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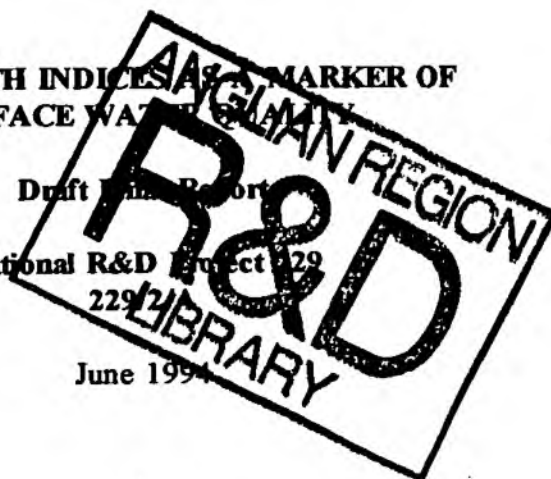
FISH HEALTH INDICES AS A MARKER OF
SURFACE WATER QUALITY

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Fish Health Indices as a Marker of Surface Water Quality

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EXECUTIVE SUMMARY

This report describes the findings of an epidemiological study assessing the effects of differing water quality on the health of fish populations in freshwater river systems under the jurisdiction of the National Rivers Authority (NRA). A variety of techniques for quantifying and describing the health status of fish populations were used. Suitable target waters were identified from existing NRA data using National Water Council (NWC) scores, Biological Monitoring Working Party (BMWP) scores and fisheries survey data. A total of 3613 fish from 35 sites were examined in the field for the presence of a number of easily recognisable external disease characteristics. In addition a sub-sample of 231 fish were examined in the laboratory to assess several physiological, immunological and biochemical parameters.

The overall external disease prevalence was found to be 8.2%. Using a generalised linear model (GLM) able to cope with binomial responses, there was no compelling evidence that prevalence rates differed with respect to river quality data at polluted and reference sites or with month sampled. In each case NWC class 1 rivers provided the lowest prevalence rates for each external abnormality, however, these sites were not statistically separable from other NWC 2 and 3 rivers. One variable was, however, found to be associated with prevalence rates (at the 7% level of significance) and was included in a statistical model. However, the predictions of the model were considered to be close to the thresholds of detectability in an epidemiological study. In laboratory-based studies, for the majority of 12 variables examined, differences between sites were greater than the variation between individual fish. Using one-way analysis of variance 3 parameters differed significantly (at the 5% level of significance) with different river water qualities. Of the 52 possible bivariate correlations between the 12 laboratory variables and 4 river quality variables, 2 indicated a possible "cause and effect" relationship and would warrant further investigation.

This study highlights the need to examine a suite of variables in assessing the overall health status of fish populations. Recommendations for an integrated approach to disease studies are discussed.

KEY WORDS

water quality, fish health, roach, ulceration, epidemiology, pollution, disease prevalence, external disease characteristics.

1. INTRODUCTION

1.1 Overall Project Objective

To determine the general health of fish populations within the NRA's jurisdiction and to evaluate the use of fish health as a surveillance tool for assessing changes in surface water quality and to quantify the role of fish health factors in limiting fish populations.

1.2 Specific Objectives

- (i) To evaluate techniques for quantifying and describing the health status of fish populations.
- (ii) To identify suitable target waters in the NRA regions for studies to be done. Gather information on water quality and biological surveillance as well as fish population data.
- (iii) To describe the health of fish populations from targeted waters using the techniques evaluated in (i) above.
- (iv) To use powerful statistical tests such as multi-variate analysis and multiple correlation to correlate trends in fish health with other biotic and abiotic determinands.
- (v) To assess the scope for improving and refining the techniques to develop a working model for the use of fish health as an indicator of surface water quality.
- (vi) To test the validity of the model.
- (vii) To identify and prioritise the validity of the model.
- (viii) To develop in-house expertise of fish disease as related to fresh water quality.

1.3 Background and History

The NRA has a statutory duty under the Salmon and Freshwater Fisheries Act 1975 to maintain, improve and to develop fisheries. Implicit in this duty is the need to protect fisheries within its jurisdiction from the risk of disease. In order for these duties to be carried out fully, there is a need for the NRA to assess the potential impact that water pollutants may have on the health and status of fish living in these waters.

Over the last two decades there has been increasing concern over the possible link between fish diseases and pollution (Vethaak and Rheinallt, 1990; Bucke, 1991). This stems from the concept that environmental stressors, including pollution, may bring about biochemical and physiological changes in fish that may predispose them to both infectious and non-infectious diseases alike (Wedemeyer *et al.*, 1984). As such, fish may act as useful bioindicators of a polluted environment.

The problem of aquatic pollution is an important area of research that is recognised by international bodies such as the International Council for the Exploration of the Seas (ICES), the European Inland Fisheries Advisory Commission (EIFAC) and the International Oceanographic Commission (IOC). In the United States, the Environmental Protection Agency and the National Marine Fisheries Service Research Laboratory have implemented research programmes to investigate the relationship between pollution and fish diseases.

Many of the first studies that attempted to correlate fish disease with pollution were carried out in the 1970's in the United States. These included epidemiological studies of marine fish from polluted coastal waters, using skin and liver tumours and fin rot as indications of disease. Several studies also attempted to related the prevalence of internal neoplastic lesions in freshwater fish to industrial pollution. These have been comprehensively reviewed by Mix (1986).

Since the late 1970's, there have been several large scale epidemiological surveys of fish diseases in the North Sea involving scientists from several countries, some of which are still ongoing. The results from these studies have been the subject of regular workshops held by the International Council for the Exploration of the Sea (ICES) Working Group on Pathology and Diseases of Marine Organisms. In the United Kingdom, annual workshops on the Relationships of Pollutants and Diseases in Marine Fish have been convened by the Ministry of Agriculture, Food and Fisheries (MAFF) as a result of coordinated investigations involving several research establishments. The subject of pollution in freshwater and its effects on fish was addressed in the 17th EIFAC session. This symposium concentrated on sublethal and chronic effects and the potential use of fish as bioindicators of contamination.

Many of these studies have produced results that go some way to providing evidence for a relationship between pollution and fish diseases. However, disease outbreaks are generally regarded as having a multifactorial aetiology (Vethaak and Rheinallt, 1990; Bucke, 1991) and as such it is often difficult to prove a cause and effect relationship. A large number of investigations have failed in this respect (Kurelec *et al.*, 1981; Wooten *et al.*, 1982; Bucke *et al.*, 1983; Sloof, 1983; McVicar *et al.*, 1988 and Möller 1979, 1981, 1984, 1985, 1988, 1990). Despite these studies there is growing evidence for such a relationship and current opinion favours this (Vethaak and Rheinallt, 1990).

Current approaches to fish disease studies is tending to concentrate on a multidisciplinary approach to fish disease studies encompassing the use of biomarkers as indicators of contaminant exposure. That is measuring changes at the molecular, biochemical and cellular level in wild populations in contaminated areas, and relating these to changes at the whole organism, population and community level. The changes at subcellular and cellular level can provide evidence of direct toxicant exposure whilst changes at higher levels of biological organisation provide evidence of long term sub-lethal effects. Thus, the aim of this investigation is to provide a multi-disciplinary approach to the potential uses of fish health factors as indicators of overall differences in water quality.

2. INDICES OF POLLUTION STRESS IN FISH

2.1 Molecular and Biochemical Indices

There has been extensive study since the early 1970s into the biochemical responses of fish to adverse physical and chemical stimuli (Burns, 1976; Stegeman, 1978; Addison and Edwards, 1988). These approaches using both biochemical and molecular biological approaches have looked at pollutant effects at simple levels of biological organisation, before they are ultimately expressed at higher biological levels such as population and community structure. The advantage of these approaches is that these "bioindicators" can act as early warning signals in the management of aquatic systems which are exposed to environmental stressors before long term damage at population level occur (Adams *et al.*, 1990). Figure 2.1 provides a simplified overview of the interaction of these "bioindicators" in fish.

2.1.1 Deoxyribonucleic acid (DNA) Adducts

Fish are exposed to a wide variety of xenobiotic compounds which they are able to biotransform, via a series of complex biochemical pathways, into metabolites which are more easily excreted. A small proportion of metabolites, especially polycyclic aromatic hydrocarbons (PAHs) bind to DNA and other macromolecules in the cell to form adducts through which they may exert carcinogenic and genotoxic effects.

DNA adduct formation has been proposed as useful parameter for assessing the genotoxic properties of environmental pollutants. Many of these are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage (Shugart, 1990), and if the damage goes uncorrected may result in subsequent adverse effects to the organism (Malins and Haimanot, 1991). For example, activation of proto-oncogenes may occur as a result of DNA-adducts which may culminate in the appearance of overt pathological disease such as neoplasia (Walton *et al.*, 1984; McMahon *et al.*, 1990).

