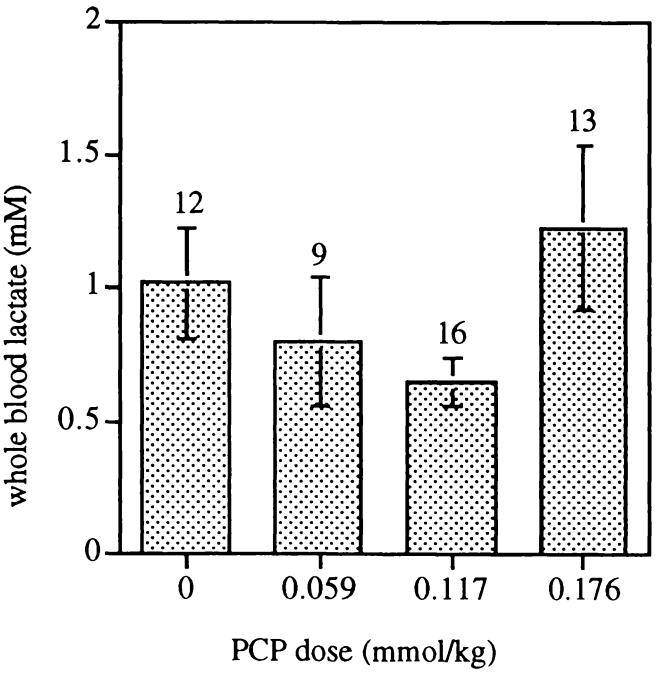


Figure 7.4 The effect of increasing doses of PCP on the blood lactate concentration (mean ± SE) of common bully. The differences are not significant. Also shown are the total number of analyses per group above the error bars.

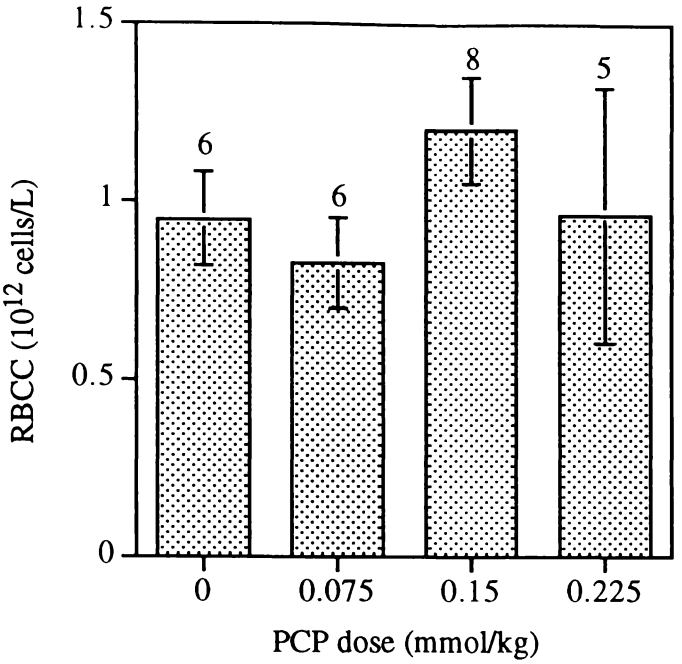


Figure 7.5 The effect of increasing dose of PCP on the total red blood cell count (mean ± SE) of inanga. The differences are not significant. The number of samples per group is shown above each error bar.

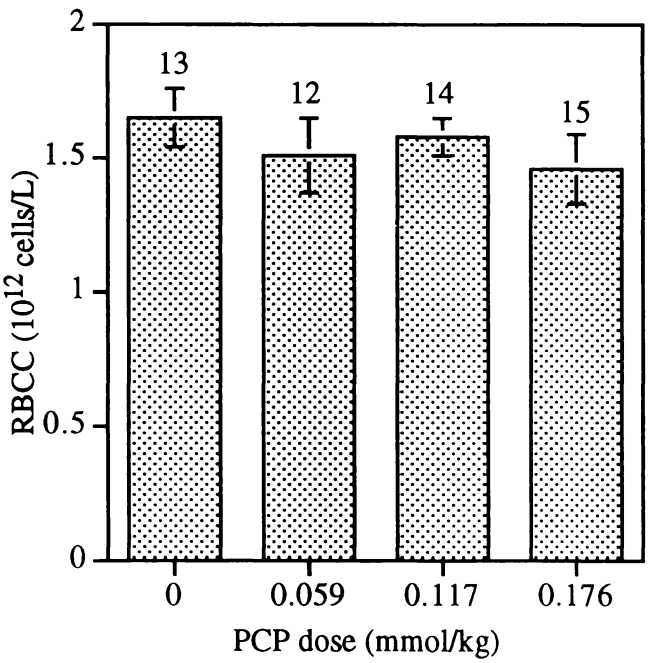


Figure 7.6 The effect of increasing dose of PCP on the total red blood cell count (mean ± SE) of common bully. The differences are not significant. The number of samples per group is shown above the error bars.

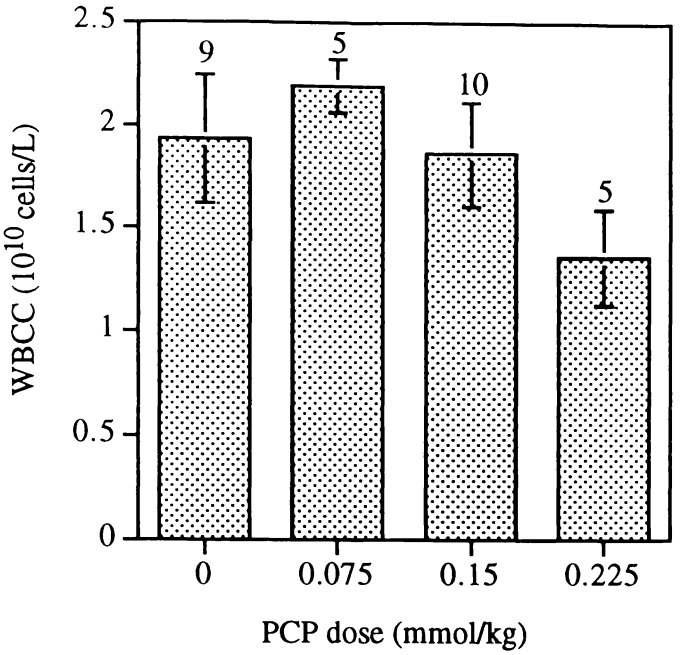


Figure 7.7 The effect of increasing dose of PCP on the total white blood cell count (mean ± SE) of inanga. The differences are not significant. The number of fish per group is shown above the error bars.

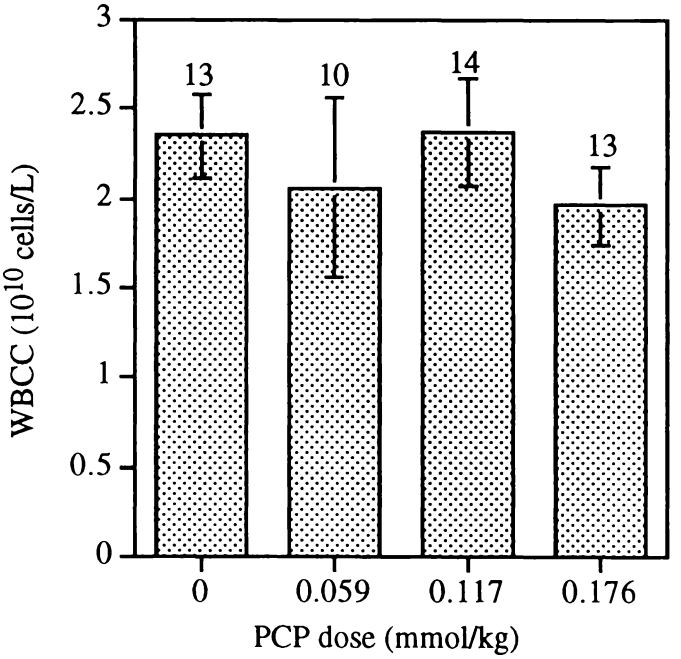


Figure 7.8 The effect of increasing PCP dose on the total white blood cell count (mean ± SE) of common bully. The number of samples per group is shown above the error bars. The differences are not significant.

7.3.4 Total white blood cell count

The total white blood cell number for inanga was not significantly affected by exposure to PCP (Figure 7.7). The total white cell number for common bully was unaffected by PCP in all treatment groups, with insignificant decreases at the low and high doses (Figure 7.8).

7.3.5 Differential white blood cell count

The percentage of lymphocytes for inanga was not significantly affected after exposure to PCP (Figure 7.9). The percentage of thrombocytes tended to increase with higher PCP doses (from approximately 8% in the control to about 16% in the high dose group), but these increases were also not significant. The percentage of granulocytes showed a mixed and insignificant reaction to PCP.

The numbers of lymphocytes, granulocytes and thrombocytes showed no significant changes when common bully were exposed to PCP (Figure 7.10). The consistency when counting common bully leucocytes from the same slide three times, but from different areas of the slide, was quite reproducible. The percentages of lymphocytes were 55, 57 and 57%, thrombocytes were 13, 14 and 11%, and granulocytes were 32, 29 and 32%, respectively.

7.3.6 Uridine 5'-diphosphoglucuronyl transferase activity

The UDPGT reaction was linear up to 20 min for both inanga and common bully (Figures 7.11 and 7.12), and it was linear when up to 0.72 mg and 0.76 mg protein was incorporated into the assay, for inanga and bully, respectively (Figures 7.13 and 7.14).

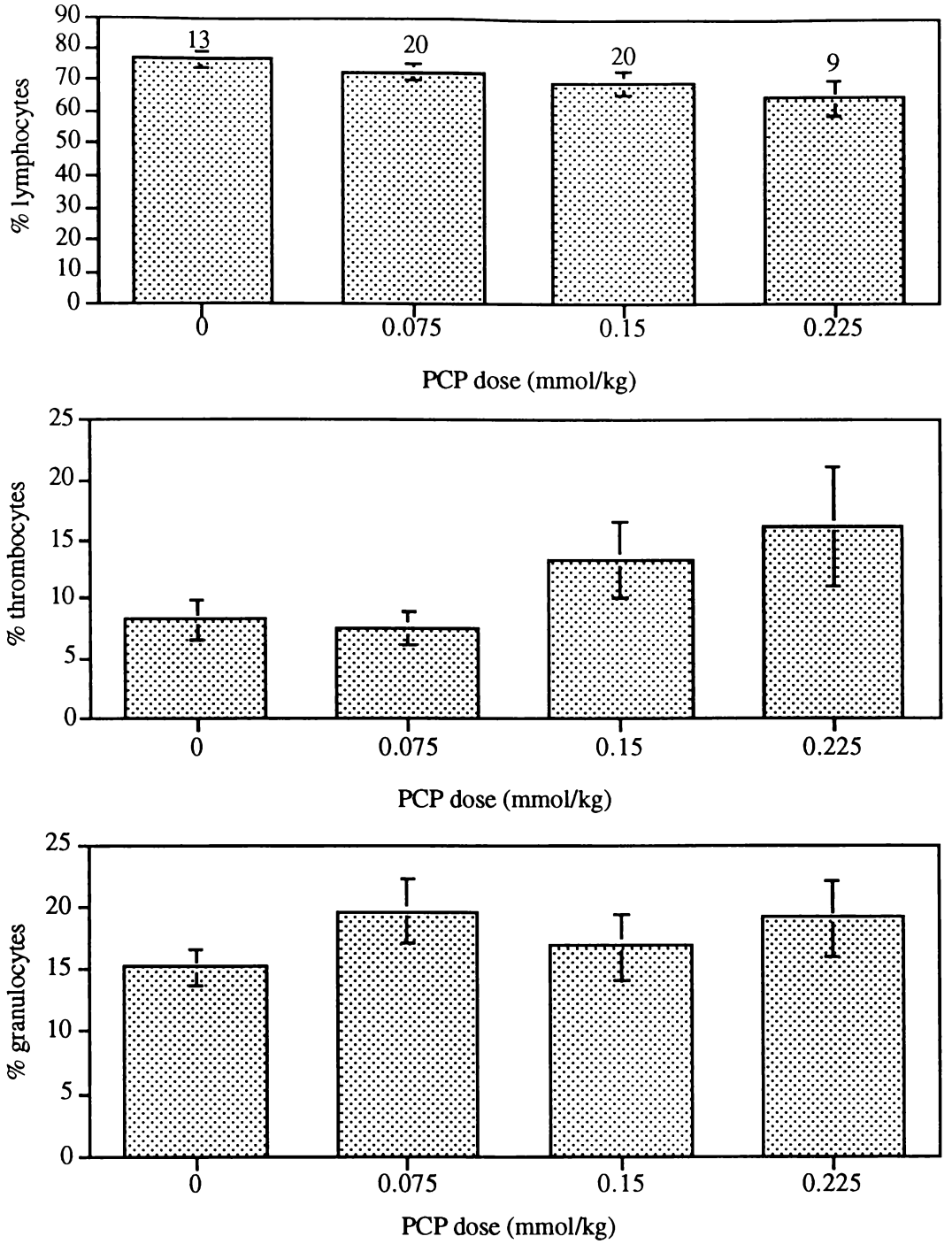


Figure 7.9 The effect of increasing doses of PCP on the differential white blood cell count of inanga (mean ± SE). The number of samples that were counted per group is shown above the error bars for the lymphocytes. None of the differences were significant.

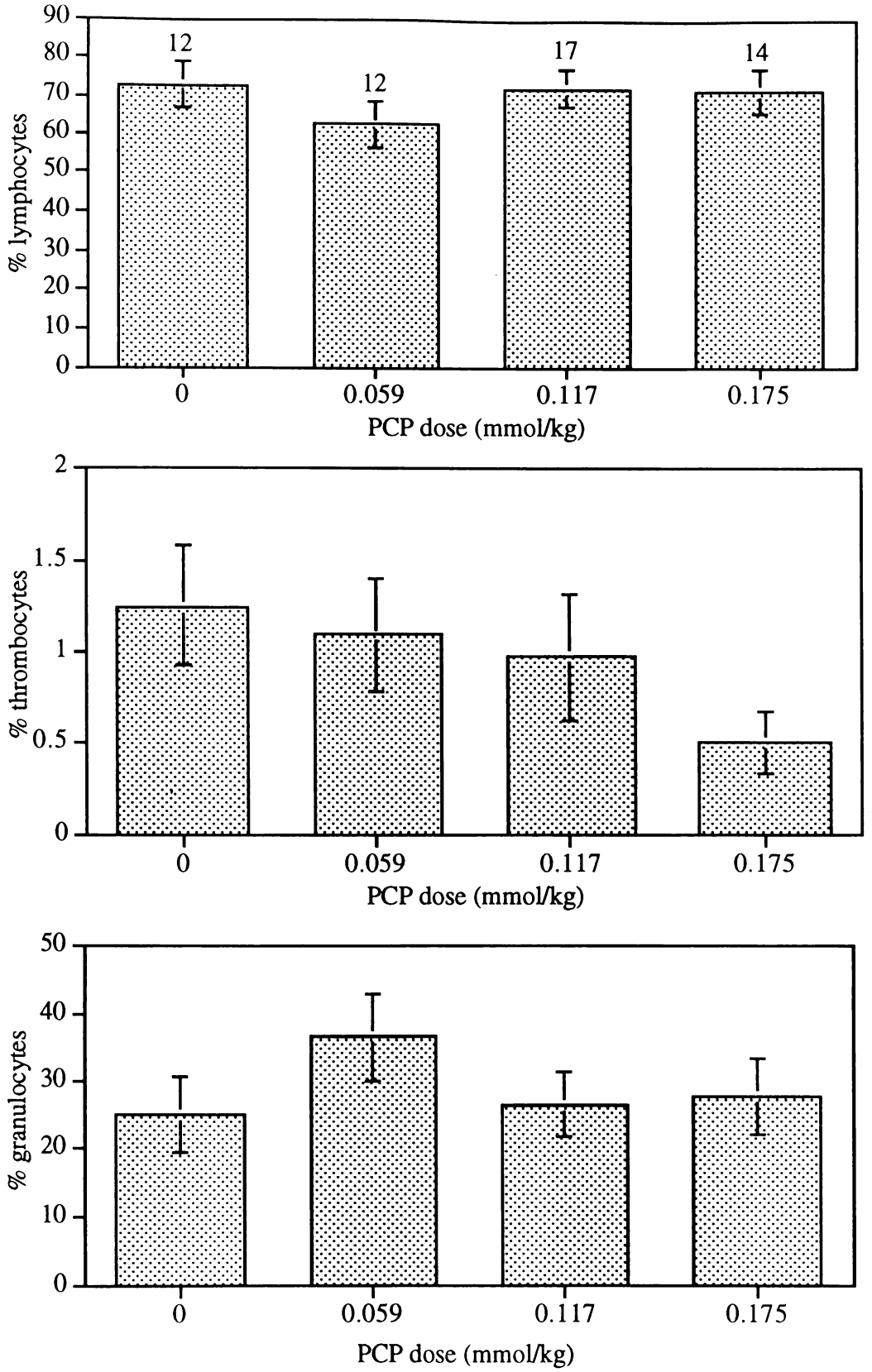


Figure 7.10 The effect of increasing doses of PCP on the differential white blood cell count of common bully (mean ± SE). The number of samples that were counted per group is shown above the error bars for the lymphocytes. There were no significant differences in the treatment groups compared to the controls.

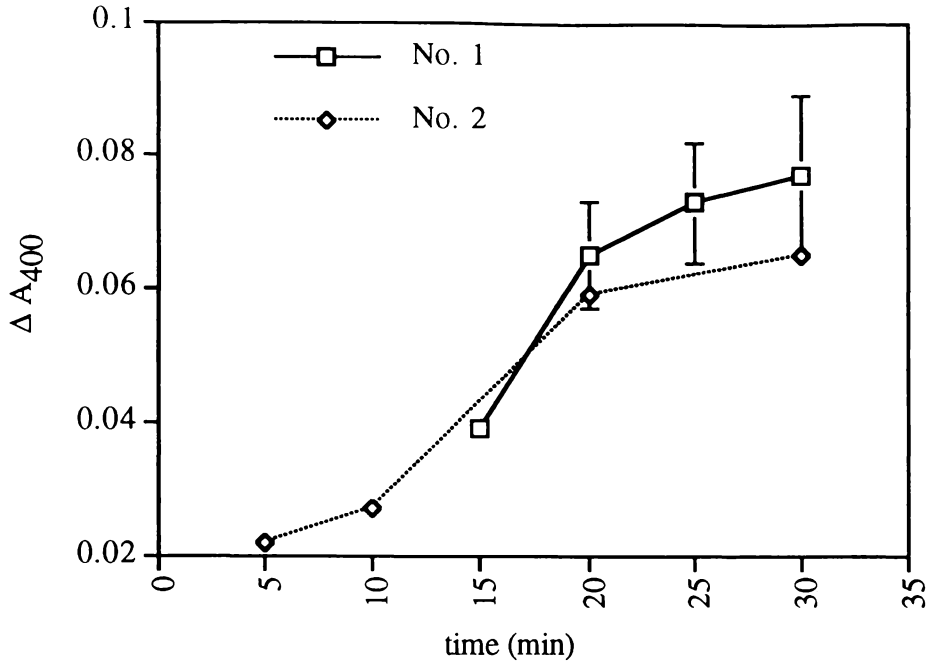


Figure 7.11 Linearity of the UDPGT assay up to 20 min using two different inanga post-mitochondrial fractions. No. 1 was done in triplicate and shows the standard deviations, and No. 2 was done in duplicate.

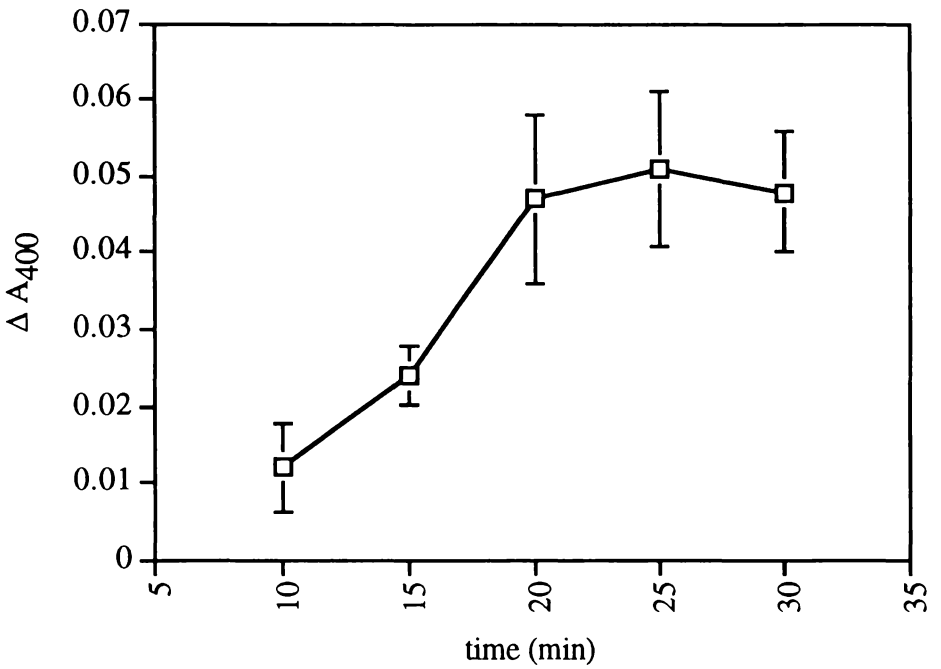


Figure 7.12 Linearity of the triplicate UDPGT assay (± SD) for common bully up to 20 min.

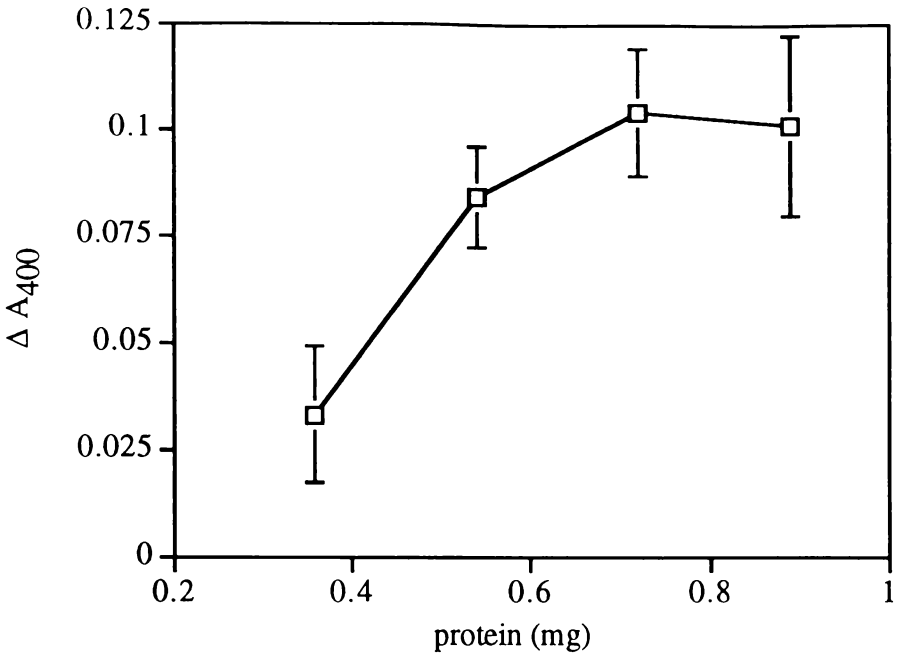


Figure 7.13 Linearity of a triplicate UDPGT assay (± SD) for inanga when using increasing protein content in the assay, and an incubation period of 20 min.

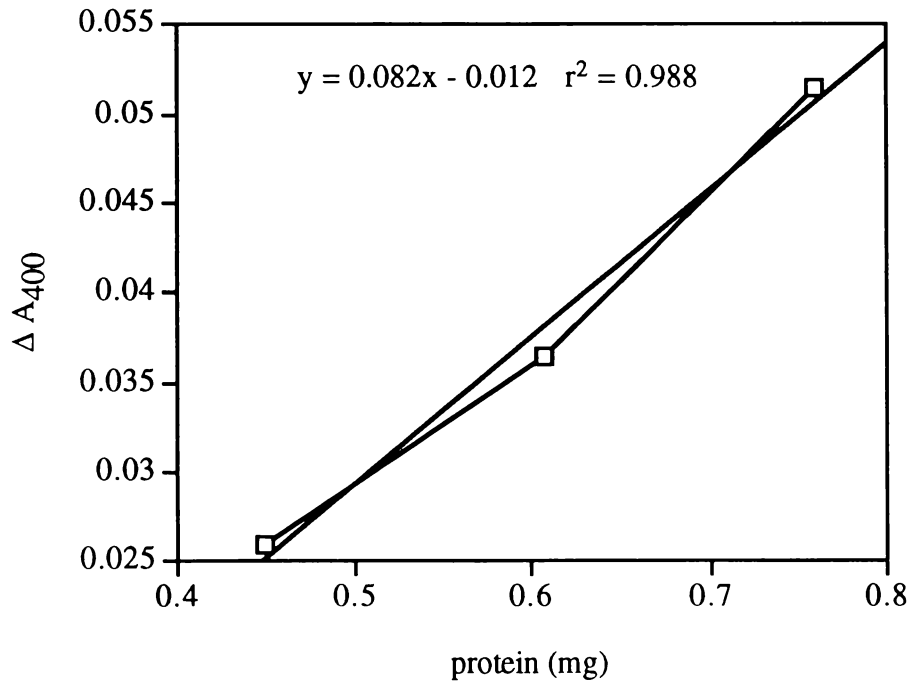


Figure 7.14. Linearity of the duplicate UDPGT assay in common bully with increasing amount of protein and an incubation period of 20 min.

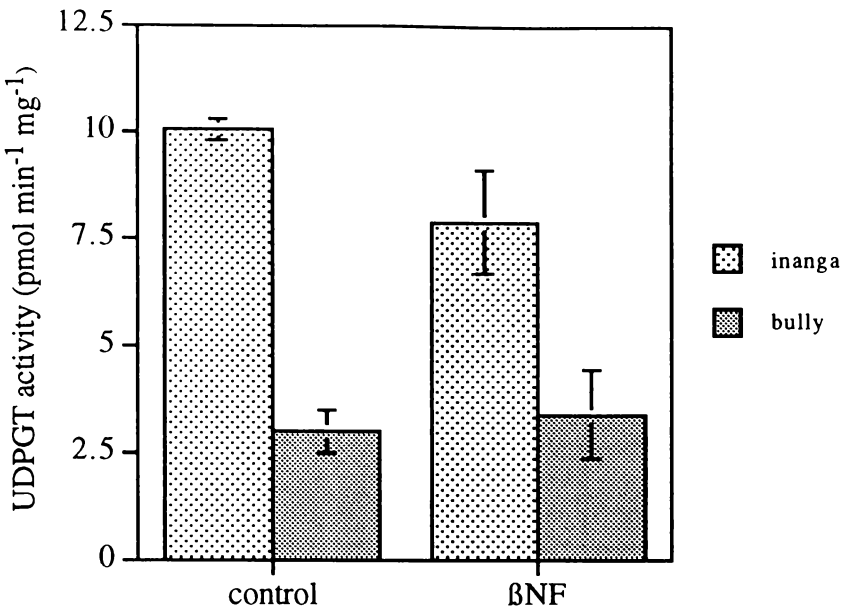


Figure 7.15 The effects of ß-naphthoflavone (ßNF) on the UDPGT activities (± SE) of inanga and bully injected at dosages of 100 mg/kg ßNF. The differences between control and ßNF-injected fish are not significant. Four fish per group were analysed.

The effects of ß-naphthoflavone (100 mg/kg or 0.367 mmol/kg) on the UDPGT activities of inanga and bully are shown in Figure 7.15. The average activity for bully was virtually unchanged, while the activity for inanga was not significantly affected (p = 0.101 and 0.132, respectively).

PCP significantly repressed the average UDPGT activity of inanga (Figure 7.16), from about 9 pmol min^{-1} mg^{-1} to about 5 pmol min^{-1} mg^{-1}, as analysed by regression analysis (p = 0.041). The sample size (n = 5 - 10) was, however, too small to perform Bartlett's test. The UDPGT activities of PCP-exposed common bully were virtually unchanged compared to the control and were close to 4 pmol min^{-1} mg^{-1} in all treatment groups (Figure 7.17).

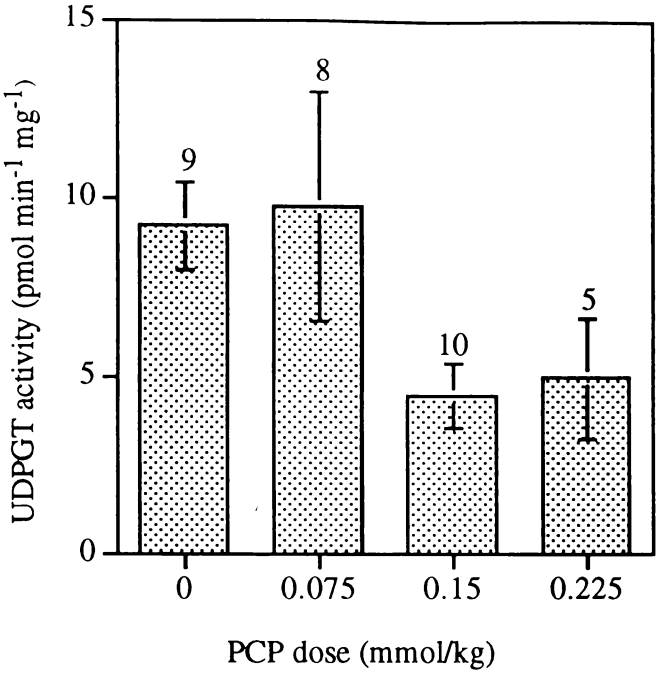


Figure 7.16 The effects of increasing PCP dose on the UDPGT activities (mean ± SE) of inanga. The number of analyses is indicated above each error bar. The decrease is significant (p = 0.041) by regression analysis but not by ANOVA.

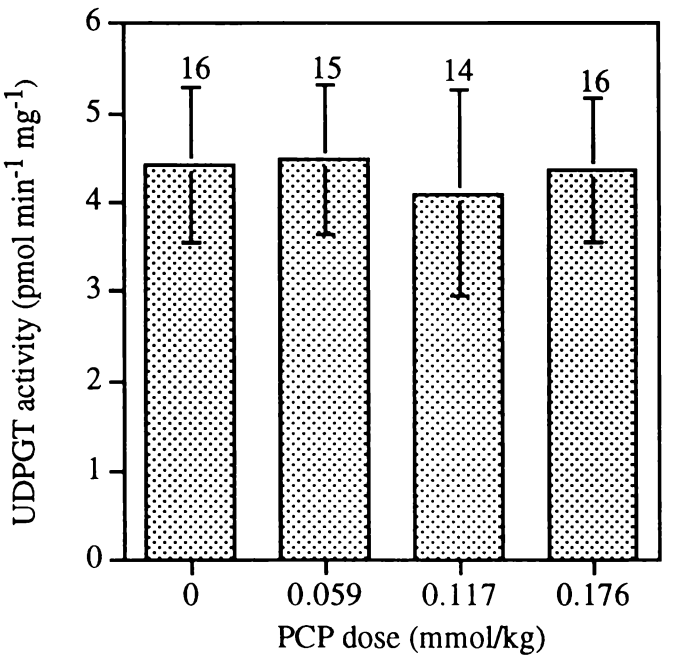


Figure 7.17 The effects of increasing PCP dose on the UDPGT activities (mean ± SE) of common bully. The number of analyses is shown above each error bar. There are no significant differences between the groups.

7.3.7 Ethoxyresorufin-*O*-deethylase activity

The EROD reaction was linear for up to 42 min for inanga (Figure 7.18) and up to 70 min for common bully (Figure 7.19). Some of the common bully samples had very low EROD activity, but the activity was detectable when compared to a sample that had been boiled for three minutes (sample No. 4 versus sample No. 5 in Figure 7.19). The reaction was linear up to 0.69 mg for inanga (Figure 7.20), and up to 0.6 mg protein in the assay for bully (Figure 7.21).

There was no significant effect of exposure to 100 mg/kg of ß-naphthoflavone on average EROD activity of both inanga and bully (Figure 7.22) (p = 0.370 and 0.121, respectively, as analysed by unpaired t-tests).

The average EROD activities for all PCP exposed inanga were not significantly affected (Figure 7.23) (p = 0.085 by regression analysis). When results from mature inanga were removed the profile remained similar and the p-value decreased slightly (p = 0.062 by regression; data not shown). The level of increase between the control and the high dose was, however, higher than that caused by ßNF (2.46-fold increase versus 1.55-fold increase with ßNF).

The average EROD activities of common bully exposed to PCP was not significantly different from controls and varied in a manner apparently unrelated to the dose (i.e. higher in low, lower in medium and higher in high dose) (Figure 7.24). If the mature common bully females were excluded from the analysis, the result was not changed appreciably (not shown).

7.3.8 Heat shock protein 70

Initial trials with the N27 antibody in inanga were able to detect, after Western blotting and chemiluminescent detection, a single band for gill and liver tissue at 72 kDa and no

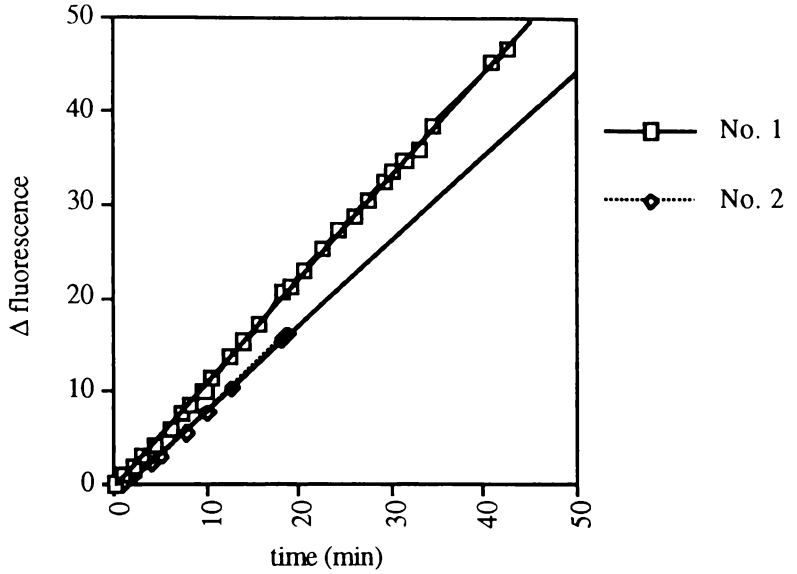


Figure 7.18 Linearity of the EROD assay for two different inanga samples up to 42 min.

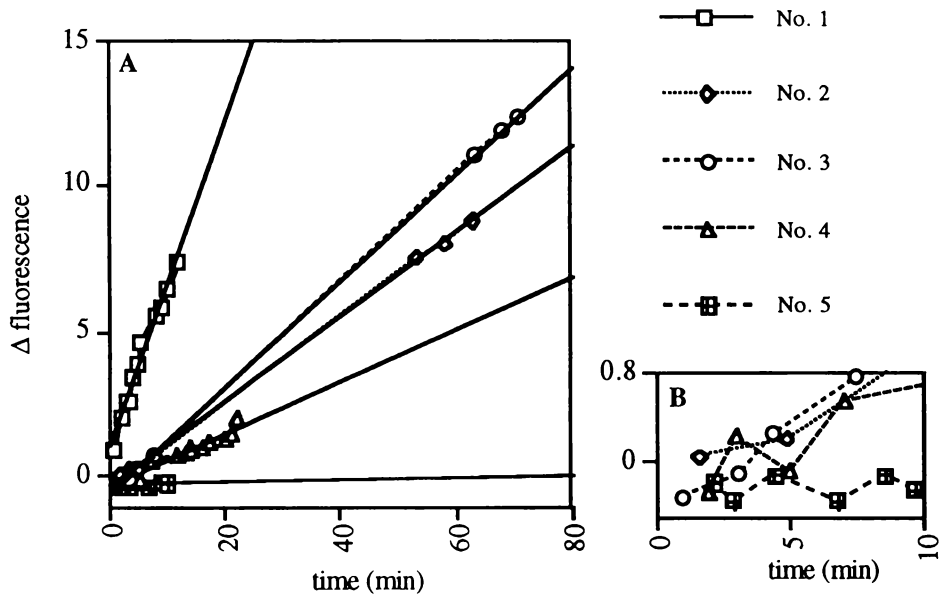


Figure 7.19A Linearity for up to 70 min of the EROD assay for common bully shown with four samples with different levels of activities (No. 1-4) and one sample which had been boiled for three min (No. 5). **B** The same graph using identical data as in A for the first 10 min.

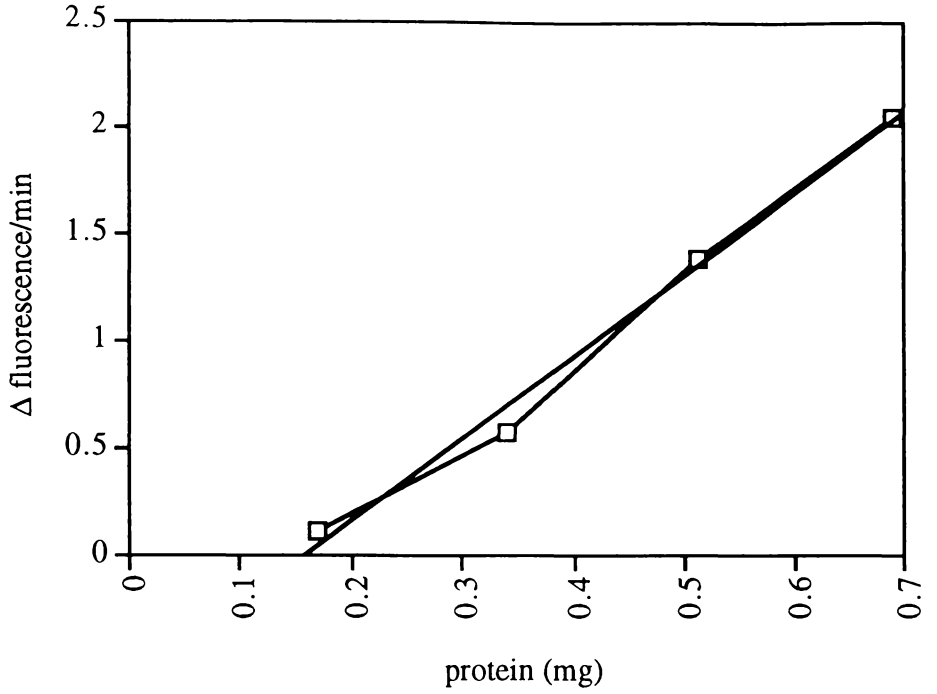


Figure 7.20 Linearity of the EROD assay for inanga with increasing amounts of protein in the assay.

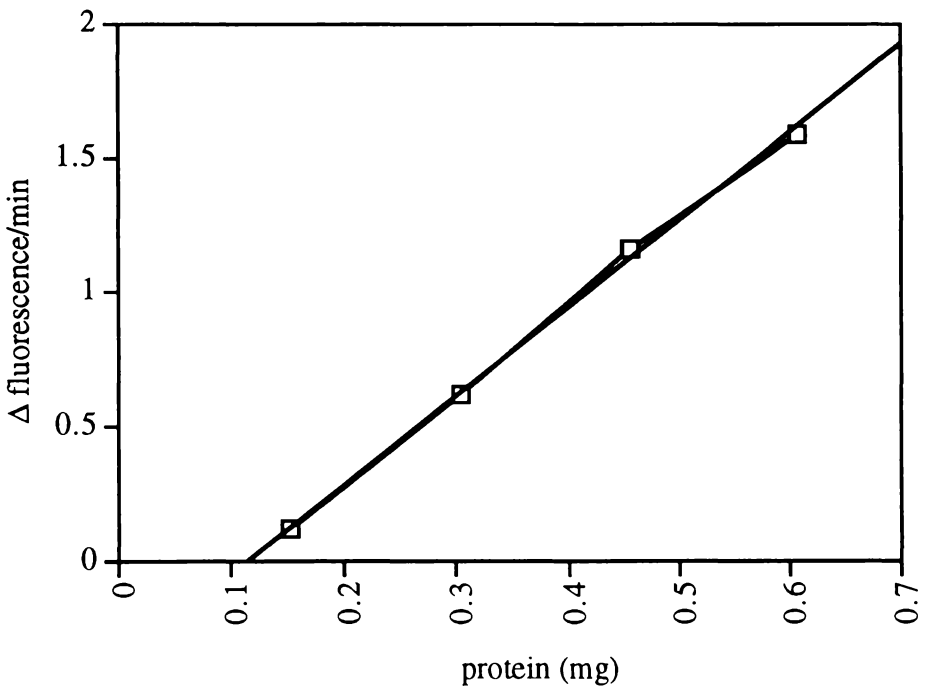


Figure 7.21 Linearity of the EROD assay for common bully with increasing protein contents in the assay.