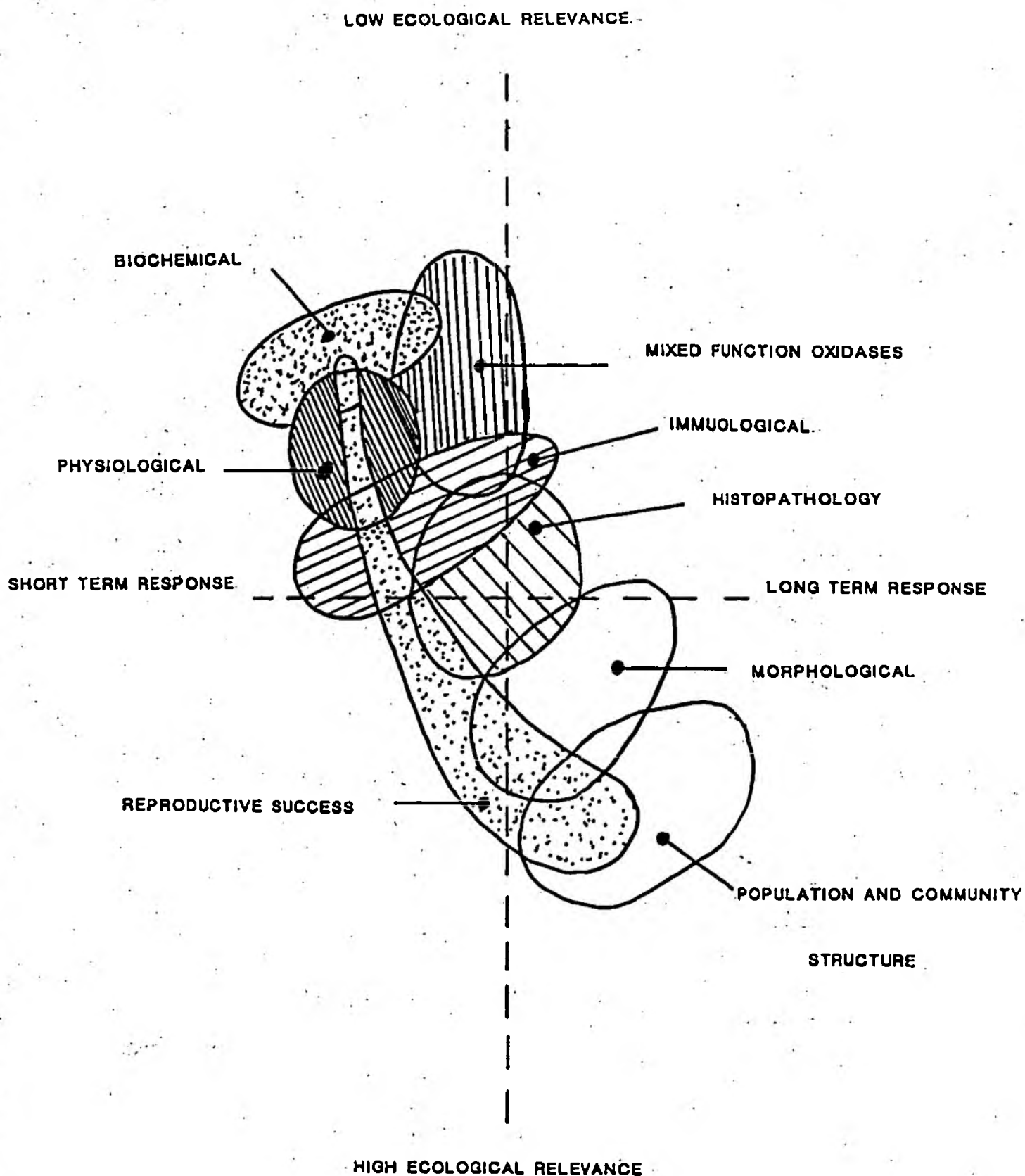


Figure 2.1 Response of "bioindicators" in fish to pollution stress over time and ecological relevance (Adapted from Adams *et al.*, 1989)

Recent developments such as radiolabelled phosphate (^{32}P) postlabelling analysis techniques have enabled the sensitive detection of PAH-related DNA adducts (Randerath *et al.*, 1981; Gupta *et al.*, 1982; Varanasi *et al.*, 1989b; Maccubin *et al.*, 1990; Kurelec *et al.*, 1990).

A summary of laboratory and field studies examining the effects of pollutants upon DNA-adduct formation are listed in Table 2.1.

Table 2.1 **Effects of pollution stress on DNA adduct formation**

Species	Expt	Exposure to:	Effect	Ref.
English sole	Lab	benzo[a]pyrene (BaP)	DNA adduct formation	Varanasi <i>et al</i> 1986
Mosquito fish	Lab	BaP	DNA adduct formation	Batel <i>et al</i> 1985
English sole and Winter flounder	Field	PAHs in sediment	↑ DNA adduct formation	Varanasi <i>et al</i> 1989

↑ = increase in DNA adduct formation

2.1.2 DNA Strand Breakage

DNA strand breakage occurs when metabolites of PAHs and other xenobiotics damage DNA by causing breaks in single strands of the molecule. It has been suggested that this form of damage may be a precursor of tumour formation (Walton *et al.*, 1984; McMahon *et al.*, 1990).

DNA strand breakages can be demonstrated by alkali unwinding assays (Shugart, 1988a,b). This method has been used to demonstrate DNA damage in a number of fish species by exposure to a range of pollutants (Table 2.2).

The use of DNA strand breakage has potential for monitoring certain classes pollutants but requires further evaluation at present (Adams *et al.*, 1990).

Table 2.2 Effects of pollution stress on DNA damage

Fish	Expt.	Exposure to:	Effect	Ref.
Fathead minnow	Lab	BaP	liver DNA damage	Shugart 1988ab
Mosquito fish	Lab	BaP	liver DNA damage	Batel <i>et al</i> 1985
Red breast sunfish	Field	Pulp mill effluent	700-1000% ↑ DNA strand breakage	Adams <i>et al</i> 1993

↑ = increase in DNA strand breakage

2.1.3 Ribonucleic acid-Deoxyribonucleic (RNA-DNA) Ratios

Growth rates can be an excellent indicator of the health of a population since it ultimately incorporates all of the biological, physical and chemical parameters acting on an organism (Bulow, 1970). The ratio of RNA:DNA has been correlated to growth rate since an increase of RNA is an indication of increases in body proteins (Bulow *et al.*, 1981).

RNA:DNA ratio analysis has been applied to natural and experimental populations of fish (Bulow 1970). For example, RNA:DNA ratios were found to be correlated to the growth rate of Lake trout fry exposed to varying concentrations of polychlorinated biphenyls (PCB's) (Passino, 1984). Similarly, growth rates (measured by changes in weight or length, which were mirrored by changes in RNA:DNA ratio) of yellow perch exposed to high levels of cadmium (Cd) and zinc (Zn) were slower than fish from a control site (Kearns and Atchinson, 1979).

These studies have give an indication of how these methods can be successfully applied to field situations, such as measurement of fry RNA:DNA in the field, where it might otherwise be difficult to measure growth using conventional methods.

2.1.4 Metallothionein

Metallothionein (MTN) measurements have been proposed as a biological indicator of metal stress in a number of species (Hamilton and Merhle, 1986) and MTN concentration may be a good measure of the extent of recent exposure of aquatic organisms to certain metals.

Metallothioneins serves a homeostatic function for the essential metals copper and zinc, and also a detoxification function for metals such as cadmium (Cd) and mercury (Hg) (Hamilton *et al.*, 1987). They are found in the liver, kidney and gill tissues of all fish (Takeda and Shimizu, 1982; Petering *et al.*, 1990).

The value of MTN as an exposure indicator to heavy metal contamination of natural freshwater ecosystems has been comprehensively reviewed (Klaverkamp *et al.* 1984; Garvey, 1990; Benson *et al.*, 1990) and there use as being demonstrated by a number of laboratory and field studies (Table 2.3).

Table 2.3 Effects of metal contamination on MTN induction

Fish	Metal	Exposure	Effect	Ref.
Carp	Cd	ip inj	MTN identified in liver	Kito <i>et al</i> 1982ab
Eel	Cd	Water borne	10 fold ↑ in MTN in gill and liver	Noel-Lambot <i>et al</i> 1978
Rainbow trout	Cu, Zn & Cd	Polluted lake	↑ MTN	Roch <i>et al</i> 1982
Coho salmon	Cu	Water bourne	↑ MTN	Buckley <i>et al</i> 1982
Fathead minnow	Various	Water bourne	↑ MTN	Benson & Birge, 1985
White sucker	Heavy metals	Polluted lake	↑ MTN	Duncan & Klaverkamp, 1980, 1983; Klaverkamp <i>et al</i> 1984

↑ = increase in metallothionein (MTN) concentration; Cd, cadmium; Cu, copper; Zn, zinc; ip inj, intraperitoneal injection.

MTN levels have been demonstrated to be influenced by additional factors. For example, sexual differences and maturation state has been found to influence MTN levels (Olsson *et al.*, 1987; Overnell *et al.*, 1987) as well as factors such as temperature and salinity.

2.1.5 Stress proteins

One of the early cellular events in response to pollution stress involves changes in the expression of certain genes. The induction or increased synthesis of stress or heat-shock proteins (hsp) is part of the cells's attempt to protect itself. The induction of many of these proteins is pollutant-specific and as such stress proteins satisfy many of the conditions of ideal candidates for developing a integrated bioindicator strategy for measuring pollution stress (Sanders, 1990). For example, stress proteins of varying molecular size and of varying function have been identified in all organisms and have been found to be induced by a wide variety of environmental pollutants (Sanders, 1990).

2.1.6 Reproductive Hormones

Reproduction in fish is influenced by a number of different factors and involves several hormones interacting with one another in different and complex ways. Both environmental and physical stress have been shown to effect hormonal levels of fish, potentially limiting reproductive success (Schreck and Lorz, 1978; Billard *et al.*, 1981; Pickering *et al.*, 1987).