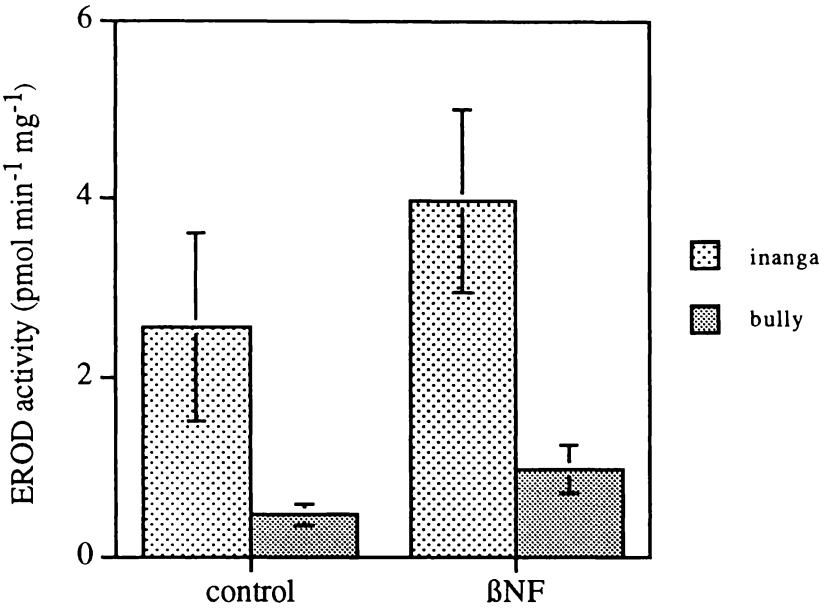


Figure 7.22 The effect of treatment with ß-naphthoflavone (ßNF) at 100 mg/kg on the EROD activities (mean ± SE) of inanga and common bully. The increases in activity were not significant. The number of samples was 5 per group.

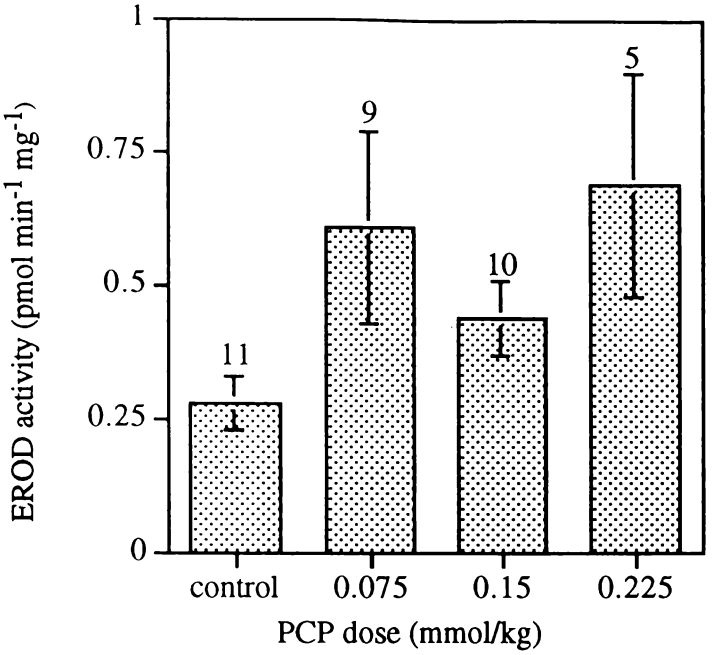


Figure 7.23 EROD activities (mean ± SE) of inanga when exposed to PCP at the doses shown on the x-axis. The increase in activity is not significant. The number of duplicate analyses per group is also shown above error bars.

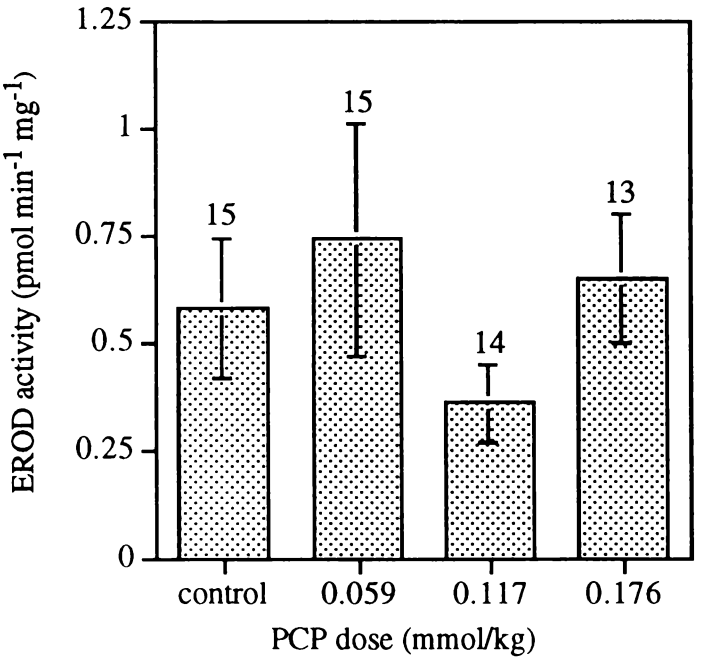


Figure 7.24 EROD activities (mean ± SE) of common bully when exposed to PCP at the doses shown on the x-axis. The number of duplicate analyses per group is also shown above the error bars. The differences are not statistically significant.

bands for muscle and brain tissue. The C92 antibody recognised a band at 112 kDa and later at 125 kDa in gill and muscle tissue, but not in brain or liver. Because of the molecular weight of the bands detected by C92, and because the C92 antibody had not previously recognised an inducible hsp70 in nonmammalian tissue (Sanders et al. 1994), the use of the C92 antibody was abandoned, and all other studies were performed with the N27 antibody.

N27 recognised a single band at approximately 72 kDa in both inanga and bully gill tissue. When a dilution series of gill extracts from control and heat-shocked fish were run on the same gel it was shown that the heat shock treatment had induced hsp70, as recognised by N27, in both inanga and common bully. The increase varied from 12 to 31% at different dilutions over the controls for inanga and from 27 to 160% at different dilutions over the control for common bully (Figures 7.25 and 7.26). The data were derived from the densitometric analysis of films shown in Figures 7.27 and 7.28.

The responses of hsp70 for PCP-exposed inanga are shown in Figures 7.29 (densitometric analysis) and 7.30 (Western blot). The hsp70 content was not significantly affected by exposure to PCP relative to controls.

There was a nonsignificant trend ($p = 0.0563$ by regression analysis) towards a dose-dependent increase in hsp70 for common bully gill tissue when exposed to PCP. The level of increase was, however, of similar magnitude to that caused by heat shock (99% increase between control and high dose for bully, compared to a 27 to 160% increase caused by heat shock, shown in Figure 7.26).

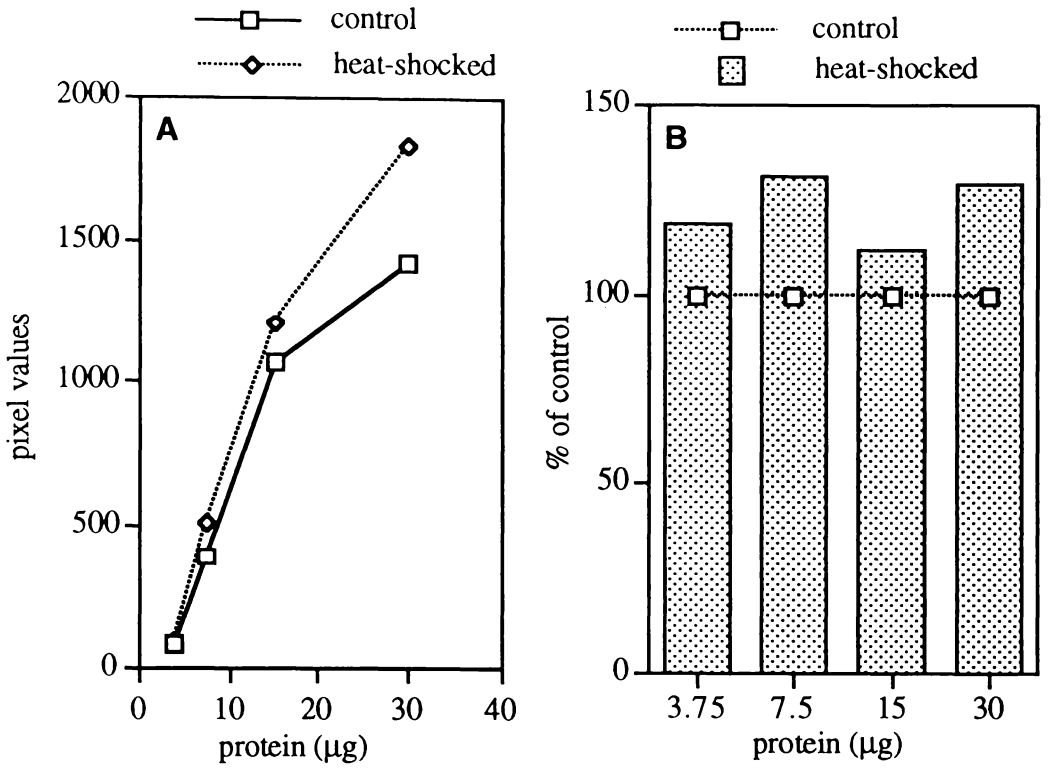


Figure 7.25 Hsp70 in control and heat-shocked inanga gill tissue. (A) shows the linear increase in detected hsp70 with increasing protein loading, and (B) shows the increase in hsp70 in heat-shocked samples over the control samples.

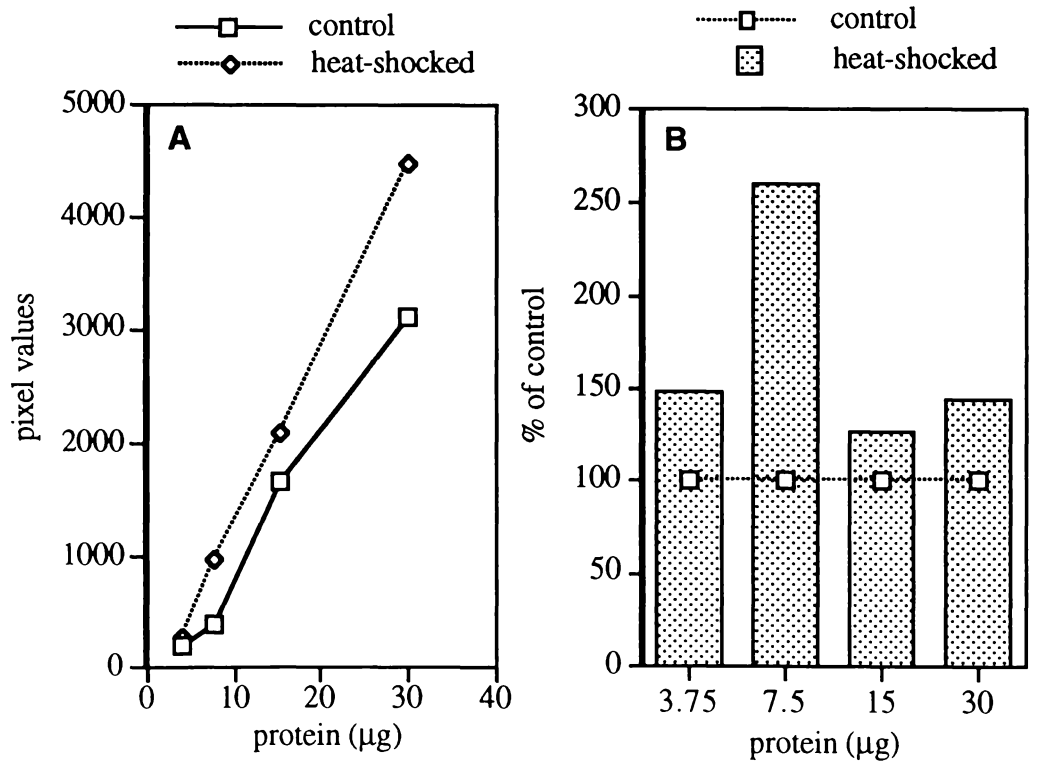


Figure 7.26 Hsp70 in control and heat-shocked common bully gill tissue. (A) shows the linear increase in detected hsp70 with increasing protein loading, and (B) shows the increase in hsp70 in heat-shocked samples over the control samples.

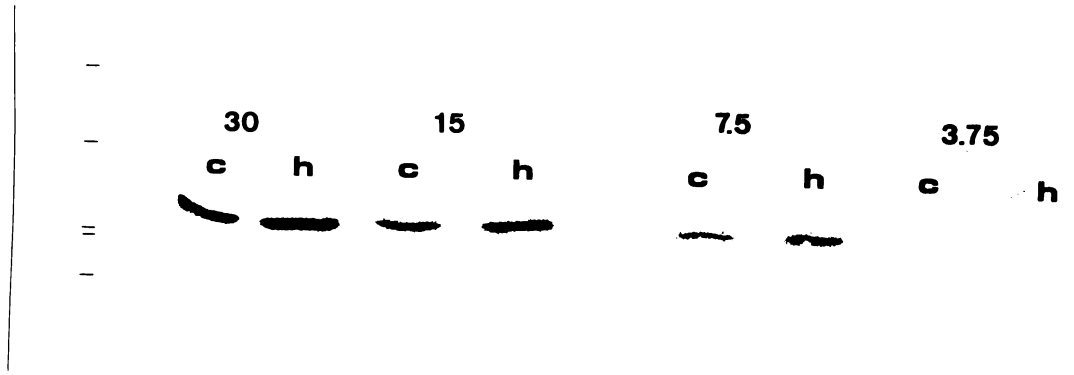


Figure 7.27 The effect of heat shock on the hsp70 of inanga gill tissue as recognised by the N27 antibody. C and h denote control and heat-shocked fish, and the numbers indicate the amount (μg) of protein loaded onto each lane. The film was exposed by chemiluminescence for 5 seconds. The molecular weight markers on the left are 112, 84, 66, 63 and 52.5 kDa from top to bottom.

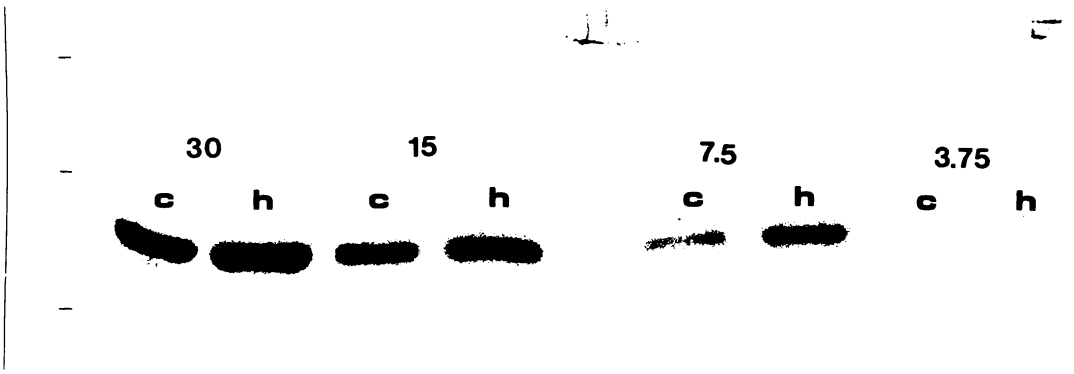


Figure 7.28 Induction of hsp70 by heat shock as recognised by the N27 antibody in common bully gill tissue. C and h denotes control and heat-shocked samples, and the numbers indicate the amount of protein (μg) loaded onto each lane. The film was exposed by chemiluminescence for 15 seconds. The molecular weight markers are 112, 84 and 66 kDa on the left side, from top to bottom.

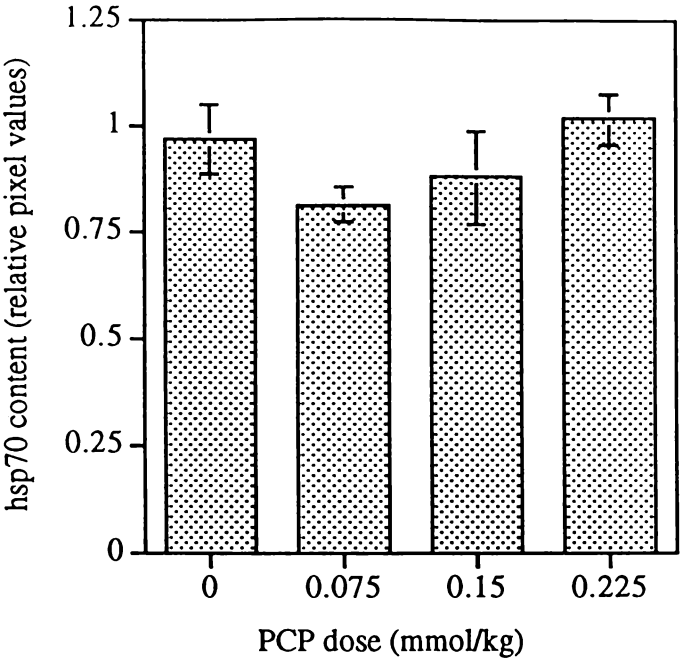


Figure 7.29 The relative amount of hsp70 (± SE) in inanga gill tissue as a response to increasing doses of PCP. There are no significant differences between the experimental groups. Each bar is based on four lanes, each a pooled sample from three fish. The pixel values are based on the film of a blot shown in Figure 7.30.

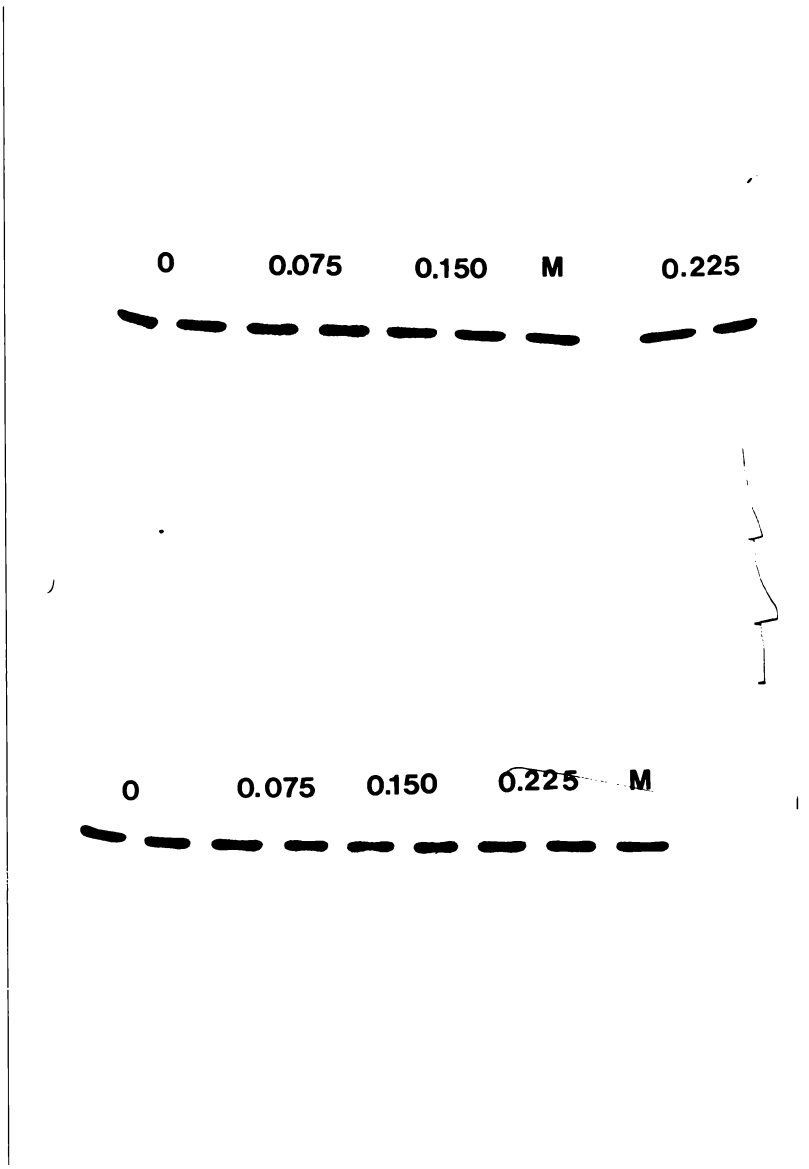


Figure 7.30 The effect of increasing PCP on hsp70 content in inanga gill tissue. The PCP doses (mmol/kg) are indicated in the figure over two lanes. Each lane consisted of a pooled sample from 3 fish. 'M' is the marker band to which the other bands were compared during densitometric analyses.

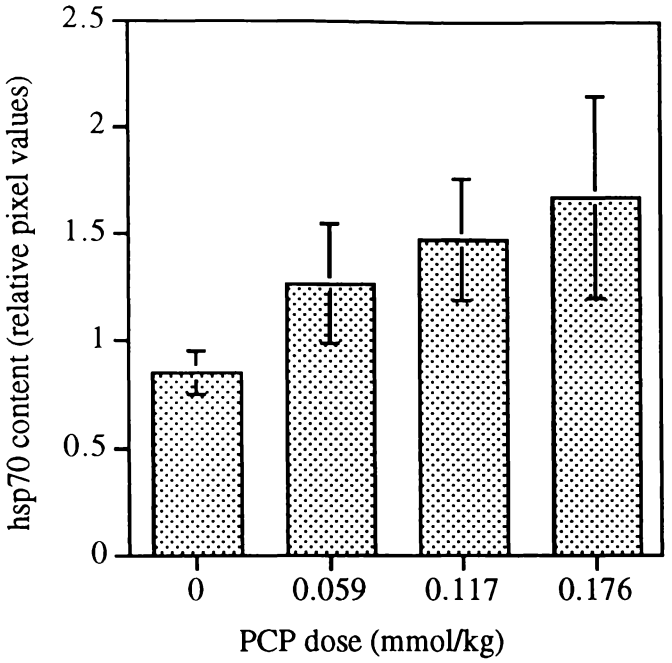


Figure 7.31 The relative amount of hsp70 (± SE) in common bully gill tissue as a response to increasing doses of PCP. The increase is not significant (as analysed by ANOVA ($p = 0.3553$) and regression ($p = 0.0563$)). Each bar is based on six lanes, each a pooled sample from three fish. The pixel values are based on the film of a blot shown in Figure 7.32.

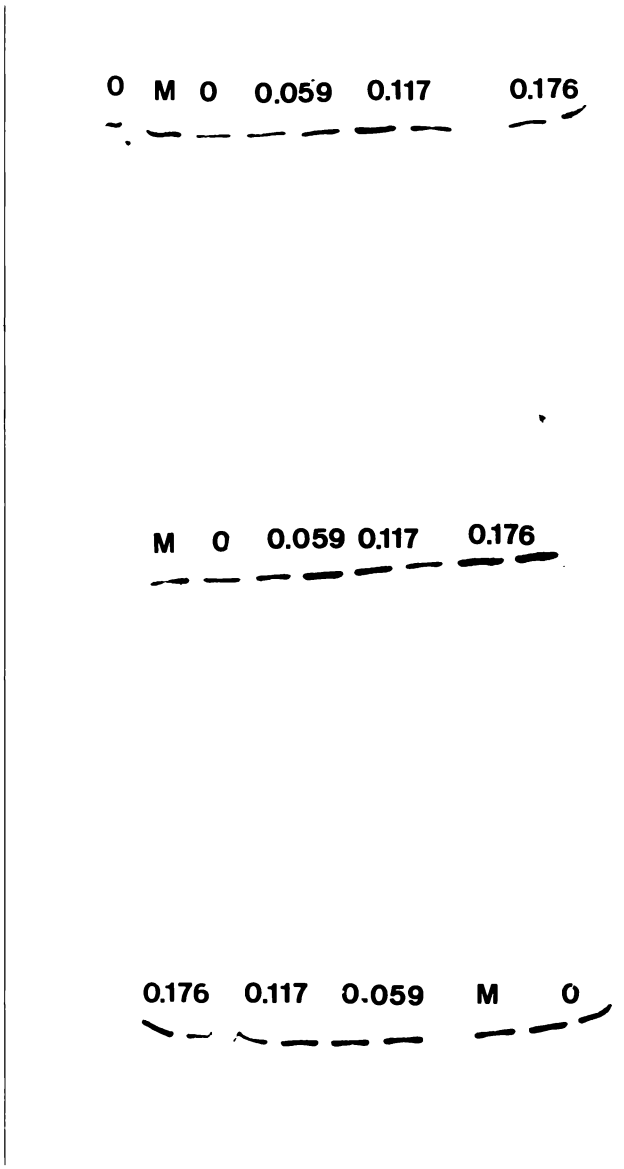


Figure 7.32 The effect of increasing doses of PCP (mmol/kg) on the hsp70 content in common bully gill tissue. The doses are indicated over two lanes in the figure. Each lane consisted of a pooled sample from 3 fish. The marker (M) sample was a control sample that was included in each gel and the pixel values of all other lanes were related to the marker value.

CHAPTER 8

DISCUSSION FOR EARLY LIFE STAGE TESTS

8.1 Developmental rates and size at hatch for control group eggs

The developmental stages of all the five tested species occurred through a sequence similar to that reported for inanga (Benzie 1968) and rainbow trout (Knight 1963), with some differences in the timing of the developmental events (Table 5.2). The developmental rates and sizes of hatched larvae in the control groups of all tested species is compared in this section to what has been reported in previous studies.

The incubation period to 50% hatch at 18°C was 10-11 days for synthetic and solvent control smelt in this study. This is somewhat shorter than what has been reported by both Mora and Boubée (1993) and McCarter et al. (1995). For instance, at the same incubation temperature (18°C) it took 17 days to 50% hatch (Mora and Boubée 1993). Furthermore, McCarter et al. (1995) observed that at 14 and 20°C it took almost 19 and 14 days to 50% hatch, respectively. The reason for smelt eggs having shorter incubation periods in this study than those reported elsewhere is not known with certainty, but it is possible that a combination of both the incubation conditions and hatching triggers in the present study were especially conducive for the hatching of smelt. For instance, it is conceivable that exposing the smelt eggs to pipetting and microscope light on a daily basis may have been potential triggers. It was often noticed that the pipetting of smelt eggs at an advanced developmental stage appeared to induce many smelt eggs to hatch during the process. Constant aeration and genetic differences may have also, in part, contributed to the observed shorter incubation times. With respect to the size at hatch, riverine stocks of smelt hatchlings have been reported to be about 6 mm (McCarter 1994), which is slightly longer than the 4.8 - 5.6 mm measured for control smelt at hatch in this study.

The incubation period to 50% hatch for inanga eggs maintained in water at 18°C was between 15 and 26 days (Table 5.2). This is a relatively broad range, which may be related to the fact that the oxygen content was not lowered to trigger hatching, as would have been the case if the eggs had been transferred from air incubation to water, as was done by Mitchell (1989) and Benzie (1968). Both Benzie (1968) and Mitchell (1989) found that inanga eggs were ready to fully hatch when transferred from air incubation to water after 10 days of development at 18° and 17°C, respectively. In contrast, when inanga eggs were incubated in water at 18°C, the mean incubation time (average time-to-hatch) was 13.5 ± 0.6 days (Carlberg 1993). McCarter (1994) reported 50% hatch of inanga between 14 and 28 days under conditions of unknown temperature and incubation method. It seems, therefore, that incubation in air either speeds up the development or provides a stronger signal for hatching upon re-immersion in water. Some events of embryonic development in inanga at 17°C described by Benzie (1968) apparently took place earlier, such as the blastopore closing at 12 h, whereas inanga eggs in this study, incubated at 18°C had only reached 50% epiboly at 24 h. However, the early eyed stage was reached at a similar time, 3.25 days for Benzie (1968) and 3-4 days in this study. The lengths of newly hatched control inanga larvae in this study were 6.62 - 7.30 mm which were similar to what has been reported previously (5.8 - 8 mm; Mitchell 1989; McCarter 1994).

Control koaro eggs took between 24 and 37 days to reach 50% hatch at 13°C. West (1989) incubated fertilised koaro eggs at the same temperature, and hatching took place between 18 and 33 days. Similarly to inanga, the wide range of incubation periods seen in the present study for koaro may have been a result of a lack of a suitable trigger for hatching. The likelihood that this trigger is a lowered oxygen concentration, as it may be for inanga, is not high because the highest success rate for incubation of koaro eggs was in water and not on filters (West 1989). The trigger for hatching, therefore, remains uncertain and requires further study. It must also be recognised that delayed

hatching may be the result of an insufficient level of hatching enzyme activity, as mentioned in Section 3.3.4.

Common bully eggs had the shortest incubation to time-of-hatch among the tested species; 50% hatch occurred between days 7 and 10 at 18°C. This is similar to the results of a study by McCarter (1994) where 50% hatch occurred on day 7 when incubated at unknown temperature. The lengths of control bullies in this study (2.8 - 3.1 mm) are slightly less than those reported (3.4-3.8 mm) by McCarter (1994).

In one study that characterised the development of rainbow trout by (Knight 1963), it was found that at 12°C, the blastoderm was formed in 24 h, the eyes were pigmented at 12 days, and peak hatching occurred on the 23rd day. Willis (1994) incubated 11 batches of rainbow trout eggs (derived from a different hatchery than those used in the present study) at 13 °C, and the 50% hatch occurred between days 23 and 30 after fertilisation. These results are similar to those observed in this study, where 50% hatch occurred between 25 and 29 days at 13°C.

One interesting observation with regard to the timing of hatch was the fact that rainbow trout hatched within a more narrow time frame, as shown by a small CV for time-to-hatch, when compared to the native fish eggs. This may, as pointed out for inanga and koaro, be a function of a lack of strong triggers for hatching in these species. More research is necessary to clarify these aspects of their development.

8.2 Survival of control eggs during tests and incubations in clean water

Overall, the conditions for incubating the fish eggs appeared to be suitable as indicated by the low mortality rates among the control eggs (Table 5.1). Importantly, the high degree of success obtained with the incubations of control eggs, both synthetic water and solvent controls, allowed a more stringent statistical analysis of post-acute test results to be performed. A less than 10% mortality in control groups made it easier to

detect small increases in mortality in exposed groups, and no Abbott's correction had to be employed in most cases. In particular, the conditions employed during the toxicity tests caused no elevated mortality of controls, with the exception of some batches of inanga and bully eggs tested at the 24 h stage. As mentioned earlier, when testing eggs at life stages as early as 24 h, it was difficult to determine which eggs were truly viable prior to initiating the toxicity tests, since eggs with apparently normal morphology in some cases died soon thereafter.

Despite the problems with some 24 h test mortalities, the overall procedures for incubating the eggs were not detrimental for the control eggs and were probably near-optimal. These conditions could be used as a starting point for additional studies into the optimisation of rearing conditions for these native species.

8.3 The acute effects of PCP

PCP was acutely toxic to fish eggs tested in this study at LC50 values between varied between 30 and 1043 ppb for all species and all life stages (summarised in Table 5.3). Newly hatched larvae were always the most sensitive life stage, with LC50 values varying between 30 and 104 ppb. This is in agreement with other studies, where fish eggs, in general, have been found to be more resistant to toxicants than larvae (Niimi and LaHam 1975; Middaugh and Dean 1977). This has also been shown to be the case with PCP as the toxicant in a previous investigation of fathead minnows by Holcombe et al. (1982). In the current studies, the variation in LC50 values between species at the same developmental stages was generally less than when comparing the LC50 values between early life stages within the same species. The exception to this was common bully, which did not show such a large degree of variation in the sensitivity to PCP between the different early life stages.

The reason for the significant variation in sensitivity between different life stages is likely to be at least partly due to the protective properties of the chorion (McKim 1985).

The fact that the common bully eggs did not seem to have a differential sensitivity to PCP between the different life stages to the degree that the other species did, may have been related to the fact that the eggs collected in the field had been manually detached at their bases. There may have been, therefore, a small opening for toxicant entry at the base of the eggs. However, it is also possible that common bully do not inherently posses the variation in sensitivity to PCP between the different embryonic and larval stages as was seen with the other native fish examined, regardless of any damage that may have been caused by the detachment of the eggs at their bases.

The role of the chorion in regulating the amount of chemical that gains entry into fish eggs has been studied, but the evidence has been inconclusive overall. It appears that toxicant entry into eggs is dependent both on the nature of the toxicant and the fish species being investigated (Weis and Weis 1989). There is certainly some entry of chemicals into eggs (Blaxter 1969), but the extent of uptake and the sensitivity of the embryos may differ. For instance, Marty et al. (1990) and Michibata et al. (1987) showed that all stages of fish eggs absorbed equal amounts of toxicants (*n*-nitroso compounds and cadmium, respectively), but that there was a differential sensitivity of the embryos to the toxicants. In contrast to these findings, Carls and Rice (1988) showed lower uptake rates of hydrocarbons into embryos than into larvae of walleye pollock (*Theragra chaleogramma*), and it was suggested that this was the primary reason for the embryos being less sensitive than the larvae. Since the internal levels of PCP of the eggs or its distribution within the embryo were not measured in this study, the ability to make firm conclusions with respect to the reasons for the differential sensitivities to PCP exposure between the different early life stages is limited.

The LC50 values of newly hatched smelt, inanga, koaro, common bully and rainbow trout in this study (Table 5.3) compare well to what has been reported for PCP and juvenile fish in the literature: 58-140 ppb for Atlantic salmon (Burridge and Haya 1990), 43 - 65 ppb for pike, lake trout and whitefish (Oikari 1987), and 218 ppb for

American flagfish (Smith et al. 1991). Smelt eggs were especially difficult to test at the 24 h stage, since mortality could not be definitely ascertained after completion of the PCP exposure at 3 days after fertilisation, and consequently no 48 h LC50 was determined for this stage for smelt.

The LC50 values reported here were arrived at using different temperatures for koaro and rainbow trout (13°C) than for the three other species (18°C). This was done out of necessity to keep the eggs in temperatures that were presumed to be near optimal for each species. The effect of this on the LC50 values was not studied and no prediction can be made whether some LC50 values would change substantially with a change in temperature (Sprague 1995). Willis et al. (1995) tested the toxicity of PCP on *Simocephalus vetulus* at two temperatures (16 and 22°C), and found no significant differences. Control survival was high (≥90%) at both temperatures. Rainbow trout eggs tested at the 24 h, early eyed, late eyed and hatched larval stages were more sensitive to PCP at 13°C than at 7°C, and again control survival was similar at both temperatures (Willis 1994). Asano et al. (1969) found that a temperature increase of 10°C doubled the toxicity of PCP (of unknown purity) to carp. Further research into the optimal incubation temperatures of the tested species and PCP toxicity at various temperatures is needed.

In one study of the early life stages of rainbow trout incubated at 10°C, the LC50 values for PCP (97% purity) were 3000, 1300, 3000, 480, 32 and 18 ppb for 0 h eggs, 24 h eggs, early eyed eggs, late eyed eggs, sac fry and early fry, respectively (Van Leeuwen et al. 1985). Willis (1994), using pure PCP at 13°C, obtained 48 h LC50 values of 969, 1351, 317 and 124 ppb for rainbow trout eggs at the 24 h, early eyed, late eyed and yolk sac fry stages, respectively. These values (Van Leeuwen et al. 1985; Willis 1994) are similar to the values obtained for rainbow trout in this study, indicating that the toxicity test results obtained with rainbow trout eggs were comparable to those found in other studies. In addition, this suggests that the results

obtained with the early life stages of native fish are also accurate, and that the use of rainbow trout as a reference organism was warranted. In the 5 cases where more than one flow-through toxicity test could be performed with the same developmental stages of native fish eggs and larvae, the resulting LC50 values also agreed fairly well with each other, further supporting the reliability and accuracy of the methods used for toxicity testing in this study.

8.4 The sublethal effects of PCP

8.4.1 Post-test hatch and mortality

With few exceptions, large proportions of eggs died shortly after termination of the acute toxicity tests at concentrations close to their LC50. This suggests that PCP either caused irreparable damage to the embryos, or that PCP inside the eggs continued to exert toxic effects on the eggs even after transfer to clean water (von Westernhagen 1988). Although the mortality rates levelled off in PCP-exposed eggs within a few days after the acute tests, eggs still continued to display mortality but at a lower rate until all the eggs had either hatched or died.

Interestingly, some of the survival curves appear to be similar to classical first-order decay curves (i.e. exponential decrease), with initial high rates of decay followed by increasingly lower rates of decay. For instance, koaro eggs exposed to 800 ppb PCP at the early eyed stage (Figure 5.8A) had an initial log-linear survival curve between day 0 and the 10th day (log % survival plotted by regression against the number of days post-test) with a slope of -6.5x (r^2 = 0.96) and between 10 days post-test to time-of-hatch the slope was -1.4x (r^2 = 0.92). It is tempting to speculate that the first of these two regression lines actually reflected the high rate of mortality due to the initial acute 48 h PCP exposure and the second line, the lower rate due to PCP that had been taken up by the eggs (presumably by the egg yolk) and then slowly released. However, some alternative scenarios are possible. For instance, it is conceivable that the lower rate starting after 10 days seen with late eyed koaro eggs may also have been due to

previous irreparable damage to the developing livers or other tissues, immuno-suppressive effects, neurological/hormonal or a combination of these which led to the observed effect.

When comparing the mortalities caused by acute exposure (post-test LC50) and the total mortalities that occurred during both the toxicity testing and subsequent incubation in clean water until hatch (termed the final LC50) (Table 5.4), it was found that the additional delayed mortality usually caused the final LC50 value to be almost half the post-test LC50. Two exceptions to this situation occurred when testing 24 h common bully and 24 h rainbow trout, where the ratios were 11.6 and 4.1, respectively. These were, therefore, especially sensitive after transfer to clean water which is possibly a result of extremely detrimental terata occurring in these eggs. This is in agreement with the review by von Westernhagen (1988) who notes that exposing eggs to toxicants prior to closure of the blastopore causes more severe reduction in hatching success than when more advanced stages are exposed.

The percentage of eggs that did hatch successfully after PCP exposure was, with few exceptions, inversely correlated to the PCP concentrations of the acute tests. The % viable hatch after exposure was lower still, which will be expounded upon further when discussing teratology (Section 8.4.4).

8.4.2 Growth

In agreement with the proposed toxic mechanism of PCP, and results previously reported in the literature (Webb and Brett 1973; Hodson and Blunt 1981; Cleveland et al. 1982; Holcombe et al. 1982; Dominguez and Chapman 1984; Brown et al. 1987; Samis et al. 1993), PCP had a detrimental effect on embryonic growth in this study (on 24 h, early eyed and late eyed eggs) with few exceptions. PCP-exposed eggs which hatched later than the controls, and thus had more time to grow, still produced smaller larvae. Even though growth responded in a consistent manner for all species, it proved

to be not as sensitive an indicator of PCP toxicity as when compared with either teratology or successful hatch. In contrast to the consistent reduction in growth due to PCP exposure of eggs, when performing toxicity tests at the larval stages, the larvae did not generally react to PCP exposure, with the exception of rainbow trout, which did show a reduction in growth. This lack of reduction in growth due to PCP exposure may be partially related to the fact that at the time of measurement of the larvae, it was only 4 days after the exposures had started, and, thus, even a total arrest in growth in the PCP exposed groups may not have been detectable when compared to control larvae.