It is known that chemical pollutants can cause increases in plasma cortisol levels. Furthermore, elevations of cortisol have been shown to lower levels of other reproductive hormones including gonadotropin, 11-ketotestosterone and testosterone in rainbow trout, possibly effecting reproductive success (Pickering *et al.*, 1987, Pickering *et al.*, 1989).

Laboratory experiments have demonstrated reduced hormonal levels in fish exposed to PCBs. Some of these are listed in Table 2.4.

Table 2.4 Effects of exposure to various pollutants on the reproductive state of fish

Fish	Exposure to:	Effect	Ref.
Brook trout	Cadmium	Impaired clearance of testosterone and 11-ketotestosterone	Sangalang & Freeman 1974
Carp & Trout	PCBs	↓ oestrogen, androgen & corticoid	Sivarajah <i>et al</i> 1977ab

↓ = decrease in hormonal levels.

In addition, numerous field studies have demonstrated reduced hormonal levels and impaired reproduction in fish following exposure to environmental pollution (Pickering, 1981). A number of other factors can effect reproduction such as temperature, photoperiod, food availability and nutritional state and disease status (Billard *et al.*, 1981).

Measurement of reproductive hormones as a reliable indication of pollution stress is best limited to species in which the normal reproductive endocrine cycle is known.

2.1.7 Mixed Function Oxidases (MFO)

Organisms exposed to pollutants including PCBs and PAHs accumulate them within cells and tissues (Varanasi *et al.*, 1985, 1987, 1989c). Aquatic organisms can either incorporate these directly from the water and sediments or indirectly through the food chain.

Mixed function oxidases (MFOs), located principally in the liver are detoxification enzymes, collectively termed the cytochrome P-450 dependent mono-oxygenases. Their presence has been examined as a potential indicator of water quality (Jimenez *et al.*, 1990). Under normal

conditions these enzyme complexes are present at low activity. However, when fish are exposed to certain pollutants, these levels of activity rise sharply in order to degrade the pollutants (Addison, 1984). One advantage of this function is that they are induced by specific chemicals and as such they are potentially very useful biomonitors of "hot spot" contaminants. In addition, induction of teleost cytochrome P-450 is brought about by only a few compounds, principally PAHs and PCBs. This selectivity can be regarded as an advantage in the field as they are now thought to be reliable indicators of pollution (Addison, 1984). Several reviews on their use have been written (Stegeman 1980, 1981; Addison 1984; Stegeman *et al.* 1990).

Experimental exposure of fish to PCB mixtures and PAHs indicate that cytochrome P-450 induction occurs very rapidly and may remain high for long periods after exposure (See Table 2.6).

Table 2.6 **Effects of laboratory-based exposure of fish to PCBs on MFO activity**

Fish	Exposure to:	Effect	Ref.
Brook trout	PCBs	↑ ECOD activity*	Addison <i>et al</i> 1981
Rainbow trout	PCB	↑AHH, ECOD & EROD activity**	Elcombe & Lech 1978

↑ = increase in activity

* ECOD activity measured from day 2 to week 3

** AHH (Aryl Hydrocarbon Hydroxylase), ECOD & EROD activity measured from day 4-7 to week 3.

In addition, numerous field studies have demonstrated the use of MFO as bioindicators, especially in areas contaminated by oil spills (Table 2.7).

Table 2.7 Effects of field exposure of various pollutants on hepatic MFO activity in fish

Fish	Exposed to:	Effect	Ref:
Killifish	Oil spill	↑ MO activity	Burns 1976
Various species	Industrial effluent	↑ MO activity	Ahokas <i>et al</i> 1976
Brown trout	Petroleum	↑ MO activity	Payne & Penrose 1975
Various species	sewage outfall	↑ MO activity	Spies <i>et al</i> 1982
Nase	PCB in Rhone PCB in Rhine	↑ EROD activity	Monod & Vindimian 1990
Lake trout	PAHs	MO induction	Luxon <i>et al</i> 1987
Benthic fish species	PCBs	demonstration of Cyt P450E isozyme	Stegeman & Kloepper-Sams 1987
Killifish	PCBs	EROD activity correlated to tissue and sediment PCB levels	Elskus & Stegeman 1990
Spot	PAH	Cyt P450E detected using immunoassays	Van Veld 1990

* several studies have demonstrated the use of polyclonal and monoclonal antibodies to detect isozymes of cytochrome P-450 (Schoor *et al.* 1991; Varanasi *et al.* 1986).

↑ = increase in activity; Cyt, cytochrome; MO, mono-oxygenase

Induction of MFOs has been shown to be influenced by a large number of other factors which have to be taken into consideration. These include tributyltin chloride (TBT) (Fent, 1991; Fent and Stegeman, 1991), season and reproductive hormones (Luxon *et al.*, 1987), sex differences and temperature (Jimenez and Burtis, 1988). These factors have been reviewed by Stegeman and Kloepper-Sams (1987).

Hepatic mixed function oxidases in fish are informative markers of exposure, but the role of environmental, physiological and toxicological factors on MFO response must be understood to interpret the bioindicator response (Jimenez *et al.*, 1990).

2.2 Physiological Indicators

Physiological parameters applied to fish health offer advantages in environmental studies since it is widely accepted that excessive environmental stress causes a variety of detectable changes in the blood, tissues and organs of fish (Bouck, 1984)(See Table 2.8). Therefore, measurement of fish health by these means could provide an early warning of excessive environmental stress and would do so faster and cheaper than measuring the population response.

Problem associated with physiological indices are the lack of normal baseline values and the fact that fish physiology is influenced by a variety of factors including season, spawning condition etc. In addition, capture stress resulting in the release of catecholamine and stress-induced corticosteroids precludes the use of a number of sensitive, early indicators of environmental stress (Pickering, 1981).

Table 2.8. Physiological indices of pollution stress in fish

Fish	Exposure to:	Expt.	Effect	Ref.
Rainbow trout	Paper & Pulp mill effluent (10 day)	Field	↓ Hct, Hgb, plasma glucose, protein & urea	Tana & Nikunen (1986)
Coho salmon	Pulp mill effluent (12h & 25 day)	Lab	12 h; No change in RBC counts & Hct. ↑ in RBC and plasma glucose. 25 day; ↓ Hct & plasma glucose; ↑ RBC	McLeay (1973)
Coho salmon	Dehydroabietic acid (DHAA)(from pulpmill effluent)(6-48 h)	Lab	No changes in Hct & RBC counts. ↓ in WBC after 24h	Iwama <i>et al</i> (1976)
Coho salmon & Rainbow trout	Pulpmill effluent (24 h)	Lab	↓ in WBC	McLeay & Gordon (1977)
Rainbow trout	Rhine water (18 month)	Lab	↓ growth rates & Hgb; ↑ LSI, kidney size & blood glucose. Histological changes observed in liver & spleen	Poels <i>et al</i> (1980)
Rainbow trout	Dehydroabietic acid (DHAA) 1.2 mg/l for 4 days (acute) 20 µg/l for 30 days (chronic)	Lab	Acute; ↓ LSI, ↑ Hct, Hgb, plasma protein. Impaired liver function. Chronic; little effect	Oikari <i>et al</i> (1983)
Rainbow trout	Bleached pulpmill effluent (10 day)	Field	↑ Hgb, ↓ plasma protein.	Oikari <i>et al</i> (1985)
Atlantic salmon	Haematotoxic effluent	Field	hyperbilirubinaemia	Everall <i>et al</i> (1991; 1992)
Redbreast sunfish	Various	Field	↑ EROD & cytochrome P-450 activity. ↑ LSI but ↓ functional liver tissue. ↓ in RNA:DNA ratio & ↑ in MMCs.	Adams <i>et al</i> (1993)

↑ = increase; ↓ = decrease; Hct, haematocrit; Hgb, haemoglobin; LSI, liver somatic index; MMC, melano-macrophage centre; RBC, red blood cell count; TBC, total blood cell count; WBC, white blood cell count.

2.2.1 Haematological parameters

It is well established that stressed and diseased fish exhibit characteristic changes in the number and composition of circulating blood cells. Although these changes are non-specific, measurements of haematocrit, leucocrit, differential and total cell counts and haemoglobin are useful indicators of chronic stress. Comprehensive reviews on the effects of environmental and pollution stress on blood biochemistry of fish have been published (Wedemeyer and McLeay, 1981; Lockhart and Metner, 1984).

Plasma enzymes (measured by histochemical techniques), such as leucine amino naphthylamidase (LAN), a component of lysosomes partly responsible for autolysis during cell death has been used to examine the effects of pollution stress on fish (Bouck *et al.*, 1975, 1978; Bouck, 1984). In addition, the enzyme erythrocyte δ -amino levulinic acid dehydratase has been used as a short-term indicator of harmful exposure of fish to heavy metals (Hodson *et al.*, 1977).

2.2.2 Immunological Assays

It has been proposed that assays of immune function are potentially superior to chemical analysis as indicators of pollutant exposure since they provide evidence of a link between environmental challenge and health status (Weeks *et al.*, 1986a,b; 1987a,b; Anderson, 1990). Assays involving the immune system can be divided into several areas:

Non-specific assays

These include assays for serum components such as C-reactive protein (CRP), an acute phase protein which increases in concentration in response to inflammation and tissue necrosis (Ramos and Smith 1978). A CRP-like molecule has been identified in rainbow trout (Winkelhake and Chang, 1982) as well as in roach (David Hoole, pers. comm.). The use of CRP as a potential bioindicator of pollution stress has not been examined.