The detrimental effect on growth caused by PCP exposure of eggs is most likely due to energy starvation of the embryos. PCP has been strongly suggested to cause a redirection of available energy to either reparation of tissue damage or detoxification instead of towards growth of the embryo (Johansen et al. 1987; Giesy and Graney 1989; Bodammer 1993). In addition, the efficiency of yolk utilisation would likely be decreased and this would also lead to a reduction in the energy that would be directed towards growth (Dave 1984; Blaxter 1988). Indeed, reductions of yolk sac resorption efficiency have been observed as a consequence of PCP exposure in rainbow trout (Hodson and Blunt 1981). In addition to a decreased efficiency of nutrient utilisation, the effect would be compounded by an increase in respiration rate. This has been shown to take place with another uncoupling agent, 2,4-dinitrophenol. This agent caused herring (*Clupea harengus*) embryos to increase their respiration by 30-400% above the controls, and the embryos utilised more carbohydrate in one day than during the entire developmental period (Stelzer et al. 1971). When exposing embryos at a late stage (e.g. late eyed) in this investigation, some of the hatched larvae were normal in appearance, but were shorter and thinner. Furthermore, exposure to PCP at an early stage can cause segments of the trunk to be omitted, concomitantly leading to shortened larvae (Rosenthal and Alderdice 1976). This could also have been the case for many of the dwarfed and clearly malformed larvae observed in this study.

Reduced growth of early life stages of fish in response to PCP has been reported in numerous previous studies (Webb and Brett 1973; Hodson and Blunt 1981; Cleveland et al. 1982; Holcombe et al. 1982; Dominguez and Chapman 1984; Brown et al. 1987; Samis et al. 1993) as well as with rats (Schwetz et al. 1974; Schwetz et al. 1978). Food conversion efficiency was also negatively affected by PCP in juvenile largemouth bass (Johansen et al. 1987) and in sockeye salmon (Webb and Brett 1973). The measuring of growth of embryonic stages of fish in this study was an easily determined experimental variable and the ability to interpret results was not limited by inconsistencies in feeding rates which has been encountered with studies of adult fish (Woltering 1984).

The issue of size-selective mortality of larger or smaller larvae has previously been discussed in the literature (Van Leeuwen et al. 1986; Ingersoll et al. 1990). The concern is that a different size of hatched fish larvae may actually be the result of a situation where certain sizes of embryos die prior to hatching due to differential size-dependent sensitivities. In this investigation, a change in the size of hatched larvae of a treatment group was considered to be a valid observation, regardless of whether this had been a direct result of PCP exposure, or whether certain sizes of embryos had a higher tendency to die prior to hatching. Even though the mechanism related to a change in larval size may differ, it is nonetheless a final result of PCP exposure.

When two batches of eggs of the same species were tested at the same stages, the results with respect to growth were generally in agreement with each other. However, in some cases, e.g. when exposing 24 h koaro, the two batches responded quite differently. These two koaro batches were also different from each other when studying other endpoints, such as malformations (Figure 5.17A and B), cranial malformations (Figure 5.23A and B), swimming performance (Figure 5.27A) and time-to-hatch (Figure 5.13C), but not when measuring post-test mortality (Figure 5.7A

and B). This discrepancy may be the result of a slightly different developmental rate for the two batches, as evidenced by different incubation periods (post-test to time-of-hatch) for the control eggs of the two batches (Figure 5.13C). A different developmental rate may have caused the two batches of eggs to be at slightly different stages when the PCP exposure was initiated. Thus, one batch may already have passed some sensitive developmental stage, that would not be apparent without studying the processes of gastrulation and neurulation in histological samples of the eggs. Gastrulation is known to be especially sensitive to pollutants (von Westernhagen 1988). In the case of koaro, the genetic heterogeneity of the egg batches was potentially less than with other tested species, except common bully, because each batch of koaro was derived from only one female and 2 - 3 males. (The egg batches from the other species examined were always a mixture of several females and males, in order to obtain sufficient numbers of eggs for each test). This homogeneity of the koaro eggs could explain differences in the developmental rates of the two batches. Another possibility is that the quality of the eggs were different, either because of parental health or the timing of fertilisation after ovulation.

8.4.3 Time-to-hatch

There was no consistent relationship between PCP exposure and time-to-hatch amongst the five species examined. Compared to controls, the tendencies were for smelt and koaro to hatch later when exposed to PCP, while inanga were unresponsive and common bully tended to hatch earlier. Rainbow trout showed a mixed response, with some cases of early hatching compared to the controls, and others with late hatching.

The biological significance of the parameter time-to-hatch and its ramifications with respect to interpretation of the toxicity tests can be examined from several different viewpoints. The reasons for the differential effects of PCP exposure on the time-to-hatch may be due to two different metabolic and physiological effects of PCP, both working in seemingly opposite directions with respect to control of hatching:

1) Because of the uncoupling effect of PCP on oxidative phosphorylation, there should be an increased metabolic rate in the exposed embryos. Increased metabolic rates increase the oxygen demand, which may cause shortages of oxygen in the embryos. As already mentioned, oxygen deficiency may serve as a trigger for hatching (Jobling 1995), and this may have been the cause of early hatching in some groups of eggs (i.e. bully).
2) On the other hand, the uncoupling of oxidative phosphorylation and the resulting reduction in ATP synthesis may also cause a generalised energy deficiency, which in turn may have led to a decrease in the physical activity levels of the embryos. Inactivity of developing embryos may well be a cause for delayed hatching, since hatching is dependent on the distribution of hatching enzymes in the eggs (von Westernhagen 1988). In support of this scenario, the presence of empty egg shells with punctiform holes, indicative of inactive embryos, was often observed in this study for the species that hatched later than the controls (smelt and koaro), but was, however, not quantified.

Other factors unrelated to PCP exposure may also affect the observed time-to-hatch. It is known, for instance, that both darkness and high oxygen levels in incubation water can potentially suppress hatching (Jobling 1995), and both of these conditions were present during the incubations. However, the conditions were identical for both the control and PCP-treatment groups, and therefore should not have significantly influenced the effects on hatching seen after PCP exposure.

Overall, the parameter time-to-hatch with the native fish species examined here was not consistent as an indicator of exposure to PCP, and thus, time-to-hatch appeared to be of somewhat limited value. It is possible, however, that certain species react in a consistent manner when exposed at certain life stages. For instance, in this study, PCP-exposed rainbow trout eggs hatched later than the controls when tested at the 24 h and early eyed stages, but earlier when tested at the late eyed stage. This trend was also

seen with rainbow trout by Willis (1994) at the same life stages. In addition, two previous studies showed that PCP caused delayed hatching in rainbow trout. In these investigations, the trout eggs were exposed to PCP either continuously after fertilisation (Hodson and Blunt 1981) or starting 24 h after fertilisation (Dominguez and Chapman 1984).

No change in the time-to-hatch was seen when comparing solvent and synthetic water control groups to each other, except with 3 tests involving 24 h koaro, 24 h inanga and 24 h rainbow trout. In these groups, the solvent control larvae either hatched earlier or later than the synthetic water controls, and therefore, the effect of ethanol in the tests was not consistent. In addition, the time-span of hatching (from the start of hatching to hatch completion, using the coefficient of variation) in almost all cases was not affected by PCP exposure in comparison to controls, except those of late eyed rainbow trout and 24 h koaro. It appears overall, therefore, that PCP did not cause a higher variability in the incubation times of the exposed embryos to time-of-hatch.

8.4.4 Teratology

PCP has been classified as a cytotoxic agent (Manson and Wise 1991), that can cause both embryo-lethality, malformations and massive cell death within the exposed embryos. Sites of high proliferative activity in the embryo are particularly susceptible to cell death after exposure to cytotoxic teratogens, and the frequency and morphological pattern of the malformations are related to the localisation and extent of necrosis in embryonic organ precursors. A full spectrum of malformations can be induced by these agents, and the site most likely to be affected is primarily determined by the time of exposure. Those organ rudiments undergoing rapid proliferation at the time of exposure are likely to be the sites of future malformations. Developmental processes that may be disturbed as a result of toxicant exposure at early embryonic stages include: cell and tissue differentiation, excessive or inadequate programmed cell death, cell movement, cellular communication and disrupted metabolism (Weis and

Weis 1989). Many of the resulting malformations may involve reduction deformities, or missing elements, presumably because insufficient cells were available to form the organ rudiment. Malformations are often induced at doses of a toxicant that cause death in a significant portion of the offspring (Manson and Wise 1991). Effects of PCP previously seen in early life stages of fish are swollen abdomens, developmental arrest, pericardial edema and associated secondary tube hearts (Helmstetter and Alden 1995).

It is clear from Figures 4.1 and 5.15 - 5.20, that exposure to pure PCP was correlated with a wide range of abnormalities in the developing embryos. With few exceptions, the terata were more severe and numerous when exposure occurred at early stages (i.e. 24 h and early eyed) than at later stages. This is in agreement with the hypothesis that has been proposed by Weis and Weis (1989) and Sharp (1990) whereby exposure to toxicants during early developmental stages is associated with the most severe effects.

The observed pattern of deformities resulting from PCP exposure in this investigation were similar regardless of the tested species. Teratology was, together with successful hatch, the parameter that most often was associated with the lowest LOEC for each species and each life stage. The significance of most of the observed deformations for future survival is marked, as most would have prevented normal swimming, and thus predator avoidance or food capture by the larvae (von Westernhagen 1988). Due to the complex nature of the behaviour, which requires integration of both the brain and neuromuscular system, swimming is likely to be one of the most easily affected parameters. This is supported by this parameter being one of the more sensitive indicators of exposure in koaro and inanga larvae (Section 8.4.6).

The teratogenicity indices (Table 5.5) suggested, however, that PCP was an agent with relatively low teratogenic potential, since the indices were <1.5 (Bantle et al. 1989). Only teratogenicity indices in excess of 1.5 are considered to be indicative of teratogenic agents. This is in agreement with the findings of Schwetz et al. (1974) and

Mayura et al. (1991) who showed that PCP was not a potent teratogen for early life stages of rat.

It must, however, be re-emphasised that the abnormalities were assessed only after the larvae had hatched. It is possible that the results may have been qualitatively and quantitatively different if they had been assessed at earlier stages, since some mortality had undoubtedly occurred in eggs that were grossly malformed during incubations in clean water after their earlier PCP exposures. The extent to which this affected the final numbers and distribution of abnormality types is uncertain. This is in part due to the fact that the extent of any selective mortality is not known. Another matter that is relevant to how the results are interpreted is that some effects, although appearing to be teratogenic, actually may have been of physiological origin (e.g. neuromuscular) rather than derived from a true developmental origin (Weis and Weis 1989).

The relatively high incidence of terata in control inanga eggs tested at the early eyed stage (Figure 5.16B) is possibly the result of using over-ripe eggs for fertilisation. Fertilised over-ripe eggs have been shown to have a higher incidence of malformations than freshly ovulated eggs (Laale 1981; Blaxter 1988). This one batch of inanga was the only batch during this investigation with a high incidence of malformations in the control larvae.

8.4.5 Cranial malformations

Cranial malformations were also numerous and, in general, increased in proportion to the levels of PCP exposure. The response was, however, a slightly less sensitive indicator than whole body morphology. Cranial malformations in response to PCP have previously been reported for early life stages of rats and fish (Schwetz et al. 1974; Schwetz et al. 1978; Dominguez and Chapman 1984). The mechanisms involved in the development of cranial abnormalities due to PCP exposure and even normal cranial development are, however, uncharacterised at this time. Common bully controls had a

fairly high incidence of malformations (1.7 - 11.8%). This could potentially be due to their crania being slightly more fragile than those of the other tested species and, thus, could have been more easily damaged during the staining process. PCP-exposed bullies may therefore have been slightly more susceptible to cranial malformations as some of the malformations (i.e. missing cartilages) would have potentially made their crania susceptible to damage during staining.

8.4.6 Swimming performance

In this study, the swimming performance of hatched koaro and common bully larvae generally decreased in response to increasing PCP concentrations. Swimming performance was not examined for the other tested species. Low physical activity levels of fish larvae exposed to PCP have also been observed in a number of other studies with rainbow trout, fathead minnows and medaka (Hodson and Blunt 1981; Holcombe et al. 1982; Dominguez and Chapman 1984; Helmstetter and Alden 1995). In contrast to the results of these studies, higher activity levels were reported by Brown et al. (1987) with largemouth bass, and no effect was reported at PCP levels less than the LC50 for sockeye salmon (Webb and Brett 1973). Weis and Weis (1989) have suggested that swimming performance, amongst other behavioural parameters, could potentially be a very sensitive indicator of toxicant exposure. In support of this, Little et al. (1993) have indicated that they may allow the detection of the effects of toxicants at 0.1 - 5% of the corresponding LC50 values.

Decreased swimming performance can be a result of either neurological disorders, failure to inflate swim bladders, the narcotic effects of a toxicant, and/or disruption of normal aerobic metabolism (von Westernhagen 1988; Weis and Weis 1989; Marty et al. 1990). In this study, PCP initially seemed to activate fish larvae placed in PCP solution (i.e. more rapid swimming movements), but by the end of 48 hours of exposure, the larvae were less physically active than control larvae. It seems likely, therefore, that the impairment of swimming performance was not related to the narcotic

effects of PCP, but rather to one of the other above mentioned causes. In a number of instances, the larvae were physically unable to swim normally because of axial deformities, caused by PCP exposure.

8.4.7 Heart rates

PCP had a statistically significant depressant effect on the heart rates of smelt exposed at the 24 h and early eyed stages. However, the depressant effect after exposure was only temporary and the embryos recovered within 6 days to the levels of control embryos. In contrast to the depressive effect on heart rates due to PCP exposures at the 24 h and early eyed stages, the lack of response at the late eyed and hatched larval stages may be in part due to the smaller differences in heart rates between the progressively later developmental stages, thus making effects more difficult to detect. A slowed heart rate and reduced circulation have also been observed in medaka eggs exposed to pure PCP (Helmstetter and Alden 1995).

Although the measurement of heart rates may be an easy and sensitive end point for early stages of development, the relevance of reduced heart rates for the future survival of embryos is unclear. For example, McKim (1985) notes that an observed or suspected consequence of alteration in heart rate is retarded development, although the opposite may be equally true; that a retarded development may cause a reduced heart rate. Similarly, the reduced heart rate may be a reflection of a depressed metabolic rate because of PCP exposure.

8.5 The feasibility and utility of using early life stages of native New Zealand fish species for toxicity testing

The availability of native fish eggs somewhat limited the number and the scope of experiments that could be performed during the course of this study. It would, for instance, have been of interest to perform additional tests at lower PCP concentrations to get a more detailed understanding of the sublethal effects of PCP. However, as an

'egg-saving' measure, it was necessary to keep eggs from acute toxicity tests for the follow-up studies rather than undertake separate tests that would have enabled a more complete characterisation of the sublethal PCP effects.

The relative scarcity of fertilised eggs may be less problematic in future studies as more knowledge becomes available on the optimised techniques for obtaining the eggs of native fish species. A natural limitation on the availability of eggs was the seasonality of the breeding cycle of fish. As mentioned in Section 5.1, mature smelt were available in April - July, inanga in July - September, koaro in March - April, and common bully in October - December at the sites where these fish were captured for this study. Rainbow trout eggs from the Ngongotaha Hatchery were commercially available between mid-May to the end of August.

Furthermore, additional information is required to enable the accurate assessment of ripeness of eggs in koaro, inanga, smelt and common bully. Data from various fish species has shown that there can be a wide range in the time that eggs are viable after ovulation; i.e. from as little as a few tens of minutes to up to 2 weeks (Kjørsvik et al. 1990; Barnabé 1994). This aspect was not studied systematically in the native fish species, although the release of eggs during ovulation of inanga and koaro was checked as often as was possible after injection with the GnRH analog in Ovaprim.

The variable success in fertilisation of apparently ripe eggs, and the variable quality of the fertilised eggs, were most likely due to either unovulated or over-ripe eggs. Interestingly, fertilisation rate alone may not necessarily be indicative of egg quality, since the fertilisation rate is maintained longer than is the ability for the eggs to develop normally (Kjørsvik et al. 1990). In addition to the possible nonoptimal conditions of fertilisation and ripeness affecting the overall quality of eggs, fungal contamination may also have had a partial role in determining egg quality. To minimise fungal contamination, it was necessary to clean the eggs on a daily basis. Fungal growth

appeared to invade already dead or dying eggs, and eventually this would have seriously affected other eggs within the same incubation container if left unchecked. Healthy and live eggs could normally resist fungal infection if no other eggs in the group were overgrown with fungus. The fungal contamination was probably caused by *Saprolegnia*, which is almost never a primary cause of disease in fish and fish eggs (Bylund and Fagerholm 1987).

Collection of fertilised eggs from natural spawns at locations where eggs are easy to find, may in the future prove to be a practical option for species such as common bully. However, one must be certain that no other related species are located in the same habitat, which potentially may cause problems in identifying the correct species used for testing. The quality of common bully eggs probably suffered to some degree as a consequence of transport and detachment from the substrates. Although common bully eggs collected in the field in this study became infected by fungus if left uncleaned, it did not seem more serious than for eggs of the other species. Inanga and smelt eggs may also be found in the wild for future studies, but this was not attempted in this investigation. Collection of natural spawns for koaro was not an option during the course of these investigations, since eggs of this species had not previously been found in the field.

Of the sublethal effects studied with the five tested species, the occurrence of abnormalities, swimming performance and successful hatch appeared to be the most sensitive, whereas cranial malformations, time-to-hatch and growth were less responsive to PCP. As shown in Table 5.6, the lowest LOEC achieved by studying sublethal effects was often shared by several end points. This is partially a reflection of the fact that the scarcity of eggs prevented several tests being performed at even lower sublethal PCP concentrations. As a consequence of this, the lowest LOEC values were also usually higher than the acute LC50 values determined from hatched larvae, and the LOEC values were often the lowest concentrations used in the tests. Further studies

may establish whether sublethal effects may occur at PCP levels lower than those tested in the current study.

It was vitally important to confirm that native fish species reacted in a manner similar to what has been reported in the literature for other PCP-exposed fish and animals. With respect to PCP exposure, it can be concluded that early life stages of the native fish responded in a similar manner, both quantitatively and qualitatively, to what has been reported for other tested species, and that using a species such as rainbow trout as a reference test species was warranted. Although one goal may have been to determine whether native fish may have been a better test organism, i.e. have a more pronounced sensitivity to PCP at some life stage, it is also important to have confirmed that this is probably not the case. It must be recognised that this provides an initial understanding of the sensitivity of the tested species, but that the situation may potentially be different if one were to use a different toxicant or other native fish species. Because of the threatened or endangered status of some native fish species, it is crucial to obtain further information on pollutants that have the potential to be detrimental to the health of New Zealand native fish and which thus represent an additional burden on their already stressed populations.

The use of the four native species for toxicity testing had some technical drawbacks. The main hindrance was the difficulty in obtaining fertilised eggs, and the lack of knowledge of the optimal rearing conditions for these eggs. In addition, some species may be more suited to laboratory-based manipulations than others; for example, inanga in this and another study were easily maintained, and resilient to manipulation by injections and other handling (Mitchell 1989). Similarly, koaro appeared to be as well suited for production of fertilised eggs as inanga, but further research is required in order to optimise incubation conditions for the eggs. Unfortunately, adult smelt caught in the field proved to be an unreliable source of fertilised eggs, and the adults cannot be easily manipulated to ovulate. Finally, common bully eggs collected in the field were a

good source of eggs, but the disadvantage was that they had to be removed from a substrate, causing a limited degree of damage.