Assays with/without Antigenic Stimulation

The mitogenic response involves measuring the ability of lymphocytes to proliferate in response to a mitogen such as bacterial lipopolysaccharide (LPS). Immunosuppression in fish has been demonstrated by demonstrating decreases in the mitogenic responses of rainbow trout exposed to dioxins (Spitsbergen *et al.*, 1986).

Additionally, macrophages are an important part of the cellular immune system of fish and act as a first line of defence in the phagocytosis of foreign antigens (Ellis, 1977). Macrophage activity has been found to be affected by toxic contaminants and a number of different macrophage assays have been developed to evaluate immune function in fish. Such methods can be applied as bioindicators of fish health and the effects of exposure to environmental stress (Scott and Klesius, 1981; Weeks and Warriner, 1984, 1988; Weeks *et al.*, 1986a,b; 1987a,b; Warriner *et al.*, 1988; Dierickx and Van de Vyver, 1991)(See Table 2.9).

Table 2.9 Effects of pollution stress on macrophage activity of fish exposed to various pollutants

Fish	Exposure to:	Effect	Ref:
Spot & Hogchoker	River polluted with PAHs	↓ chemotaxis ↓ phagocytosis ↓ pinocytosis ↓ chemiluminescence	Weeks <i>et al</i> 1986ab
Rainbow trout	Cu & Al	↓ chemiluminescence	Elasser <i>et al</i> 1986
Various estuarine species	TBT	↓ chemiluminescence	Wishkovsky <i>et al</i> 1989
Toadfish	PAHs	↓ phagocytosis	Seeley & Weeks-Perkins 1991
Dab	Cd	↓ chemotaxis	Hutchinson (pers. comm.)
Dab	Contaminated sediment	↓ chemotaxis	Field (pers. comm.)

↓ = decreased activity; Al = aluminium; Cd = cadmium; Cu = copper; TBT = tributyltin.

A variety of factors have been demonstrated to effect macrophage activity and it is important to consider these in any study (Angelidis *et al.*, 1987, 1988; Sheldon and Blazer, 1991; Blazer, 1991).

Specific Indicators of Immune Response

Investigations of the effects of pollution stress on the specific immune response of fish requires immunisation of the fish with bacterial antigens such as *Aeromonas salmonicida* or *Vibrio anguillarum* cells followed by measuring the production of specific antibodies to the antigen by techniques such as enzyme-linked immunosorbent assay (ELISA) (Bucke *et al.*, 1989) and enzyme-linked immuno-spot assay (ELISPOT) (Davidson *et al.*, 1992). Many studies have demonstrated that environmental stress and pollution alters disease resistance and the humoral immune response to particular antigen (Wedemeyer, 1970; Bucke *et al.*, 1989; Secombes *et al.*, 1991).

Melano Macrophage Centres

Melano-macrophage centres (MMCs) are discrete macrophage aggregates containing pigments such as haemosiderin, melanin and lipofuscin. They are present in the spleen, liver and kidney of fish (Aguis, 1979, 1981; Roberts, 1989); their numbers in fish vary according to age (Brown and George 1985), nutritional status, starvation (Agius and Roberts 1981; Micale and Perdichizzi, 1990) and disease (Roberts 1989). Despite these factors, they are still considered to be sensitive indicators of fish health (Kranz and Peters, 1984; Wolke *et al.*, 1985) and have been shown to increase in frequency in response to pollutants in both laboratory and field studies (Kranz and Gercken, 1987)(See Table 2.10).

Table 2.10 Effects of laboratory and field exposure of pollutants on the melano-macrophage centres of fish

Fish	Expt.	Exposure to:	Ref.
Dab	Lab	sewage sludge	Secombes <i>et al</i> 1991
Plaice	Field	Oil spill	Haensly <i>et al</i> 1982
Yellowfin sole Quillback rockfish	Field	Petroleum	Khan & Nag 1993
Plaice	Lab	potassium dichromate	Kranz & Gercken 1987
Roach Gudgeon	Field	<i>Ligula intestinalis</i> infection	Taylor & Hoole 1989
Whitemouth croaker	Field	Polluted river	Macchi <i>et al</i> 1992

2.2.3 Body burdens/Bioaccumulation

The ability of aquatic organisms to bioaccumulate environment xenobiotics is well established (Sonstegard and Leatherland, 1984; Varanasi *et al.*, 1992). Heavy metal and pesticide residues in tissue samples give a good indication of the pollution levels to which populations are being exposed (Stein *et al.*, 1984, 1987).

2.3 Morphological Indicators

Morphological indicators of pollution stress in fish offers an advantage in that many of the indices eg length and weight measurements and the prevalence of gross morphological lesions are all externally visible and can easily be recorded whilst sampling in the field.

2.3.1 Organismic Indices

The condition factor and organismic indices such as liver somatic indices (LSI), spleno-somatic indices (SSI) and gonado-somatic indices (GSI) are a general indicator of the overall health of fish and can reflect the integrated effect of both nutrition and metabolism induced by stress (Fagerlund *et al.*, 1981; Slooff, 1983; Adams *et al.*, 1985, 1990).

2.3.2 External and Internal Indicators

Pollution has often been described as being a major trigger for the development of certain diseases of fish eg skeletal deformity, fin erosion, ulcerations and tumours (Lindesjö and Thulin, 1990; Khan and Thulin, 1991). In several studies, correlations have been made between certain gross morphological lesions and the presence of specific pollutants (Sonstegard, 1977; Murchelano, 1982; Malins *et al.*, 1985; Murchelano and Wolke, 1985; Lindesjö and Thulin, 1987).

The prevalence of these bioindicators, along with the degree of severity or damage incurred by an organism or tissue from environmental pollutants has been integrated to provide a quantitative index of fish health in the field by several workers. Both the Health Assessment Index (HAI) described by Adams *et al.* (1993) and the Index of Biotic Integrity (IBI) described by Karr (1991) have been used to assess the general health status of fish populations in the field. Both indices have been able to characterise fish health in various aquatic ecosystem systems and offer a simple, proven and inexpensive means of rapidly assessing fish health.

Lesions (eg necrosis, ulceration, haemorrhage)

External lesions are characteristic of many fish diseases and are generally of an infectious aetiology, often presenting similar clinical and histopathological pictures (Roberts, 1989). Although of infectious aetiology, stress-related factors are often implicated in outbreaks, such as temperature, season, spawning and poor water quality (Owen, 1988). Causative agents include bacteria such as *Aeromonas salmonicida* subspecies *salmonicida*, *achromogenes* and *masoucida*, motile *Aeromonas* species such as *A. hydrophila* and *A. sobria* and *Pseudomonas* species (Austin and Austin, 1989; Inglis *et al.*, 1993), virus such as Spring Viraemia of Carp (SVC) (Roberts, 1989) and ectoparasites such as black spot (*Posthodiplostomum minimum*) (Steedman, 1991), leeches (*Piscicola geometrica*) and Crustacean ectoparasites such as *Argulus* species (Roberts, 1989).

Tumours

The rapid development of tumours in fish may be attributed to the relatively poor ability at repairing DNA adducts (See 2.1.1)(Walton *et al.*, 1984; McMahon *et al.*, 1990). Fish may therefore act as an early warning system for the detection of carcinogens in the aquatic environment (Tyler and Everett, 1993).

There are few reports of neoplasia in fish especially species of freshwater fish (Bucke and Feist, 1985). The presence of neoplasia is generally restricted to epidermal papillomas and hyperplasias of unknown aetiology (Mawdesley-Thomas, 1975; Andrew and Bucke, 1982; Tyler and Everett, 1993). There are many reports of other neoplasias especially of the liver such as hepatocellular carcinoma in bottom-dwelling marine fish, where strong association between tumours and pollution (Malin *et al.*, 1984, 1987, 1988; Bowser *et al.*, 1990, 1991; Murchelano and Wolfe, 1991; Harshbarger and Clark, 1990).

Vertebral deformities

Numerous skeletal deformities have been described in teleost fish (Poynton, 1987). The use of skeletal deformities for monitoring pollution has been considered by Bengtsson (1979). There is generally an increase in the prevalence of skeletal deformities such as curvature (scoliosis and lordosis), spondylitis and spondylosis in fish exposed to pollution stress and these physiological diagnostic methods have been frequently used to detect the toxic effects of heavy metal exposure in both experimental and field studies (Bengtsson and Larsson, 1986).

Fin erosion

Fin rot and fin erosion are common pathological conditions of the fins of teleost fish. Although the aetiological agent is generally attributed to the bacterium *Flexibacter* spp. (Post, 1983), during the last ten years, fin rot and erosion has been increasingly associated with environmental pollution (Cross, 1984; Thulin *et al.*, 1988; Lindesjö and Thulin, 1990).

2.3.2 Histopathology

The use of histopathology in assessing fish exposed to pollutant stress has been studied by Johnson and Bergman (1984) and Hinton and Lauren, 1990).

The potential uses of histopathology is generally characterised as being either diagnosis or monitoring. Diagnostic histopathology involves the identification of the cause of observed adverse population effects, whereas histopathology used for the purpose of monitoring, involves warning of any impending contaminant-related environmental degradation.

The prevalence of specific lesions in organs of fish such as the liver, spleen and kidney proved useful indicators of exposure to chemical pollutants in a number of fish species (Hinton and Lauren, 1990; Bowser *et al.*, 1990; Bucke, 1991). For example, liver histopathology is good bioindicator since the liver integrates both biochemical and physiological functions which when altered may produce bioindicators indicative of exposure to pollutant stress (Hinton & Lauren, 1990).

2.4 Other indicators

Populations of living organisms have evolved through a process of adaptation to the environmental changes they experience. Environmental change therefore acts on both individuals and their offspring. If changes in growth, survival and reproductive rates of fish occur, higher levels of biological organisation may be affected. For example, at the species level, reduced recruitment of fish to succeeding life stages may result in a population decline. In addition, at the community or ecosystem level, ultimately species diversity may be affected.

2.4.1 Population indicators

The status of fish populations is a reflection of the overall condition of the aquatic environment in which the population resides. As such, fish population characteristics can be used as an indicator of environmental health (Kerr and Dickie, 1984). However, simple and inexpensive methods enabling fish population responses to environmental degradation are lacking.

The impact of environmental degradation on the reproductive competence, fecundity and condition factors have been assessed by Munkittrick and Dixon (1989). Population characteristics were used to examine ecosystems exposed to pollution stress for evidence of long-term damage. When used alongside biochemical indicators such studies were considered to be a powerful tool for health assessment.

2.4.2 Community indicators

Most changes in phenotypic characteristics can be detected by examining gross-indicators of population structure. Fisheries exploitation can successfully measured by mortality, growth, age-structure and mortality these measurements have to measured over a long period of time (Kerr and Dickie, 1984).

The use of community indices of pollution stress requires the ability to readily detect population changes before holistic monitoring can be used successfully. There is always a time lag between pollution events and detection of effect at population. As such these indices offer a retrospective view of the health of fisheries.

3. METHODS AND MATERIALS

3.1 Selection of Target Species

Different species of fish may be differentially susceptible to diseases. Therefore, it was important to select a species that could be found in abundance at all study sites in order for meaningful comparisons to be made. For this reason roach (*Rutilus rutilus* L.) were selected as the main species for study. Roach also have the advantage that previous disease studies by Hotchin and Williams (1982) and Owen (1989) also centred on this species. At sites where other species such as chub (*Leusiscus cephalus* L.) were found in reasonable abundance, they were also captured for examination.

In addition, in response to the concern of anglers about barbel (*Barbus barbus* L.) with tumours and other lesions in certain rivers of Southern England (Legge, 1989), barbel from the River Lee, a tributary of the River Thames were examined during this investigation.

3.2 Selection of Study Sites

Study sites were selected to fulfil several fundamental criteria:-

- A high level of background data on existing fish populations including numbers, species diversity, biomass and age structure and disease status.
- The target species for study should be present in numbers that can be relatively easily sampled.
- There should be a high level of background data on chemical water quality (NWC scores) and biological water quality (BMWP scores).
- It should show well characterised pollution sources and a measurable gradient of pollution.
- Ideally there will be a compounded stretches of river (eg. sections between weirs) to prevent fish movement away from the pollution source.
- Angling pressure on the water should be minimal.
- The site should be easily accessible for sampling purposes.

No one site was identified that fulfilled all of these criteria resulting in the need to target several sites.

3.2.1 Description of Study Sites

Willowbrook (NRA Anglian Region)

The Willowbrook arises as three small streams near Corby, Northamptonshire which join at the first of a series of three lakes along the length of the water course. The catchment is approximately 9000 hectares of largely arable farmland. These lakes act as a series of settlement lakes to some extent, with water quality improving from Class 3 (above and immediately below first lake), to Class 1B below the third lake. Here a trout fishery is supported. Pollution problems include organic loading from Corby sewage treatment works (STW) and heavy metal discharge from a steel works. Roach dominate below the first lake but become subordinate to chub below the third lake.

Soham and Burwell Lodes (NRA Anglian)

The Soham and Burwell Lodes are situated principally in arable farmland. The main pollution problems in the Soham Lode are organic loading from Newmarket and Soham STWs. Water quality is NWC Class 3 and it has failed to meet Fisheries Ecosystem Class 3. By contrast, water quality in the Burwell Lode is good and this can act as a control.

Cut Off Channel (NRA Anglian)

Water quality in the Cut Off Channel is good, being classified Class 1B/2 with no major pollution problems. As such it useful as a control system in the study.

River Nene (NRA Anglian)

Water quality on the River Nene suffers from industrial pollution sources and effluent from STWs. One such site identified on the river is a stretch below Broadholme STW. Water quality is NWC Class 2/3 at this point.

River Cam (NRA Anglian)

The catchment of the River Cam is predominately rural and includes high grade agricultural land. Water quality is generally good being Class 1B or better for much of its length. However local pollution problems, mainly from STWs reduce water quality to Class 3 in some short sections. This is evident below Cambridge as a result of discharge from the Cambridge STW.

Wiltshire Ray (NRA Thames)

The river Ray (Wiltshire) is a truly urban river with its catchment dominated by the Swindon conurbation. Water quality varies from Class 3 to Class 2A along its length, and under dry conditions maximum consented discharge would constitute 80% of the river flow. It has also suffered from a large number of sporadic pollution incidents, mainly chemical, oil and sewage.

Blackwater (NRA Thames)

This is an urban river running through Aldershot, Farnborough, Camberley and Yately. Water quality is Class 3 in the upper reaches, improving to Class 2 at its confluence with the Loddon. Pollution problems consist of organic loading from several major STWs along its length and cadmium in the upper reaches from a plating works.

River Lee (NRA Thames)

The Kings Weir on the River Lee, Essex, was identified as a special case for investigation following reports of barbel exhibiting external abnormalities. Claims from the angling press linked these to potential water quality problems. The River Lee at this point receives effluent from Rye Meads and East Hyde STWs and possibly leachate from infill sites. The section at Kings Weir is run as a private day ticket fishery. Water quality at this point is good, being Class 1B.

River Tame (NRA Severn Trent)

The River Tame and its tributaries drain most of the West Midlands conurbation. In the upper reaches it is grossly polluted (NWC Class 4) but improves to class 2/3 at its confluence with the Trent. Known sources of pollution include heavy metals from old mine workings, leachate from industrial waste, road run off and organic loading from STWs. Problems are further exacerbated by the fact that it is subject to flash flooding resulting in the re-suspension of organic sediments during periods of high flow. Purification lakes at Lea Marston have now led to an improvement in the lower reaches indicated by the reestablishment of coarse fish populations over the last 20 years. Populations above the lakes remain poor however.

River Avon (NRA Severn Trent)

The River Avon at Evesham in Warwickshire was identified as a potential study site because of readily identifiable disease problems. This section is run as a private day ticket fishery and is quite heavily fished. It contains chub and roach which have been reported as having a high incidence of external lesions. The river is classified NWC class 2 at this point.

3.2.2 Water Quality and Fish Population Data

Both fisheries and chemical and biological data relating to water quality at the selected sites listed above are routinely collected by the NRA. This data was used during the course of the project.

3.3 Fishing Techniques

Standard fishing techniques used by the NRA were adopted (Coles *et al.*, 1985). For most sites electro-fishing techniques were employed. However, sites on the River Cam and River Nene were seine netted because of the nature of the two rivers. No attempt was made to carry out population type surveys since this data is already collected by NRA fisheries teams. Therefore electro-fishing was carried out on a roving basis without the use of stop nets.

All fish captured were measured, scaled and prevalence of disease and ectoparasitic infestation recorded. Table 3.1 describes the bioindicators examined. Each was recorded as being either present or absent on each individual. Biopses of external abnormalities present on barbel from the River Lee were fixed in 3% glutaraldehyde in 0.1M cacodylate buffer at pH7.2 for transmission electron microscopy (TEM). Randomly selected sub-samples of approximately 10 roach, between 14 and 20 cm in length, were taken back to the laboratory for further detailed examination. The remainder of the fish were returned to the water.

Table 3.1 Description of external disease indicators

Symptoms	Description
Ulceration	Scale loss leading to a localised necrotic lesion of the skin. These ranged from superficial damage of the epithelium to deep wounds exposing the musculature.
Fin rot	Degeneration of the interray tissue at the trailing edge.
Haemorrhage	Bleeding from any part of the vasculature any where on the fish
Petechiae	Small blood spots form pinprick to pinhead size.
Mouth damage	Any damage to the mouth region, angler caused or otherwise.
Deformities	Obvious skeletal or fin ray deformities resulting in changes in body shape
Hyperplasia	Thickening of the epithelium over individual scales similar to carp pox
Healed ulcers	Any previous damage to the body signified by whorling scale patterns or scar tissue
External parasites	Any external parasites were recorded

3.4 Laboratory Procedures

3.4.1 Post Mortem Procedures

Samples of live fish were transported back to the Fisheries Laboratory (FL) in sealed polythene bags filled with oxygen and kept overnight in a flow through tank system. The fish were processed individually, the following day. Fish were gently caught in a mesh net with as little disturbance as possible and humanely killed with a lethal dose of benzocaine (1mg/ml). The post mortem procedure is summarized in Table 3.2.