The lowest acute LC50 value of 30 ppb obtained in this study was for inanga larvae, with the larvae of the other species having LC50 values of 31 - 104 ppb (Table 5.3). Common bully exposed to PCP at the 24 h stage were sensitive during the follow-up period, with a final (at hatch) LC50 of 16 ppb, and a NOEC for sublethal effects of 25 ppb (Tables 5.4 and 5.6). These values can be compared to the known concentrations of PCP in the New Zealand aquatic environment. For example, the Waipa stream with up to 655 ppb PCP (NTG 1992) far exceeds levels that could be considered safe for any life stage of the tested species. In contrast, Lake Rotorua had PCP concentrations of less than 0.04 ppb, whereas the Puarenga stream had levels of 3.62 ppb PCP or less (Gifford et al. 1995). The Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in the USA, employs certain guidelines to assess the risk of estimated environmental concentrations (EEC) (Touart 1995). For instance, an EEC less than 1/10 of acute LC50 is considered to be of no risk, whereas the risk becomes unacceptable if the EEC is more than 1/20 than the acute LC50 for an endangered species. There is a presumption of a risk that may be mitigated by restricted use when the concentration is more than 1/10 of LC50, but less than 1/2 of LC50. In light of this, the concentration of the Puarenga stream (3.62 ppb) would pose a risk that could be mitigated by restricted use if compared to the acute LC50 for inanga (30 ppb). The Rotorua area is, however, only one site where the concentrations of PCP have been measured, and the potential for contamination of other aquatic areas in New Zealand is very real. It is, therefore, possible that these and other native fish species in New Zealand are affected by PCP contamination in waterways. Finally, the stress associated with other inputs of pollution, the effects of deforestation, introduction of exotic fish species and other modifications to the habitats of native fish, could act in either an additive or synergistic manner on the native fish species. The combined effects of all

these stresses, on species that are already either threatened or endangered, is likely to be significant.

CHAPTER 9

DISCUSSION FOR THE ADULT FISH INVESTIGATIONS

9.1 LD50 values for inanga and common bully

Inanga and common bully did not appear to be more sensitive than other species when injected intraperitoneally with PCP. The LD50 values for inanga were 0.27 and 0.32 mmol/kg, and for common bully, 0.234 mmol/kg. These LD50 values are similar to what has been reported for rainbow trout (0.14 mmol/kg; Hodson et al. 1984) and for some mammalian species (0.101-1.12 mmol/kg; Ahlborg and Thunberg 1980; USEPA 1988). Pascoe (1983) classifies a toxicant that is able to cause this degree of toxicity to be an "extremely toxic" compound. Interestingly, for inanga, a PCP concentration equivalent to 75% of the LD50 caused 40% mortality whereas the 25 and 50% levels did not cause elevated mortality compared to the control. This is in contrast to what was seen with common bully where the 75% PCP exposure level did not have any excess mortality compared to controls.

The distributions of males and females, and of immature and mature fish were fairly equal for both the inanga and common bully within the control and experimental groups. In addition, the weights of the fish receiving different doses of PCP were statistically equal. This suggests that the sexual maturity and size of the fish should have had no appreciable effect on the sublethal effects observed in response to PCP. Although most aquatic species have been exposed to toxicants by the aqueous phase, in an interesting study reported by Hodson et al. (1984) rainbow trout were exposed to PCP by both the aqueous phase and intraperitoneal (i.p.) injections. The LD50 (i.p.) was 0.14 mmol/kg, as mentioned above, and the corresponding LC50 value was 160 ppb. Hodson et al. (1984) also correlated rainbow trout LC50 and LD50 values for several other toxicants. When the log octanol/water coefficient (log P) was incorporated into the equation, the correlation coefficient was significant ($r^2 = 0.82$). Unfortunately, the equation computed by Hodson et al. (1984) for converting LD50

values to LC50 values ($LD50/logP = 0.3483 + 1.03 \times 10^{-4} \times LC50^2$) was not suitable for the PCP LD50 values obtained in this study, due to the high log P of PCP (5.12), which would have necessitated taking the square root of a negative number. Thus, no estimation can be given for the LC50 values that would correspond to the LD50 (i.p.) values obtained in this study for inanga and common bully.

Exposure of adult inanga and common bully to PCP by intraperitoneal injection was a practical alternative to aqueous exposure. In addition, as Hodson et al. (1984) has pointed out, intraperitoneal injections are faster, simpler and less expensive than traditional LC50 bioassays. The environmental cost of disposing of large amounts of contaminated water was also avoided. With a maximum loading of 0.7 g fish/L exposure water, the acute and sublethal testing of all the fish (inanga and common bully) in this investigation would have required about 2000 L of test solution, if no changes were made during the four exposure days. With respect to experimental considerations, the injection procedure was not associated with significant mortality in the control groups of either common bully or inanga.

9.2 Sublethal effects

9.2.1 Haematology of inanga and bully

The mean blood glucose levels of control inanga and common bully in this study (2.93 and 4.01 mM, or 53 and 72 mg/dL, respectively), compare well to values reported in the literature for other fish species. The range of glucose levels reported for other teleosts has been found to be between 0.79 and 115 mg/dL (Ferrando and Andreu-Moliner 1991; Folmar 1993; Johansen et al. 1994; Sunyer et al. 1995; Neilson 1996; Roche and Bogé 1996). The method for glucose determination was indicated to be reliable and accurate even with 5 µL whole blood, as indicated by a small coefficient of variation for the replicates (11%) and by the human blood glucose values being within the normal range.

The mean blood concentrations of lactate in this study of control inanga and common bully (1.48 and 1.02 mM or 13.3 and 9.2 mg/dL, respectively) also compare well to levels determined in control fish from other studies. The range has been reported to vary between 3.15 and 25 mg/dL (Ferrando and Andreu-Moliner 1991; Johansen et al. 1994; Pankhurst and Dedual 1994; Kennedy et al. 1995; Neilson 1996). The method of lactate determination was fairly accurate, as evidenced by normal concentrations for human blood, and standard curves with high r-values, but the reproducibility was low, as indicated by the relatively large coefficients of variation for the two triplicate analyses of common bully blood.

The effects of PCP on glucose and lactate of inanga and common bully were mostly insignificant, except for a significant decrease of glucose for inanga exposed to 0.075 and 0.150 mmol/kg PCP. The levels of glucose were 55 and 56% of the control in the low and medium doses, respectively, for inanga in this study. McDonald and Milligan (1992) classify a >50% drop in glucose levels as severe hypoglycaemia, and they doubt that this would occur in fish, due to their need to maintain glucose levels within a certain concentration range for their nervous systems to function properly. It is conceivable that the exhaustion of hepatic glycogen reserves and subsequent failure to maintain plasma glucose levels may have contributed to the high mortality among inanga exposed to the high doses of PCP (75% of LD50) in this study, as has been suggested by Thomas et al. (1981) in another study of PCP effects on fish.

The most common and easily explained general effect on fish blood glucose in response to stress in general in fish is an increase due to the mobilisation of glycogen or other energy reserves (Hille 1982). A similar situation has been suggested to occur in male rats exposed to PCP, which showed an increase rather than a decrease in glucose levels (Knudsen et al. 1974). In addition, Thomas et al. (1981) found an increase in plasma glucose levels in response to PCP exposure in striped mullet, with a concurrent decrease in liver glycogen levels. Their results suggested that plasma glucose levels

could not be maintained once the liver glycogen levels had fallen to about 5% of the normal values. The same authors observed alternating hypoglycaemia and hyperglycaemia in the sandworm (*Neanthes virens*) in response to PCP. The response of blood glucose levels to PCP may, therefore, be time-dependent phenomena, where the glucose levels are dependent on the mobilisation of different energy reserves of the fish. The reasons for a decrease of the glucose and possibly other energy reserves seen in inanga is likely to be that PCP caused an uncoupling of the oxidative phosphorylation of ADP to ATP yet still allowing a high rate of electron flow to occur in the mitochondria. This would have led to a higher demand for substrate from the citric acid cycle and the glycolytic pathways, in agreement with the mechanisms proposed by Boström and Johansson (1972).

The glucose levels of common bully were not affected in this study by PCP exposure which may have been due to a slightly different energy status or a different metabolic strategy for controlling the blood glucose levels of the fish when compared to inanga. It was observed during detoxification enzyme studies that bully livers often contained large amounts of lipids which were not present in inanga livers. In fish, the liver may function as a storage site for lipids, which may be utilised for energy supply, whereas glycogen stores are small and would not last for long during starvation (Dave et al. 1975). After glycogen depletion, the fish would maintain glucose levels through oxidation of lipids and protein catabolism (Thomas et al. 1981; Folmar 1993). Thus, it is conceivable that inanga placed a greater reliance on glycogen for maintenance of blood glucose and possessed less fat reserves which would have prevented them from being able to survive a prolonged period of toxicant-induced stress or increased energy demand. Higher fat reserves of common bully did not, however, appear to provide protection against the acute effects of PCP, since the LD50 for common bully was somewhat lower (0.23 mM) than that of inanga (0.30 mM).

It is known that anaesthesia and the stress resulting from both attempting to capture fish and other manipulations, may have an effect on glucose and lactate levels as well as on other blood parameters of fish (Hattingh and van Pletzen 1974; Pickering et al. 1982; Korcock et al 1988; Bollard et al. 1993; Roche and Bogé 1996). These factors should, however, not be contributing significantly to any bias in the results of this study. For instance, because no anaesthesia was used, and because the fish were sampled in random order, equal stress would have been present in all treatment groups. It is possible, however, that the prolonged sampling periods that were necessary for sampling all of the fish may have introduced a slightly higher variability between individuals within the same treatment groups. This would be due to having all the fish in the same aquarium, and sampling them in random order from the different treatment groups. Due to the large number of fish examined and the time-consuming manipulations that were required, it took 10 - 14 hours to sample all fish for both the bully and the inanga experiments. This may have made it slightly more difficult to distinguish between background 'noise', i.e. variability due to different lengths of time required for sampling (varying levels of associated stress), and actual variation due only to PCP exposure.

The effects of PCP on lactate levels were mixed and statistically insignificant, with a decrease at the low and medium doses and a slight increase at the high dose for common bully. There was a slight increase in lactate at the medium dose for inanga (and no effect at the low dose and no sample for the high dose). Increases in lactate content of the blood have been attributed to an increase in anaerobic metabolism, as a consequence of internal hypoxia (McDonald and Milligan 1992; Philip et al. 1995). This would not be an unexpected result as the metabolic rate is likely to increase in PCP-exposed fish (Kim et al. 1996; Wang and Widdows 1993). In support of this scenario, Wang and Widdows (1993) showed that an increased oxygen consumption in PCP-exposed sea mussels was accompanied by a major shift to anaerobic pathways, as determined by the levels of succinate and lactate. In addition, if the fish are in a state of

internal hypoxia, they may switch from using aerobic red muscles for locomotion, to anaerobic white muscles (Davison 1983; Goolish 1991). Anaerobic white muscles utilise glycogen for energy, with the concurrent production of lactate (Goolish 1991). Inanga may be more susceptible to relying on white muscle due to their high level of swimming activity, as opposed to bully which tended to remain motionless on the bottom of the fish tanks. Increases in lactate may also be due, in part, to increased secretion of corticosteroid hormones due to stress (Bollard et al. 1993; Johansen et al. 1994).

The average red blood cell counts found in control inanga and common bully were 0.95 and 1.65 x 10^{12} cells/L, respectively. These values are similar to those reported for control teleosts in the literature which have ranged between 0.5 and 2.6 x 10^{12} cells/L (Mulcahy 1970; Blaxhall 1972; Hattingh and van Pletzen 1974; McCarthy et al. 1975; McLeay 1975; Johansson-Sjöbeck et al. 1978; Clark et al. 1979; Matthiessen 1981; Pickering et al 1982; Dick and Dixon 1985; Gill et al. 1991; Neilson 1996; Shakoori et al. 1996). The haematocrit (Hct) values of fish, which is a measure of the volume of erythrocytes to the total blood volume, may vary even more: the icefish (*Chaenocephalus aceratus*) have Hct values close to zero, and active species like scrombrids and thunnids have Hct values of up to 40 - 50%. The majority of fish, though, have Hct values around 15 - 30% (Jobling 1995). The range of RBCC may also vary widely within a particular species, due to the RBCC value being dependent on several factors such as erythropoiesis (in response to relatively long periods of low oxygen tension), general activity levels, cortisol concentration, erythrocyte turnover or haemoconcentration (due to decreased blood volumes).

The red blood cells showed an inconsistent response to PCP in both inanga and common bully and, therefore, cannot be considered as a sensitive indicator of exposure (Figures 7.5 and 7.6). The lack of a consistent response may in part be due to the relatively large variation between individual fish in their RBCC. This result is in

agreement with Schwetz et al. (1978) who found no change in the RBCC in rats exposed to PCP, and in contrast to results of Knudsen et al. (1974) who found an increase in the RBCC in male rats exposed to PCP. It must, however, be mentioned that other important measures of erythrocyte function, such as haemoglobin content and haematocrit, were not measured due to the limited volumes of blood collected, and, therefore, effects on parameters such as these cannot be excluded. As an example, cortisol does not seem to affect the RBCC of fish (Johansson-Sjöbeck et al. 1978), but low oxygen in the environment stimulates erythropoiesis of fish (Blaxhall 1972; Fänge 1992). The results from the inanga and common bully indicate that the fish may have been stressed due to PCP-mediated cortisol elevation (since cortisol does not seem to affect RBCC), but were not appreciably affected by internal hypoxia as was suggested for the lactate results (since hypoxia should increase the RBCC).

The average total white blood cell counts in control inanga and common bully were 1.93 and 2.35 x 10^{10} cells/L, respectively. These values are within the broad range for the number of leucocytes for control teleosts as reported in the literature: 0.021-13.7 x 10^{10} cells/L (Mulcahy 1970; McCarthy et al. 1975; McLeay 1975; Hlavek and Bulkley 1980; Matthiessen 1981; Dick and Dixon 1985; Angelids et al. 1987; Fänge 1992; Neilson 1996; Shakoori et al. 1996). The variable changes in total WBCC in response to PCP were insignificant in this study, but it is notable that the inanga WBCC was 1.36 x 10^{10} cells/L at the highest dose (compared to 1.93 x 10^{10} cells/L for the control group). This has consequences for the inanga differential count, where an insignificant decrease in the percentage of lymphocytes becomes significant (p <0.0001) when calculated from absolute numbers of lymphocytes (1.48 x 10^{10} cells/L in controls versus 0.88 x 10^{10} cells/L in the high dose group). In addition, the absolute numbers of inanga thrombocytes were 0.22 x 10^{10} cells/L in the high dose group compared to 0.16 x 10^{10} cells/L in the control group, but this difference was not statistically significant. The overall decrease in WBCC, therefore, may have been largely due to the decrease in inanga lymphocyte numbers.

Common bully showed no consistent changes in their total WBCC or differential counts in response to PCP, although the number of lymphocytes again appeared to correlate with the total WBCC. The thrombocyte numbers were 0.024 x 10^{10} cells/L in high dose group compared to 0.041 x 10^{10} cells/L in the control group, although this was not statistically significant ($p = 0.268$).

The results for inanga appear similar to those of Dick and Dixon (1985), who found lymphocytopenia (a decrease in the number of lymphocytes) and thrombocytosis (an increase in the number of thrombocytes) after acute exposure of rainbow trout to copper. It appeared that this effect was mediated through corticosteroid secretion stimulated by adrenocorticotropin, and may, therefore, be a generalised response to stress (Dick and Dixon 1985). Indeed, cortisol has been found to cause lymphocytopenia, which is thought to occur either because of immunosuppression, or through lymphocytes leaving the peripheral blood (Johansson-Sjöbeck et al. 1978, Angelids et al. 1987). In support of this scenario, PCP has been found to cause an elevation of cortisol in carp (*Cyprinus carpio*) exposed to 100 and 500 ppb (Hanke et al. 1983), and in striped mullet exposed to 100 and 200 ppb (Thomas et al. 1981).

Schwetz et al. (1978) found no effects of PCP on total and differential white blood cell counts when rats were exposed to PCP, but both Anderson and Brubacher (1993) and Roszell and Anderson (1993) demonstrated immunosuppressive effects of PCP, *in vitro*, when using fish phagocytes. It is clear that further investigations would be required to clarify the nature of PCP toxic effects on the immune systems of fish.

The percentages of lymphocytes of control bully and inanga (74 and 77%, respectively) are similar to what has been found in numerous other studies (Mulcahy 1970; Blaxhall 1972; McCarthy et al. 1975; Hlavek and Bulkley 1980; Pickering et al. 1982; Dick and Dixon 1985; Angelids et al. 1987; Fänge 1992). Some researchers have, however,

found fewer lymphocytes (40-60%) in control groups (Johansson-Sjöbeck et al. 1978; Ellsaesser and Clem 1986), with an overall average percentage lymphocyte count in the reviewed literature of about 70%, in close agreement with what was found for inanga and common bully. There is also a wide range of thrombocyte percentages reported; 0-56% of the total leucocyte count, with an average of about 22% (Blaxhall 1972; Ellis 1977; Johansson-Sjöbeck et al. 1978; Hlavek and Bulkley 1980; Pickering et al. 1982; Dick and Dixon 1985; Ellsaesser and Clem 1986; Angelids et al. 1987; Fänge 1992). The values obtained in this study for control inanga and bully thrombocytes are in the lower end of this spectrum; 8 and 1.25%, respectively. The possibility of misidentifying thrombocytes as lymphocytes has been pointed out by other researchers (Ellis 1977; Fänge 1992). Although much care was taken to reduce that possibility, there was the potential for this to occur in the present study as well. It is also conceivable that a portion of the thrombocytes selectively aggregated and adhered to surfaces during sample preparation, which, therefore, may have led to a reduced number of thrombocytes being identified.

The granulocytes (neutrophils, basophils and eosinophils) in this study constituted between 15 and 25% of the differential count of control inanga and bully, respectively. This is higher than is often reported in the literature for control fish (i.e. <10%, with an average of about 7%), when counted in the same fashion as in this study, i.e. classifying neutrophils, basophils and eosinophils as granulocytes (Ellis 1976; Johansson-Sjöbeck et al. 1978; Hlavek and Bulkley 1980; Pickering et al. 1982; Dick and Dixon 1985; Fänge 1992). Granulocytes are not likely to be confused with lymphocytes or thrombocytes, but there is a possibility that some cells classified as granulocytes were, in fact, monocytes. However, monocytes are said to constitute only a minute fraction (0.1-0.2%) of the blood leucocytes in fish, and may actually be lacking in some species (Ellis 1976; Fänge 1992). This is in contrast to Hine et al. (1987) who classified the numbers of monocytes in inanga as 'very common' to 'abundant', and in common bully as 'uncommon'. There is, therefore, a possibility

that the two leucocyte types were not differentiated in this study. Only a couple of basophils were seen in inanga, and no eosinophils were detected in either fish species. Both of these types of leucocytes have been reported to be scarce or completely lacking in other species of fish (Fänge 1992).

Further studies into the identification of leucocytes in inanga and bully would be beneficial, as the number of control inanga and common bully samples in this study was only 13 and 12, respectively. With respect to the ease in misidentifying subtypes of leucocytes, as reported in the literature, it could prove to be important to be consistent when counting leucocytes. The consistency when counting common bully leucocyte types (thrombocytes, lymphocytes and granulocytes) was indicated to be reproducible in this study, as evidenced by the repeat counting of the same blood sample three times (Section 7.3.5).

9.2.2 Detoxifying enzyme activities

The average UDPGT activities of control inanga and common bully were 4.42 and 9.22 pmol min^{-1} mg^{-1}, respectively (Section 7.3.6). These values are low compared to UDPGT activities (using 4-nitrophenol as a substrate and without activity-enhancing detergent) reported in the literature for other fish species, which have been reported to vary between 30 and 2670 pmol min^{-1} mg^{-1} (Clarke et al. 1991; George 1994). The same method (without detergent) for UDPGT determination with hatchery reared rainbow trout microsomes has, in a prior study, produced activities between 1110 - 1920 pmol min^{-1} mg^{-1} protein (Hannus and Gifford 1992), suggesting that the low activities obtained in this study are species specific. The UDPGT activity had not been measured in inanga and common bully prior to this study. It is also possible that the use of post-mitochondrial fractions rather than microsomes for UDPGT determinations caused a decrease in the observed activities.