Table 3.2 Summary of PM procedures

Order of PM procedures		Ref.
1	Fish killed humanely in benzocaine	FL standard protocol for fish health examination
2	Measured and weighed	
3	Blood sample taken immediately, blood smear done, remainder stored in fridge for later use	Dacie & Lewis (1984)
4	External examination of fish, shoulder scale removed for aging, skin scrape done	FL standard protocol
5	A) If bacteriology sampling, swabs taken from any external lesions, or B) If fish to be used for macrophage work, head kidney removed	A) FL standard protocol B) Secombes (1991), Weeks <i>et al</i> (1986ab)
6	Liver and spleen removed and weighed.	
7	Tissues fixed in 10% v/v neutral buffered formalin	FL standard protocol
8	Examination of gills, eyes and internal organs for parasites	FL standard protocol
9	Carcass frozen	

3.4.2 Blood Sampling and Analysis

Blood samples were taken from the caudal vein using heparinised syringes with a 25 gauge needle. The needle was inserted into the flank of the fish at a point marked by the lateral line immediately above the anal fin, and not from a point on the mid ventral line behind the anal fin. Better results were obtained in this way. Two or three scales were first removed and the area cleaned with alcohol to remove any traces of mucus which contains coagulating agents. Blood was drawn out under gentle aspiration and immediately mixed well to avoid clotting. It was then transferred to eppendorf tubes and kept in the fridge until further processing.

Plasma for protein analysis was separated by centrifugation at 4000 g at 4°C for 5 minutes (Heraeus, Biofuge 15R centrifuge) and frozen at -20°C until analysed. Any samples that showed visible signs of clotting were only analysed for blood glucose and plasma protein.

Blood films were prepared, stained using May-Grunwald-Giemsa stain and examined using the methods described by Dacie and Lewis (1984) to obtain differential blood cell counts.

Total blood counts, hematocrits, cell volumes and haemoglobin content were measured using a haematological analyser (Cosmark, model AL-871 Vet-Pack analyser). Blood was diluted for analysis with Celloton-Isotonic diluent (Cosmark) using an automated Diluter III, model 222 (Cosmark). The mean of three readings was taken.

Blood glucose levels were measured as soon after sampling as was reasonably possible, using Glucostix and a Glucometer II (blood glucose meter) model 5626 (Ames). The mean of two readings was taken.

Total plasma protein levels were determined by the biuret method using the Sigma diagnostic assay (No 541; Sigma Chemical Company).

3.4.3 Histopathology

Tissues for histological examination were excised and fixed in 10% v/v neutral buffered formalin (NBF) within 15 minutes of death. Tissues to be examined included gill, liver, kidney, spleen, gonads and any other tissue that exhibited abnormalities. They were processed using standard automated histological techniques either by the FL or commercially by the Department of Pathology, Dryburn Hospital, Durham.

Sections were stained using heamatoxylin and eosin (H&E) for routine work. Melanomacrophage aggregates were stained by Perls' Prussian blue method for the demonstration of ferric iron. An image analysis system (Macintosh Quadra 950, ColourVision software, Improvision) was used to evaluate the number of aggregates under the microscope at x100 magnification. The area of these was expressed as a percentage of the total area examined.

3.4.4 Electron Microscopy

Biopsies of any external abnormality present on barbel were fixed as described in 4.*.* and processed for transmission electron microscopy (TEM) using standard methodologies by MAFF Fish Diseases Laboratory, Weymouth (Feist, pers. comm.)

3.4.5 Macrophage Activity

Assays of cellular immune function were carried out using techniques described by Weeks *et al* (1986a,b) with only minor modifications. Macrophages were aseptically isolated from the pronephros of the roach. The external surface of the fish was first doused with alcohol

before they were dissected. Cell suspensions were obtained by gentle homogenisation in 1 ml glass homogenisers (Jencons) and standardised to a concentration of 1×10^7 viable cells/ml using a Neubauer improved haemocytometer to count the cells. All work was carried out in RPMI 1640 medium (Sigma Chemical Company). Cell viability was determined by trypan blue (0.5%) exclusion assay (Sigma Chemical Company, 1991). Cell viability was found to be in the region of 80-90%.

The chemotaxis response of the macrophages was measured using a Neuro Probe 48 Well Micro Chemotaxis Chamber (Costar, Nuclepore) as described in the Neuroprobe manual. Chambers were separated by 5 μ M millipore membranes to allow migration of the cells.

The lower wells were filled with 28.5 μ l suspension of formalin killed suspension of *Escherichia coli* ATCC 35218 (Difco) adjusted to 5×10^8 cells/ml opsonised with 3% (v/v) chicken serum (Sigma) as a chemo-attractant. This amount was found to be ideal in providing a slightly convex meniscus so as to avoid trapping air bubbles when locating the filter membrane. The upper wells were filled with 45 μ l of the kidney cell suspension. Elkay White pipette tips (Labsystems, Cat No 000 OGEL R01) were used to fill the wells since their use resulted in fewer air bubbles being introduced into the wells.

The chamber was incubated for 1 hour at room temperature to allow cell migration to occur. The filter paper was then removed and the upper surface (cells from the initial cell suspensions) was rinsed twice in 0.85% phosphate buffered saline (PBS), taking great care not to wet the under side, and passed over a rubber scraper (Costar, Nucleopore) to remove these cells. This left only the migrated cells on the under surface of the membrane. It was then fixed for 10 minutes in 100% methanol and stained with May-Grunwald-Giemsa stain as described previously. The filter membrane was rinsed well in buffer and mounted whilst still wet on to a glass microscope slide before being allowed to dry.

The migrated cells were examined under the microscope using x1000 oil immersion magnification. The total number over one transect (16 fields of view) of each well was counted and expressed as number of cells/mm².

3.4.6 Bacteriology

Standard procedures used at the FL for bacteriological examination of fish were adopted. Swabs were only taken from surface lesions since previous work at the FL has shown that systemic infections in ulcerated fish are rare (Owen, 1988). Isolation of pure bacteriological cultures was done on Tryptone Soya Agar (TSA; Oxoid) or TSA plus 5% v/v horse blood (bioMerieux) and identification made using the presumptive identification techniques routinely used at the FL. These include the following tests; gram stain, motility, oxidase, catalase, O/F reaction and API 20E and API 20NE identification systems (BioMerieux)(FL laboratory manual).

3.4.7 Parasitology

Standard procedures used at the FL were adopted. Skin scrapes from the external surface of the fish and squash preparations of eyes, gill, liver, kidney and spleen were made for microscopic examination.

3.4.8 EROD Analysis

EROD analysis of liver tissue was carried out in collaboration with David O'Hare, Derby University. Only livers from fish 15-20 cm in fork length were used in order to obtain enough material for the assay and in order to standardise the technique. These were wrapped in aluminium foil and stored in liquid nitrogen for no longer than three days before analysis.

Levels of EROD were estimated using the S9 liver fraction only. This was obtained by homogenisation in a potassium chloride (0.154M) magnesium chloride (10mM) buffer dissolved in Tris-HCL (50mM), adjusted to pH 7.4, followed by centrifugation at 10,000 g for 20 minutes at <4°C (Sorvall RC-5B centrifuge).

EROD levels were determined by direct fluorometric assay based on the method described by Burke and Mayer (1974) using 7-ethoxyresorufin as substrate. The reaction mixture consisted of 925 µl of substrate solution (1.1 mM), 50 µl S9 fraction and 25 µl NADPH (25 mg/ml; Sigma. The substrate concentration was measured on the basis of the extinction coefficient for 7-ethoxyresorufin at 482nm being 22.5 Cm²/mmol. The excitation and emission wavelengths were 530nm and 585nm respectively and EROD levels expressed as pmol/min/mg of protein.

3.4.9 PAH analysis of sediment samples

Chemical analysis of sediment samples were performed commercially by Resource Consultants Cambridge Ltd (RCC) using standard methodologies. 10-20 g sediment (collected by ...) were analysed for the PAHs listed in Table 3.3.

Table 3.3 PAHs examined in sediment samples

PAH
Phenanthrene
Fluoranthene
Pyrene
Benzo[g,h,i]perylene
Benzo[a]pyrene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Indeno[1,2,3-c,d]pyrene
Chrysene
Benz[a]anthracene

3.4.10 Statistical Analysis

Statistical analysis of field and laboratory data was performed by Robert Lacey of WRc plc, Medmenham under contract 04647.

Field data

Analysis of field data was based on a "generalised linear model" (GLM) designed for coping with a "binomial" response, r , the number of abnormal fish out of a total n examined at a particular site. r is assumed to be a binomially distributed variable with site-specific prevalence rate p , whose independence on the possible explanatory variables is modelled by an equation of the form:

$$\log_e (p/(1-p)) = b_0 + b_1x_1 + b_2x_2 + \dots + b_kx_k$$

where x_1, \dots, x_k are explanatory variables and b_0, \dots, b_k are parameters available for estimation.

This framework was used by WRc plc to accommodate both continuous explanatory variables such as length and categorical factors such as NWC class, and provides the same facilities as in multiple linear regression for testing and estimating their effects. The analysis was performed using the Rothamsted package GENSTAT.

Laboratory data

The relationship between each of the laboratory variables and the NWC river class was assessed by a one-way analysis of variance of the relevant site averages, grouped into the three river water quality classes. Equality of the three class mean was tested in the standard

way using the F-ratio. No transformations were used on any variable since it was beyond the scope of this statistical investigation.

The possible association between each of the laboratory variables and each of the four chemical determinands of river quality was assessed by inspecting the bivariate correlation coefficient and comparing it with the percentage points in a standard statistical table.

4. RESULTS

4.1 Statistical analysis of field data

The data analysed consisted of the records of individual fish examined for a number of external disease abnormalities listed in Table 3.1. A total of 3382 fish were examined during the course of this study at 35 sampling sites for roach of which 5 sites (visited 13 times) were sampled specifically for chub (Table 4.1). The sampling sites are listed in Table 4.2. Examples and descriptions of the various external disease markers observed in the field are illustrated in Figures 4.1 - 4.6.

Table 4.1 Sites and frequency at which chub were sampled

Site	Site/visit	Number of visits	NWC
Avon @ Harvington	Avon6/91;3/92;6/92 3/93	4	2
Nene @ Chettles	Nene3/91	1	2
Blackwater @ Eversley Cross	Blw3/92	2	2
Willowbrook @ Alders Farm	Wlb6/91;2/92;6/92	3	2
Willowbrook @ Woodnewton	Wlb3/91;6/92;9/92	3	1

At each of the 35 sampling site electrofishing was used to obtain a sample of n fish to be individually measured and the presence or absence of the 8 external disease abnormalities to be examined. For each site, the number of fish examined, average length, number of fish showing *each* disease symptom and the number showing *any* disease symptom were recorded for statistical analysis as well as descriptive variables for each site and fishing occasion including the month in which the fishing took place; NWC river class and the average concentrations of dissolved oxygen (DO), ammonia and biological oxygen demand (BOD). These are listed in Table 4.2.

Figure 4.1 Typical ulcer on a roach



Figure 4.2 **Developing ulcer on a roach following scale loss.**

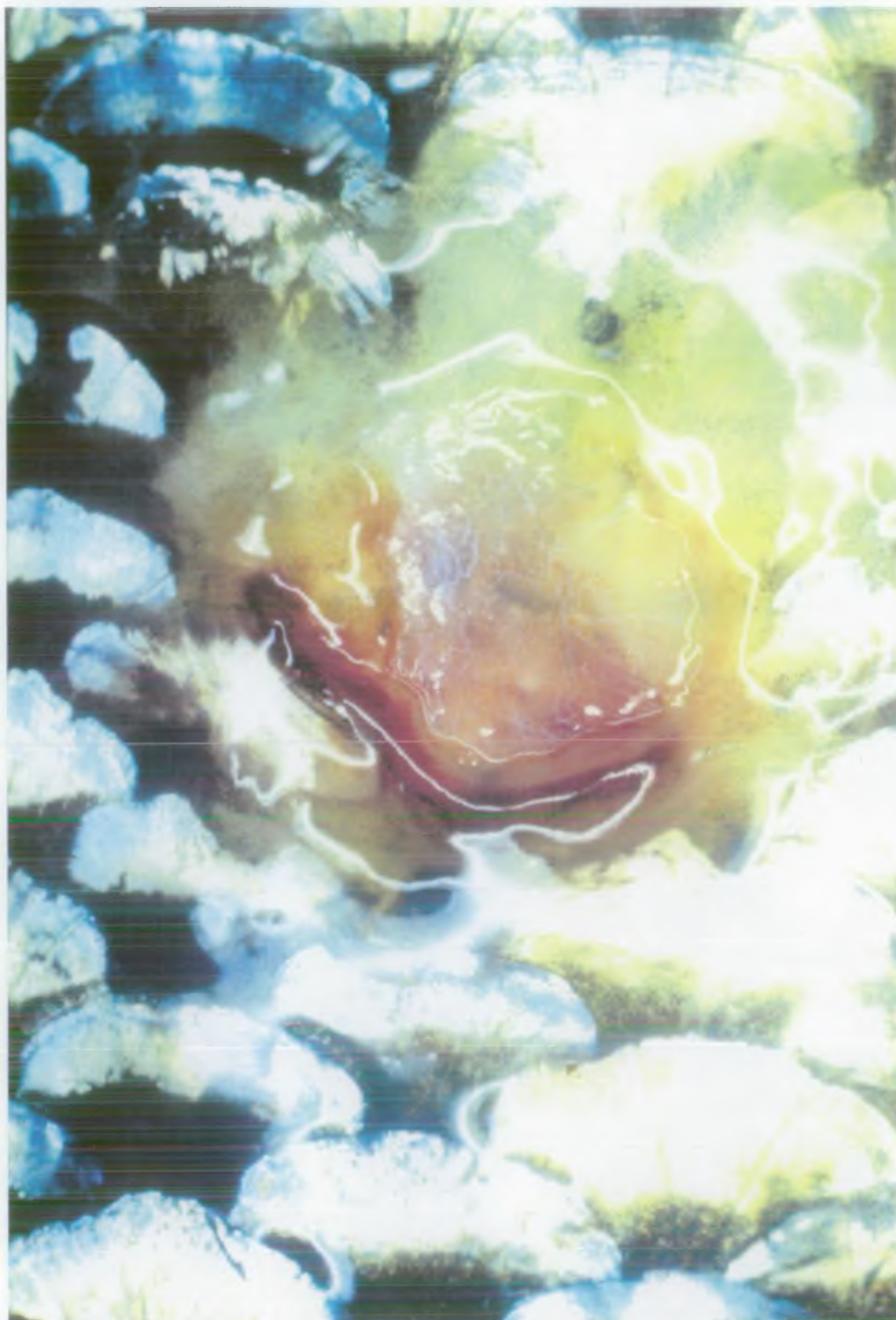


Figure 4.3 Typical ulcer on a chub. Note the whorling scale pattern indicative of a "healed" ulcer.



Figure 4.4 Haemorrhagic patches on the ventral surface of a roach.



Figure 4.5 Fin damage and haemorrhage at the base of a pectoral fin of roach.

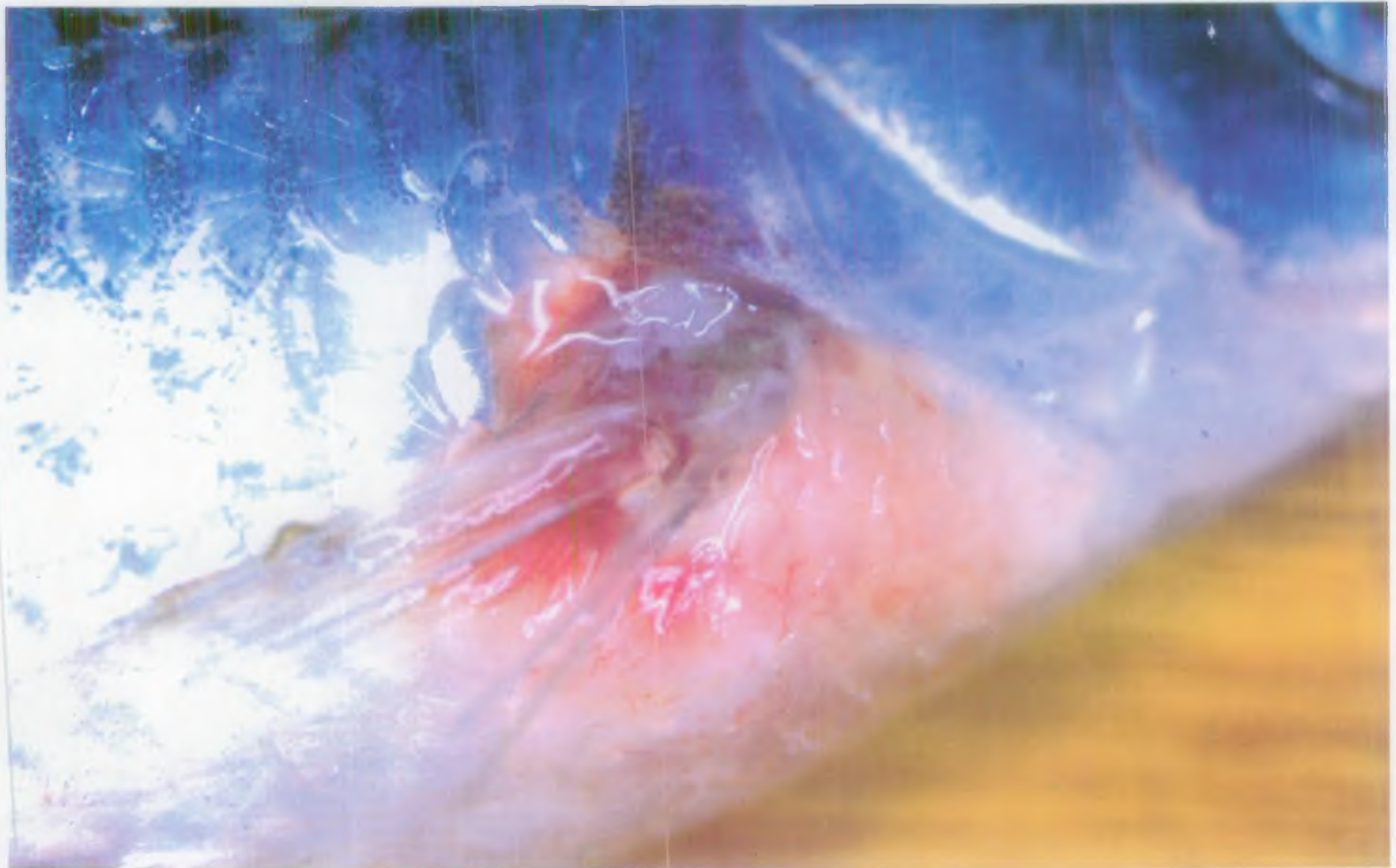


Figure 4.6 Severe infection of blackspot on a roach.