The UDPGT assays were technically difficult because of a combination of both the low absolute enzyme activities and the small sizes of the fish. The resulting small liver sizes forced the minimisation of the volumes used in the assay, and the low enzyme activities required the use of experimental conditions that would provide for maximal sensitivity. Thus, the incubation time of the assay was extended to 20 minutes, which is in the upper range of the linearity of the assay. In addition, the measurement of substrate disappearance, as is utilised in this assay, is less sensitive than measuring product formation (Lake 1987). The assay with the two studied species is close to its detection limit; lower levels of UDPGT activity in fish as small as inanga or common bully described here would not be much above the statistically defensible detection limits.

A single i.p. injection of ßNF (100 mg/kg) did not induce UDPGT activities 48 hours post-injection in either species. Indeed, in inanga, the activity was unexpectedly repressed. These levels of activity in inanga and bully are somewhat in contrast to those seen in other fish species, which had been given similar doses of ßNF and which showed modest increases in UDPGT activities of about 2-fold (Andersson et al. 1985; Förlin and Haux 1985; Celander et al. 1993). In another study, Goksøyr et al. (1987) found that rainbow trout UDPGT activity increased about 2.5-fold in response to ßNF treatment, whereas no increase or decrease in UDPGT activities was observed for cod (*Gadus morhua*). The authors concluded that the lack of response in cod may have been the result of a delayed response time of UDPGT induction to the ßNF inducer. This may also have been a possible cause for the lack of induction of UDPGT activity seen in this study.

PCP exposure was associated with no significant change of UDPGT activities in common bully, and a statistically significant reduction in activity in inanga. Statistical significance was shown even when then UDPGT activities were generally low and the sample sizes small. The PCP-exposed inanga (the medium and the high dose groups) had UDPGT activities approximately half that of the controls. Whether this level of

decrease of already low activities is biologically significant is not clear at present. Also, this result may seem unexpected since it is known that PCP is a substrate for UDPGT in rainbow trout, goldfish, striped bass and Arctic charr (*Salvelinus alpinus*) (Kobayashi 1978; Lech et al. 1978; Gates and Tjeerdema 1993; Cravedi et al. 1995). However, not all substrates are inducers of detoxification enzymes (Chambers and Yarbrough 1976). For instance, Castrén and Oikari (1987) found that PCP inhibited UDPGT activities of fish exposed for four days to 20% of the LC50 for PCP. Other studies have shown no effect of PCP on UDPGT activities in heifers (McConnell et al. 1980) and rat (Ahotupa et al. 1981), and an increase when exposing Sherman rats (Goldstein et al. 1977). In addition to induction or repression of activity, PCP can have a direct inhibitory effect on UDPGT activity. For example, Ahotupa et al. (1981) showed that PCP had an *in vitro* inhibitory effect on UDPGT activity, when using 100 x 10^{-6} M PCP (equivalent to 26.63 mg/L). For the inhibition of UDPGT activity observed in inanga, it is conceivable that sufficient quantities of PCP were present in the post-mitochondrial fractions to cause at least a partial inhibition of the activity *in vitro* in accordance with the findings of Ahotupa et al. (1981). Although the levels for *in vitro* effects of PCP in the study of Ahotupa et al. (1981) seem high, the BCF for PCP in trout between the water and the bile in Lake Rotorua was approximately 50 000 (Section 2.4; Gifford et al. 1995), and it is therefore likely that the liver bioconcentrates the internal PCP dose. This is further supported by studies where the highest internal PCP concentrations have been found in the liver of exposed fish (Glickman et al. 1977; Pruitt et al. 1977).

The EROD activities of control inanga and common bully, 0.28 and 0.65 pmol min^{-1} mg^{-1}, respectively, were also low compared to the average of 180 pmol min^{-1} mg^{-1} reported for 10 fish species listed by Sijm and Opperhuizen (1989). EROD activities comparable to those of the native New Zealand species investigated in this thesis have, however, been reported for control fish in numerous studies: Di Giulio et al. (1993) found 1.4 - 5.9 pmol min^{-1} mg^{-1} in channel catfish (*Ictalurus punctatus*); Eggens et al.

(1995) found 0.1 - 1.4 pmol min^{-1} mg^{-1} in flounder (*Platichthys flesus*); Jones et al. (1995) found 0.8 and 1.8 pmol min^{-1} mg^{-1} in post-mitochondrial fractions of eel and brown trout livers, respectively; and O'Hare et al. (1995) found 0.52 - 0.63 pmol min^{-1} mg^{-1} in roach. Of particular relevance is the study by Woodworth et al. (1996) who measured EROD activities (1 pmol min^{-1} mg^{-1}) of control Tasmanian inanga (*Galaxias maculatus*) that are similar to the results obtained in the present study.

The empirically determined molar extinction coefficient for the resorufin standard used in the EROD assay was 16.68 mM^{-1} cm^{-1}. This is within the range reported for resorufin in an interlaboratory study (Munkittrick et al. 1993). The EROD assay worked well in this study, with linear increases in fluorescence up to 70 min, well beyond what was used for routine assays (< 20 min). However, due to the variation between lots of commercially available resorufin, it is important to make direct comparisons only between experiments where the same resorufin standards have been used, and only the relative differences between treatment groups should be of importance when assessing this end point as an indicator of pollution.

The reference inducer ßNF did not significantly affect EROD activities, (1.55 and 2.13 × controls for inanga and bully, respectively). These induction responses are low compared to the 2 to 172-fold increases in EROD activity of fish reported in the literature (Andersson et al. 1985; Kloepper-Sams and Stegeman 1989; Celander et al. 1993; O'Hare et al. 1995). The reasons for a low response to ßNF in common bully and inanga may be related to the following:

1) The assaying of EROD activity 48 hours after injections may not have been at the optimal time for induction of EROD activity. Maximal responses in a variety of fish species have been reported in some cases to occur from between 3 - 4 days to 2 or 3 weeks after a single dose of ßNF (Andersson et al. 1985; Kloepper-Sams and Stegeman 1989; Celander et al. 1993; Stegeman and Hahn 1994). In contrast,

Haasch et al. (1993) found significant increases in EROD activity after 18 and 48 hours after i.p. injection of ßNF in rainbow trout.

2) Species differences in their sensitivity to specific inducers clearly exist, with some species being less likely to show induction of EROD activities than others. The ability to induce EROD activity has been shown to be correlated to the amount of Ah receptor (Andersson and Förlin 1992; Bucheli and Fent 1995). It is possible that both inanga and common bully are relatively deficient in this receptor, and that this may have been the cause of their low level of inducibility.
3) It is conceivable that both inanga and bully already possessed maximally or partially induced EROD activities at the time of collection in the field, due to past exposures to inducers present in their natural habitats. However, this is not very likely given the low levels found in common bully and inanga.
4) As for UDPGT, the post-mitochondrial fraction was used for the EROD determinations. This was shown to lower the EROD activities about 4-fold (Section 6.9.1).
5) ßNF may inhibit the EROD assay if it is present at sufficiently high concentrations in the enzyme preparation (Haasch et al. 1993). However, this claim has not been substantiated by a study of the kinetics of ßNF in fish (Haasch et al. 1993).

PCP caused no consistent response in the EROD activities of common bully, and an increase in inanga which, however, was not statistically significant. However, the increase for inanga was of the same magnitude as that caused by ßNF induction. The full spectrum of effects of PCP on phase I enzymes has been reported in the literature, i.e. either repression, no effect, or induction (McConnell et al. 1980; Ahotupa et al. 1981; Shull et al. 1986; Jones et al. 1995). It should be mentioned that inhibition of the catalytic activity of phase I detoxification enzymes may occur even if protein synthesis due to stressors has been induced. Indeed, it has been shown that the mRNA, protein content and catalytic activity of the MFO system may vary seemingly independently of each other (Haasch et al. 1993). Thus, it appears that the effects of PCP on the MFO

system have been variable in past studies, and that this variation may also apply to both inanga and common bully. However, it is important to have determined the activities of these assays with these two species, since little data is available with respect to induction by toxicants in these fish. Given the large variation in EROD activities after treatment with an inducer (2 - 172-fold with ßNF), one does not know, a priori, what the response of these fish would have been. It is also evident that further research is required with these fish with respect to detoxifying enzymes, such as the timing of induction responses, and the physiological reason for low ßNF inducibility. The induction responses may be substantially different if another toxicant is investigated.

One interesting aspect of the present study is the relative refractoriness of both common bully and inanga detoxification enzymes to PCP. One reason mentioned for refractoriness to induction, in addition to a defective Ah receptor, is the presence of fat in fish livers (Payne et al. 1987; Kloepper-Sams and Benton 1994). As mentioned in Section 6.9.1, common bully livers contained copious quantities of fat, whereas inanga livers contained no visible fat. It has been suggested that fat deposits may sequester contaminants and, therefore, reduce the effective intracellular free concentration of toxicant in cells responsible for detoxification, thus resulting in a reduction in the level of induction in the detoxification enzymes. Fat sequestering of PCP may also have been responsible for the change in the temporal profile of free PCP present in the liver, and thus the timing of optimal induction. On the other hand, common bully did show a 2.13-fold increase in EROD activity when exposed to ßNF, which does not support the above scenario about fat sequestration.

Although the same fractions of the LD50 levels for PCP were used for both species, the high dose (0.225 mmol/kg) had a high partial mortality effect on the inanga of 40%. As mentioned in the introductory section to this chapter, the two tested species may have had different slopes for their dose-response curves, causing inanga to be more vulnerable to a 75% dose of the LD50 value.

9.2.3 Heat shock protein 70

The method of detection of hsp70 was sensitive, with only 3.75 µg total protein per gel lane being required to enable detection with the enhanced chemiluminescence system. The primary antibody N27 recognised only one band in all gels with both species, even when the size of the gels was larger (16 x 14 cm) than the small gels (10 x 8 cm) used for the final analysis. One band was also seen by Abukhalaf et al. (1994) and Vélez-Granell et al. (1994) when using an antibody for both the heat-inducible and constitutive hsp70. It is, however, still possible that there were two bands that may have been separable only by using two dimensional gel systems (Fader et al. 1994). Using the same primary antibody as in the current study, Sanders et al. (1994) found double bands for some heat-shocked animals, but not for other animals, using one-dimensional SDS-PAGE. Dyer et al. (1993) also detected two bands for toxicant-exposed fathead minnows using one-dimensional SDS-PAGE and a monoclonal antibody against hsp72/73 from HeLa cells which possibly was identical to the N27 antibody used in the present study. It, therefore, appears that the ability to separate the two hsp70s in toxicant- or heat-exposed fish may be species dependent.

The N27 antibody recognises both the hsc70 and the heat-inducible hsp70. This was supported by the fact that the 70 kDa band recognised by this antibody increased in size after heat-shocking the fish. The response to heat-shock was stronger in common bully gill tissue, with up to 2.6-fold increase in the pixel values above the controls, as opposed to up to 1.31-fold increase for inanga. This is similar to the results of Abukhalaf et al. (1994), who found that heat-shocked fathead minnow hsp70 levels increased by 2.3 or 2.4-fold in different fractions of gill tissue, and those of Gutierrez and Guerriero (1995) who found a 2-fold increase in hsp70 protein in heat-shocked bovine skeletal muscle. The fact that N27 recognised both the cognate and the heat-inducible hsp70 makes this antibody less sensitive for detecting changes than if the antibody were to recognise only the heat-inducible form.

The hsp70 levels of PCP-exposed bully gill tissue were not significantly different compared to the control samples, possibly because of the variability of the response. Inanga hsp70 levels of gill tissue showed no consistent response to PCP.

With respect to the response of hsp70, it appears that common bully hsp70 was more sensitive to both heat and PCP than inanga. The potential reasons for a low level of response in hsp70 levels to inducers, as seen in inanga, have been attributed in the literature to the following two factors:

1) The first reason is the lack of an antibody specific for the heat-inducible hsp70 for use in fish (Williams et al. 1996). The monoclonal antibody C92, which was trialed in this study, should recognise only the heat-inducible hsp70. Unfortunately, the band recognised by this antibody in this study was of the wrong size (112 and 125 kDa). In support of this, this antibody has not been able to previously recognise hsp70 of nonmammalian tissue in at least one other study (Sanders et al. 1994).
2) Fish living in tidal zones or other highly variable environments may have a high natural level of hsp70, which would make it even more difficult to detect increases over the basal levels. For example, Yu et al. (1994) detected only an average hsp70 increase of 8.7% in heat-shocked salamanders, and two heat-shocked fish species which normally live in estuaries showed no increase in hsp70 levels. In addition, the thermal and pollution-related histories of exposure and genetic background of poikilotherms may have important consequences for the ability to induce hsps, and this may potentially reduce the application of this end point (Koban et al. 1987; Veldhuizen-Tsoerkan et al. 1991; Fader et al. 1994). Inanga used in this study were caught in streams close (50-200 m) to the Tasman Sea, which may have resulted in higher basal levels of hsp70, whereas the common bully were captured in the Waikato River, which may have provided a less stressful habitat (i.e. more consistent environment with no thermal, osmotic or oxygenic stresses).

For future studies with hsps in these species, it would be useful to have access to an antibody that is absolutely specific for the heat-inducible form of hsp70. Other useful strategies for studying the heat shock response are metabolic labelling of newly synthesised proteins, two-dimensional polyacrylamide gels, or ELISAs, as detailed in the literature review (Section 3.4.3). If the inanga had already been induced as discussed in point 2 above, it would probably not be very beneficial to study newly synthesised proteins in this species. Although hsp70 is the hsp that most commonly reacts to toxicant exposure, it may be useful to study other hsps that can also be induced, such as hsp60 or hsp90 (Mirkes et al. 1994).

Although PCP has been shown to induce hsps in previous studies of *Euglena gracilis* and *Escherichia coli*, the specific effect on fish hsp70 has not previously been shown (Blom et al. 1992; Barque et al. 1996). This is, therefore, the first time PCP effects on hsp70 in fish has been studied. It is also the first time hsp70 has been analysed in common bully and inanga.

The heat shock proteins are certainly an interesting and important new addition to the battery of physiological responses which could potentially aid in the monitoring of responses to pollutants. In addition, further investigations with these and other native New Zealand fish may enable this end point to be utilised for routine application and, in so doing, provide for additional knowledge on hsp induction due to toxicant exposure.

9.3 The feasibility and utility of using adult inanga and common bully as testing species

Both common bully and inanga proved to be relatively hardy, to adjust readily to living in tanks and to thrive on commercially available fish food. Their robustness allowed for high survival rates with only minor repercussions to experimental manipulations such as anaesthesia and injections. However, for measurements of biochemical or

haematological responses to toxicant exposure, their small sizes posed a potentially significant technical problem. This limited the number of different analyses that could be performed per fish, which is a disadvantage when attempting to elucidate biologically significant relationships between the various physiological changes in the fish. This limitation could be partially overcome through the pooling of samples from different fish, which was done for both the inanga and bully used in this study.

Overall, considering that inanga and common bully in the high exposure groups were exposed to 75% of their LD50 levels for PCP, it is surprising that so few parameters showed statistically significant changes compared to controls. Inanga was the more sensitive of the two species, but many trends recorded for inanga (EROD, % lymphocytes and lactate) were not statistically significant. This may be due, in part, to the variability within groups and the relatively small sample sizes. It is interesting, however, that inanga EROD and bully hsp70 reacted to PCP to the same extent as their respective positive controls, indicating that these changes may have been biologically significant, even though a statistical significance could not be shown. Inanga may have appeared more sensitive than bully because the highest PCP level was more toxic to inanga than expected.

It is conceivable that common bully and inanga did respond to PCP more fully than what was observed, but did not show maximal responses to the sublethal parameters measured on day four when they were sampled (after PCP injections). Another possibility is that the responses of the fish were weak because they already were stressed by physical factors or exposed to toxicants in the field. Although this remains a possibility, the fish that were used for experimental procedures were always disease free, and appeared to feed normally prior to initiating the tests. A certain degree of stress would, however, always be present when maintaining fish in confinement (Neilson 1996).

In conclusion, the use of the adult New Zealand fish, common bully and inanga, for assessing the physiological impact of pollutants has been introduced. Important new information on these species has been obtained and the use of these species as potential bioindicators of PCP exposure has been shown to be feasible. The sublethal effects that were shown with the model toxicant PCP in the two tested species were similar to what had been reported in previous studies. Further research with other toxicants or with field-collected fish would be beneficical for expanding the use of sublethal parameters with native fish. In addition, the parameters measured in this study could be expanded for future studies by including other important haematological and physiological analyses.

CHAPTER 10

OVERALL CONCLUSION

The results presented in this thesis can be examined from several different perspectives. First, the effects of PCP were studied using techniques that were quite different for the adult and early life stages. The ultimate significance of the end points may be viewed with respect to the consequences for the health and survival of whole fish populations assuming exposure within their habitats at similar concentrations studied here. For the early life stages, negative effects such as the reduced hatch success, increased teratology and decreased swimming performance could potentially impair the survival of whole cohorts of fish eggs and larvae, whereas the significance of other factors such as time-to-hatch and heart rate would be more uncertain. All of the responses observed in the early life stage tests were obtained at fairly high concentrations of PCP, which was partly due to experimental design.

Surprisingly few of the parameters studied in adult fish resulted in a significant effect even at high doses of PCP (up to 75% of LC50). This suggests that the parameters were not especially sensitive to PCP, and that other end points, such as adenylate charge, gene-specific expression, DNA/RNA ratio or other detoxifying enzyme activities such as AHH and ECOD may prove to be more sensitive indicators of PCP exposure.

The technical advantages and disadvantages of using the early life stages or adult fish for toxicological research is another perspective of this study. Early life stages of fish were useful for toxicity testing because of their small size and hence, requirement for only small quantities of toxicant solution. It was also advantageous to have batches of eggs with identical fertilisation and pre-exposure conditions. However, one drawback of using eggs was the limited availability of suitable eggs, and the fact that optimised techniques for incubation of eggs and the rearing of larvae were not available at the start

of this study. In fact, a substantial portion of the time required to bring the early life stage part of the study to a successful completion was utilised in the development of suitable laboratory conditions. In addition, the degree to which the chorion of the eggs may have limited the uptake of PCP into the embryos is a relatively unknown factor, although this would have resembled the natural situation. Future studies could perhaps employ sophisticated subcellular techniques to overcome this limitation and enhance the sensitivity of the detection of significant metabolic effects due to PCP exposure.

In the case of adult stage experimental procedures, the utilisation of adult fish was not limited by the availability of fish but the small sizes of bullies and inanga hampered the obtaining of samples of sufficient size for analysis. However, both inanga and common bully proved to be amenable to advanced toxicological research as evidenced in this thesis by the study of both their detoxification enzymes and heat shock proteins.

Another aspect of this study was the appropriateness of PCP as a model toxicant. It was an expectation that PCP would have produced similar effects with different species of fish, since it is a general metabolic stressor. That PCP was likely to have been a suitable reference toxicant was partially substantiated for the early life stages in this study since it exerted similar responses in all species and at similar doses. The studies with inanga and common bully suggested that the prior energy status and activity levels of the fish can influence the toxicity of PCP.

Utilising native fish species for toxicological research is important since very little of these types of investigations have been conducted in the past. Although PCP is a model toxicant with a suite of general metabolic effects it is particularly relevant given the degree of contamination at multiple sites within New Zealand that its specific effects on native flora and fauna be studied. It was entirely conceivable that any one of the species studied could have displayed significant effects at lower levels than that described for rainbow trout and thus would have easily warranted an indepth study of its potential

effects. The results of this study suggest that New Zealand native fish species are useful for this purpose, albeit technically demanding. Results from this study suggest that native New Zealand fish species are not significantly more sensitive than rainbow trout, although generalisations should not be based on only one toxicant.

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APPENDIX 1
POTTHOFF'S METHOD FOR CARTILAGE AND BONE STAINING
(Potthoff 1984)

1) The larvae were fixed in neutral buffered formalin

2) The larvae were dehydrated in an ethanol series:
   50% ethanol 1-2 days
   100% ethanol 1-2 days twice

3) The cartilages of the larvae were stained for 1 day:
   70 mL 100% ethanol
   30 mL acetic acid
   20 mg alcian blue

4) Neutralization for 1/2 day in saturated sodium borate solution (borax).

5) Bleaching for 20-40 min:
   15 mL 3% H_2O_2
   85 mL 1% KOH

6) Trypsin digestion - the larvae were digested until completely clear
   35 mL saturated borax
   65 mL MQ
   20 mg trypsin

7) The bone tissues of rainbow trout fry were stained for 1 day:
   1% KOH with alizarin

Alizarin stock:

alizarin red S, C.I. 58005, saturated solution in 50% acetic acid	5 mL
glycerol	10 mL
chloral hydrate, 1% aq	60 mL

1% KOH with alizarin: 5 mL stock to 500 mL 1% KOH

8) For storage of larvae, they were transferred through a glycerin series:
   30 parts glycerin and 70 parts 1% KOH
   60 parts glycerin and 40 parts 1% KOH
   100% glycerin with thymol for final storage

APPENDIX 2

NUMBERS OF ABNORMALITIES

species and stage	concentration (µg/L)	number hatched	total number abnormal larvae	axial	cranial	eye	caudal	other
24 h smelt	s.c.	104	2	2				
	100	68	8	7				
	200	35	14	14	3			1
	300	41	37	33	1		2	3
	400	14	12	11	1	1	2	1
e.e. smelt	s.c.	93	5	5	2			1
	100	88	19	19				
	200	70	18	17	2	1		
	300	60	33	32	1			
	400	29	21	19		1	1	2
l.e. smelt	s.c.	62	3	3				
	100	28	12	12				
	200	9	9	9				
24 h inanga	c	74	4	3		1		
	s.c.	55	3	3				
	400	34	14	11	4		1	1
	600	18	17	16	11	2		6
e.e. inanga	c	88	16	15		1		1
	s.c.	90	19	18		1		
	400	22	9	9	1			1
	600	39	16	15	3	1		1
l.e. inanga	c	56	5	5				
	s.c.	111	9	9				
	200	23	8	7		1		
	350	7	4	4		1		

24 h koaro	c	34	0					
	s.c.	97	3	3			1	
	50	85	1	1				
	150	10	8	5	5	2	4	5
	250	2	2	2		1		2
24 h koaro	c	67	4	2	2			
	s.c.	57	1	1			1	1
	50	90	1	1				1
	150	18	0					
e.e. koaro	c	73	2	2				
	s.c.	78	3	2	1			
	200	86	1	1				
	350	62	8	7	1	2	1	1
	500	20	8	7	2	1		1
	650	30	21	17	7	1	13	4
	800	11	10	8	9		4	8
e.e. koaro	c	84	1	1				1
	s.c.	93	0					
	400	84	1	1	1	1		1
	600	66	50	25	4		46	4
	800	14	13	7	4	4	5	5
	1000	11	10	6	1		8	5
l.e. koaro	c	96	0					
	s.c.	102	2	2				1
	200	74	11	9	2	1	2	4
	300	59	19	19				1
	500	9	5	3		1	1	2
24 h common bully	c	41	2	2				
	s.c.	34	2	2	1		1	2

	25	22	16	8	9		1	1
	75	0						
	125	1	1					
e.e. common bully	c	93	1	1				
	s.c.	97	1			1		1
	50	35	11	11	1			
	150	25	20	20				2
	250	17	12	12				
	350	5	5	5		1		3
l.e. common bully	c	81	3	2				1
	s.c.	90	4	3				1
	100	65	32	32				
	200	33	21	21				
24 h trout	c	63	0					
	s.c.	63	2		2			
	225	38	16	4	7	5	3	
e.e. trout	c	78	1					
	s.c.	76	0					
	250	71	5					5
	500	77	1	1				
	1000	7	1		1			
l.e. trout	c	87	0					
	s.c.	84	0					
	75	86	0					
	150	76	0					
	300	61	1	1				

e.e.=early eyed; l.e.=late eyed; c=synthetic water control; s.c.=solvent control

APPENDIX 3
NUMBERS OF ABNORMAL CRANIA

species/ stage	concen-tration (µg/L)	number of success-fully stained larvae	shape/ size	asymmetry / organization	missing	eye	total number of larvae with abnormal crania
24 hr smelt	s.c.	86					
	100	56					
	200	29	3	3	1	6	9
	300	36	7	4		6	11
	400	14	2	4		1	4
e.e. smelt	s.c.	84	1	3		4	5
	100	74	3	3	2	1	5
	200	48	3	1	4	5	6
	300	42	3	2	4	3	6
	400	33	3	2	2	5	5
l.e. smelt	s.c.	37					0
	100	21	1	1		1	1
	200	12	1			1	1
24 h inanga	c	18					0
	800	10	10	5	3	3	10
24 hr inanga	c	55		2		1	3
	s.c.	68	1	1			1
	400	35	3				3
	600	16	4	3	2	3	5
e.e. inanga	c	89	1	1	2		3
	s.c.	82					0
	400	22	1	2		1	2
	600	36	4	1		1	5
l.e. inanga	c	52					0
	s.c.	112				1	1

l.e. inanga (cont)	200	21	2				2
	350	4					0
24 h koaro	c	33	1				0
	s.c.	94		1			1
	50	82					
	150	10	1	5	2	2	5
	250	2	1	2		1	2
24 h koaro	c	96		1			1
	s.c.	92					0
	50	84	1	1	1		1
	150	18					0
e.e. koaro	c	72					0
	s.c.	71	1				1
	200	89					0
	350	59		1			1
	500	20					0
	650	25	8	6	1	2	9
	800	11	6	2	3	3	7
e.e. koaro	c	84					0
	s.c.	93					0
	400	90	2	2	1	1	2
	800	13	2	3	2	3	4
	1000	4	1	1	1	1	2
l.e. koaro	c	95		1	1		1
	s.c.	101	1		1		1
	200	74	2			1	3
	300	57		2	1		2
	500	10	3	2	1	2	3
24 h common bully	c	28	2	1	2		3
	s.c.	17	1	1	1		2
	25	8	3		3		3

24 h common bully	s.c.	13	1	1			1
	50	8	1	3	1		4
e.e. common bully	c.	58	1				1
	s.c.	73	2	1	1		3
	50	18	10	3	9		12
	150	11	2	2	8	3	11
	250	5	2	3	1	3	5
l.e. common bully	c	54	2	2			2
	s.c.	47	1		1		1
	100	39	1		1		1
	200	25	8	1	9	1	14
24 h rainbow trout	c	63					0
	s.c.	62			1	1	1
	225	26	4	2	1	4	8
e.e. trout	c	78	2	1			2
	s.c.	76					0
	250	71					0
	500	78	1				1
	1000	4	4	1			4
l.e. trout	c	84		2			2
	s.c.	82					0
	75	85	2	2			2
	150	65					0
	300	62	1	2	1	1	3

e.e.=early eyed; l.e.=late eyed; c=synthetic water control; s.c.=solvent control

APPENDIX 4
SHAW'S SOLUTION
(Klontz and Smith 1968)

Solution A:	neutral red	25 mg
	NaCl	0.9 g
	water (MQ)	100 mL
Solution B:	crystal violet	120 mg
	trisodium citrate	3.8 g
	40% formalin	0.4 mL
	water (MQ)	100 mL

- Solutions were filtered with GF/A Whatman filter before use.
- Equal volumes of solution A and B were mixed for staining.
- Discarded after one week.
- This differs from the original protocol in that the concentrations of neutral red and crystal violet were increased ten-fold.

APPENDIX 5
URIDINE 5'-DIPHOSPHOGLUCURONYL TRANSFERASE (UDPGT) ASSAY
(Castrén and Oikari 1983)

1. SOLUTIONS:

Buffer for blanks

KH_2PO_4, 0.5 M, pH 7.0	34.023 g
(adjust pH with 5 M KOH)	
K_2EDTA, 10 mM	2.0224 g
p-nitrophenol, 0.35 mM	24.4 mg
Made up to 500 mL in MQ water	

Buffer for assay

4 mg UDPGA/mL buffer for blanks = 6.34 mM

UDPGA = Uridine 5'-diphosphoglucuronic acid, triammonium salt (Sigma)

Trichloro acetic acid, TCA 3%	3 g/100 mL
NaOH, 5 M	20 g/100 mL

2 ASSAY PROCEDURE

	BLANK	ASSAY
Buffer for blank, µL	45	-
Buffer for assay, µL	-	45
Post-mitochondrial fraction, PMF, µL	90	90
Mixed and incubated for exactly 20 minutes at 25°C		
TCA, µL	400	400
Spun for 5 minutes at 13 000 g, and added to supernatant:		
NaOH, µL	45	45

- Measured at 400 nm against water using a microcuvette.
- Dilution of post-mitochondrial fraction was varied to maintain the assay in linear range.

3 CALCULATION OF UDPGT ACTIVITY:

Activity (pmol/ml/min) was calculated using the molar absorption coefficient of *p*-nitrophenol (18.8 $cm^2/\mu mol$) (Castrén and Oikari 1983):

$$(\Delta Abs \times \text{total volume (mL) in assay})/(0.0188 \times \text{min} \times \text{sample volume (mL)})$$

APPENDIX 6
ETHOXYRESORUFIN-*O*-DEETHYLASE (EROD) ASSAY
(Burke and Mayer 1974, Prough et al. 1978)

1. SOLUTIONS:

Buffer

HEPES, 0.1 M	2.383 g
Bovine serum albumin, 1.5 mg/mL(Sigma)	150 mg
$MgSO_4 \cdot 7H_2O$, 10 mM	247 mg

Adjusted pH to 7.4, final volume 100 mL

NADPH (Nicotinamide adenine dinucleotide phosphate, reduced, Sigma)

50 µM 2.5 mg/6 mL buffer

Prepared fresh prior to use

Ethoxyresorufin

Stock: 7.25 mM 1 mg/571 µL methanol:dimethylsulfoxide (1:1)

Stored in freezer (-20°C)

For assay: 5 µL stock/3.7 mL HEPES buffer (0.1 M, pH 7.4)

Concentration in assay is 0.245 µM.

Resorufin standard

Stock 5.78 mM 6.8 mg resorufin/5 mL NaOH (0.01 M)

Stored at 4°C

For assay 5 µL stock/2.88 mL HEPES buffer (0.1 M, pH 7.4)

2. EROD ASSAY PROTOCOL

- Fluorometer was set to excitation 530 nm and emission 585 nm
- 360 µL buffer with NADPH was added to microcuvette
- Microsomes or PMF 10-20 µL were added (depending on protein content)
- Cuvette was equilibrated to assay temperature
- Baseline was recorded
- The reaction was started with 10 µL ethoxyresorufin
- Cuvette contents were mixed several times during assay
- The increase in emission was recorded during linear part of reaction

APPENDIX 6 (continued)

- Resorufin was added to calibrate each assay (equivalent to 2-20 pmol for inanga and bully)

3. CALCULATION OF EROD ACTIVITY

Known amount of resorufin standard and the associated change in fluorescence were related to the change in fluorescence of the the sample divided by the length of the assay (= pmol/minute):

(ΔFluorescence during assay x pmol of standard)/(ΔFluoresence of standard x minutes of assay)

APPENDIX 7
BRADFORD ANALYSIS OF PROTEIN
(Kruger 1994)

1. SOLUTIONS:

Ovalbumin standard 100 µg/mL

Stored in freezer (-20°C)

Bradford protein reagent

Coomassie blue G250 100mg/50mL ethanol (95%)

This was mixed with 100 mL of 95% phosphoric acid

Made up to 1 L with distilled water

Standard, µL	MQ
0	100
10	90
20	80
40	60
60	40
80	20
100	0

Microsomes or postmitochondrial fraction were diluted to the range of the standards.

2. PROTEIN ASSAY

- 100 µL of standards and samples in duplicate
- 1 mL of protein reagent was added, then mixed gently but thoroughly
- After 2-60 minutes, the absorbance at 595 nm against blank was read
- Calculation of protein concentration was done from regression curve of standards

APPENDIX 8
SOLUBILISATION AND SDS-PAGE OF HEAT SHOCK PROTEINS
(Hames 1990; Lee and Dunbar 1994)

Solubilisation buffer solution

SDS, 2%	2 g
Tris base, 0.0625 M	0.75 g
Glycerol, 10%	10 mL

Filled to 100 ml and adjusted pH to 6.8

Filtered with 0.2 μm syringe filter

Sample solubilisation

- Gills were ground in liquid nitrogen using a stainless steel mortar and pestle.
- Samples were diluted in 15 volumes of hot solubilisation buffer.
- Glass tubes with samples were heated in boiling water for 30 min, and covered with foil.
- Centrifugated at 13 000 g for 15 min at 4°C

Polyacrylamide gels

Acrylamide-bisacrylamide (30:0.8):

30 g acrylamide and 0.8 g bisacrylamide
Add 100 mL MQ
Filtered through Whatman no. 1 paper, stored at 4°C in dark bottle

SDS 10% (10 g/ 100 mL MQ)

Stacking gel buffer stock (0.5 M Tris-HCl, pH 6.8):

Tris 6 g/40 mL MQ, pH adjusted to 6.8 with 1 M HCl
Filled to 100 mL
Filtered with Whatman no. 1 paper and stored at 4°C

Resolving gel stock (3.0 M Tris-HCl, pH 8.8):

Tris 36.3 g/48 mL 1 M HCl - mixed and filled to 100 mL
Filtered with Whatmans no 1 filter and stored at 4°C

APPENDIX 8 (continued)

Ammonium persulphate 1.5%:

0.15 g/10 mL MQ

Made fresh prior to use

Recipe for 7.5% gel, suitable for about 70 kDa proteins:

	Stacking gel	**Resolving gel**
Resolving gel buffer stock, mL	-	3.75
Stacking gel buffer stock, mL	5.0	-
10 % SDS, mL	0.2	0.3
MQ water, mL	11.3	16.95
Acrylamide-bisacrylamide, mL	2.5	7.5
1.5% ammonium persulphate, mL	1.0	1.5
N,N,N'N'-tetramethylethylenediamine, TEMED, µL	15	15

Loading PAGE gels

- Samples diluted to same volume and same protein content with solubilisation buffer
- ß-mercaptoethanol added to 5% (v/v) and bromophenol blue to 0.025% (w/v) to samples prior to loading
- Reservoir buffer stock: 0.25 M Tris, 1.92 M glycine, 1% SDS, pH 8.3
- (30.3 g Tris, 144 g glycine, 10 g SDS in 1 L MQ, dilute 1:10 for use)

Molecular weight (MW) -marker

- Prepared according to manufacturer's instructions (Sigma)
- The MW-marker (SDS-7B) contained 7 standard proteins with apparent molecular weights between 32 and 195 kDa conjugated to a blue dye.

Running gels

- Gels run at 4°C at 45 V until dyefront reached resolving gel, then the voltage was increased to 80-120 V.

APPENDIX 9
LOWRY ASSAY FOR HSP70 SAMPLES
(Lee and Dunbar 1994)

1. SOLUTIONS

Solution A 2% Na_2CO_3 2 g
0.1 M NaOH 0.4 g
Brught to 100 mL in MQ and stored in dark bottle

Solution B1 1% $CuSO_4 \cdot 5H_2O$ 0.5 g
Filled to 50 mL with MQ

Solution B2 2% NaK tartrate·$4H_2O$ 1g
Filled to 50 mL with MQ

Solution C 100 parts A 50 mL
1 part B1 0.5 mL
1 part B2 0.5 mL

2 M Folin-Ciocalteu's solution

BSA standard stock

- 1 mg/mL - frozen in aliquots
- Absorbance should be 0.58 at 280 nm
- Protein standards were done in duplicate or triplicate:

BSA stock (µL)	Solubilisation buffer (µL)	MQ (µL)
0	5	195
5	5	190
15	5	180
25	5	170
35	5	160
45	5	150

APPENDIX 9 (continued)

Hsp samples

- Protein samples were determined in duplicate or triplicate
- 5-30 µg protein dissolved in 5 µL solubilisation buffer (same volume as for standards)
- 195 µL MQ added

2. LOWRY PROCEDURE

- 1.0 mL of solution C was added to 200 µL of diluted protein or standard
- This was mixed and incubated for 10 min
- 50 µL of 2 M Folin-Ciocalteu's solution was added and vortexed immediately
- The absorbance was read at 750 nm after 30 min against blank
- Protein content was determined from regression curve of standards

APPENDIX 10
WESTERN BLOTTING, DETECTION AND QUANTIFICATION OF HSP70
(Harlow and Lane 1988; Dunbar 1994; Amersham's ECL Western blotting protocol)

WESTERN BLOTTING

1. Solutions

Transfer buffer:

SDS, 1%	10 g
glycine, 192 mM	14.5 g
Tris, 25 mM	2.9 g
Methanol (v/v), 20 %	200 ml
Made up to 1 L, pH 8.3	

2. Protocol

- Nitrocellulose membrane (0.2 μm; Pharmacia) was cut to size and soaked in distilled water.
- Gel, membrane, filter papers and support pads were immersed in transfer buffer.
- Transfer sandwich was assembled with transfer membrane towards the positive electrode and in close contact with the gel. Air bubbles were eliminated by rolling a glass tube over the sandwich during assembly.
- The sandwich was placed in a BRL Western blotting module, and covered with transfer buffer.
- Transfer took place at 25-30 V for 15 hrs at 4°C.
- Transfer was verified by staining gel with Coomassie, or by staining blotting membrane with India ink or Amido black.

Coomassie blue staining of gels

- Stain was 0.25% Coomassie brilliant blue R-250, 50% methanol and 10% acetic acid.
- Gel was incubated in stain for 4 hours or more.
- Destaining was performed with 5% methanol, 7.5% acetic acid.

APPENDIX 10 (continued)

India ink staining of blots

- Blot was washed in 0.4% Tween 20/phosphate buffered saline twice for 5 minutes.
- Ink solution was 100 µL of India ink in 100 mL 0.3% Tween 20/phosphate buffered saline.
- Blot was placed in ink solution for 15 minutes or longer.
- Blot was destained by washing in phosphate buffered saline.

Amido black staining of blots

- Blot was washed in three changes of 0.3% Tween 20/phosphate buffered saline for 15 minutes each.
- Amido black stain was 0.1% amido black 10-B, 45% methanol, 10% acetic acid.
- Blot was incubated for 5 minutes in amido black stain.
- The blot was destained in 90% methanol/ 2% acetic acid/ 8% water.

ANTIBODY EXPOSURE

1. Solutions

Phosphate buffered saline (PBS), pH 7.5

Na_2HPO_4	11.5 g
$NaH_2PO_4 \cdot 2H_2O$	2.96 g
NaCl	5.84 g

Made up to 1 L, pH checked.

PBS-Tween (PBS-T)

Tween-20, 0.1%	1 mL/L

Blocking agent

Nonfat dried milk 5% in PBS-T (50 g/L)

2. Procedure

- All incubations and washes on rocking or shaking platform at room temperature
- The membrane was incubated in blocking agent for one hour
- The membrane was washed in PBS-T once for 15 minutes, twice for 5 minutes
- The primary antibody N27 was diluted 1:1000 in blocking agent and the membrane was incubated for one hour
- The membrane was washed as above

APPENDIX 10 (continued)

- The secondary horseradish peroxidase-conjugated sheep antimouse Ig antibody was diluted 1:5000 in blocking agent
- The membrane was incubated for one hour with the secondary antibody
- Wash in PBS-T once for 15 minutes and 4 times for 5 minutes

DETECTION

- Equal volumes of ECL detection solutions 1 and 2 (Amersham) were mixed.
- Excess PBS-T was drained from membrane and the membrane was placed on Glad Wrap
- Detection solution was added to cover the surface of the membrane and this was incubated for one minute
- The detection solution was drained from the membrane, and the membrane wrapped in Glad Wrap
- The membrane was placed in a film cassette, all lights were turned off
- A sheet of Hyperfilm-ECL was placed on top of membrane, and the film was exposed for 15 - 60 seconds
- The film was developed using standard methods
- Several films were exposed using different exposure times to get an appropriate signal

QUANTIFICATION OF HSP70

- Molecular Dynamics scanner with ImageQuant software was used
- The film was scanned
- Identical objects (rectangles) were created around each band by copying
- The background was definded by a separate object on film background
- The pixel values of each band were quantitated
- One band was used as a marker and other values were normalised against this