Table 4.2 Sampling sites and water quality data

Sampling Site	Site/visit	DO	BOD	Ammonia	NWC
Burwell Lode @ Upware Lock	Bur2/93	11.80	3.90	0.03	1
Willowbrook @ Woodnewton	Wlb3/91	12.13	3.94	0.08	1
Hundred Foot drain @ Earith	Hfd3/93	10.12	2.60	0.09	1
Cut-Off Channel @ Eriswell	Cut3/93	10.51	2.41	0.16	1
Willowbrook @ Woodnewton	Wlb9/92	12.13	3.94	0.08	1
Willowbrook @ Woodnewton	Wlb6/92	12.13	3.94	0.08	1
River Lee @ Dobs weir	Lec5/92	NV	2.20	0.18	1
Oxford canal @ Nell Bridge	Oca8/91	NV	3.30	0.08	1
River Avon @ Harvington	Avon3/93	10.90	3.11	0.167	2
River Avon @ Harvington	Avon6/91	12.50	3.29	0.156	2
River Avon @ Harvington	Avon3/92	11.28	3.30	0.212	2
River Avon @ Harvington	Avon6/92	11.28	3.30	0.212	2
River Nene @ Chetilles	Nene3/91	8.90	3.70	0.68	2
Blackwater @ Eversley Cross	Blw3/92	8.34	2.32	0.64	2
Willowbrook @ Alders Farm	Wlb6/92	9.92	4.16	0.15	2
Willowbrook @ Alders Farm	Wlb6/91	9.73	2.97	0.22	2
Cam @ Baits Bite	Cam2/93	9.83	1.92	0.08	2
Willowbrook @ Woodnewton	Wlb2/92	9.92	4.16	1.31	2
Cam @ Baits Bite	Cam4/11	8.40	1.48	0.47	2
Willowbrook @ Alders Farm	Wlb9/92	9.92	4.16	0.15	2
Oxford canal @ Kidlington	Oca8/91	NV	4.60	0.91	2
Cam @ Horningsea	Cam2/93	7.66	2.99	0.53	3
Blackwater @ Camberley	Blw3/92	8.40	1.48	0.47	3
Willowbrook @ Deene Lake	Wlb5/92	9.86	5.77	0.34	3
River Tame @ Lea Marston Lakes	Tame8/91	6.43	5.93	2.60	3
Willowbrook @ Deene Lake	Wlb6/91	9.32	5.76	1.24	3
Soham Lode @ Soham STW far	Soham1/93	9.52	2.70	0.43	3
Blackwater @ Camberley	Blw2/93	8.40	1.48	0.47	3
River Foss @ York	Foss6/93	8.21	2.30	0.19	3
River Ray @ Swindon	Ray6/93	NV	3.90	1.25	3
Willowbrook @ Deene Lake	Wlb2/92	9.86	5.77	0.34	3
Blackwater @ Camberley	Blw10/92	8.40	1.48	0.47	3
Selby Dam @ Selby	Selb4/91	8.70	3.78	0.91	3
Selby Dam @ Selby	Selb4/93	10.12	2.96	0.65	3
Willowbrook @ Deene Lake	Wlb8/92	9.86	5.77	0.34	3

NV No value determined

Of the 3372 fish examined, the total number of fish exhibiting external disease symptoms described are shown in Table 4.3, together with the crude prevalence rates, based on the combined data from 35 sites (for roach) of which 5 sites were examined for chub. It should be noted that the "total" prevalence of any of the external disease symptoms is not the sum of the 8 specific symptoms, because a fish carrying multiple symptoms is counted only once.

Table 4.3 Total numbers of abnormal fish and crude prevalence rates

Disease condition	Total number (all sites)	Crude Prevalence* (%)
Finrot	37	1.1
Ulcer	94	2.8
Petechiae	62	1.8
Haemorrhage	91	2.7
Mouth damage	13	0.38
Deformities	5	0.15
Healed ulcer	51	1.5
Hyperplasia	7	0.21
TOTAL	277	8.2

Total number of fish examined 3382

* The prevalence of a specific condition at a specified point in time was considered to be the proportion of the fish population which exhibit that condition.

In this study, the number of diseased or abnormal fish and hence the prevalence rates for several of the external disease symptoms were considered to be too low for detailed statistical analysis to be profitable. Therefore, the 3 highest prevalence rates were considered for further investigation, these being; "total" external disease prevalence, ulceration and haemorrhage with a prevalence of 8.2%, 2.8% and 2.7% respectively.

The objective of the statistical analysis was to determine whether the prevalence of the abnormality was significantly different between the different sites and, if so, to investigate whether the differences could be explained in terms of the site descriptors and the month in which the fish were sampled. A subsidiary objective was to consider the possibility of a relationship between the prevalence of abnormality and the length of the fish. Although the survey was based on a restricted range of lengths (14 to 20cm), the variation in average length between sites, was large enough for length-standardisation to be necessary.

The 3 categories of abnormality were analysed separately but their results were similar in pattern. Due to simplicity the findings are described in relation to "total" disease prevalence and then the corresponding results for ulceration and haemorrhaging indicated.

The comparison of "total" prevalence rates between sites provided no compelling evidence to assume other than that the observed prevalence rates at different sites were random manifestations of a uniform underlying rate. Considering the possible effects of individual variables concerned with the river quality data and month sampling took place, the results of the analysis again provide no compelling evidence. The time of year appeared to have no systematic effect at all, although the survey dates due to logistical reasons were not ideal for this analysis.

Of the 4 descriptions of river quality used in this study (NWC class, DO and ammonia, BOD) the variable most plausibly associated with the prevalence data appeared to be BOD. Although it failed to achieve significance at the conventional 5% level, it was near enough (7%) to be worth considering for inclusion in a proposed statistical model. The predictions of the model incorporating BOD and length are demonstrated in Table 4.4.

Table 4.4 "Total" prevalence (%) in relation to the average length of the fish and BOD

BOD (mgml ⁻¹)	Length (cm)		
	12	16	20
1.5	4.4	6.1	8.6
3.5	5.4	7.5	10.4
5.5	6.6	9.2	12.7

It can be seen from Table 4.4 that, across the available range of BOD values chosen here (1.5 - 5.5), the influence of BOD on prevalence was less than that of "length". The relative risk associated with an increment of, for example, 4 mg BODl-l was calculated to be approximately 1.5 units (ie 50%). An effect of this size is considered to be close to the threshold of detectability in any epidemiological study such as this (Robert Lacey, pers. comm.).

The effect of NWC class on "total" external disease prevalence rate was not significant, even at the 10% level. Neither DO or ammonia offered any explanation.

Because of the particular interest in a possible connection between river quality and the disease status of fish, the above analysis was repeated with data from sites with little or no angling pressure. Estimates of the "total" disease prevalence rates, for the 3 river quality classes are demonstrated in Table 4.5. These estimates, again, do not show a consistent trend, and the wide confidence intervals obtained indicate that the observed differences may be due to chance. The possible effect of BOD also failed to achieve significance within the restricted set of sites.

Table 4.5 "Total" prevalence (%) at sampling sites at a length of 16cm in relation to NWC class

NWC Class	Estimate	95% Confidence limit	
		Lower	Upper
1	6.6	4.6	9.2
2	6.1	4.5	9.1
3	8.2	7.0	9.7

All the above results are for "total" external disease prevalence rates. For the analysis of ulceration and haemorrhage prevalence data individually it was not possible to discern any relationship with any of the river quality variables.

The chub data was analysed using the same statistical methodologies; the only difference was in the treatment due to the nesting of visits within the sampling site (this was not performed for the roach study since the larger number of sites involved were uncondusive to several visits).

For all of the external disease symptoms examined, again, as with the roach data, there were no significant variation between the prevalence rates observed on different visits to the same site.

It was considered from the analysis of the field data for roach and chub that it was not possible to associate any differences in prevalence rate with differences in river class. Although the NWC Class 1 sites generally provided the lowest prevalence rates for each abnormality considered, there were never statistically separable from any NWC Class 2 sites.

4.2 Statistical Analysis of Laboratory Data

4.2.1 Variation within and between sites

For each of the 25 sites where data was available for individual fish (See Table 4.6) a sub-sample of approximately 10 fish were taken back to the FL for further biochemical assessment. Measurements included: length (cm); weight (g); spleno-somatic index (SSI) (spleen weight/body weight as a %); liver-somatic index (LSI) (liver weight/body weight as a %); Total blood cell count; Haematocrit (Hct) (%); Mean Cell Volume (MCV); Haemoglobin (Hg)(gdl⁻¹); Serum glucose (mmoll⁻¹); serum protein (gdl⁻¹); melano-macrophage centres (MMC)(% area per section); Chemotaxis (cellsmm⁻²). In certain instances, not every measurement was recorded for every fish. The total number of fish examined was 231.

Table 4.6 Sites with laboratory data for individual fish

River	Visit	Number of fish
Burwell Lode	Bur2/93	10
Cut-off Channel	Cutoff3/93	12
Willowbrook	Wlb9/92	7
Willowbrook	Wlb6/92	8
Blackwater	Bwc3/92	8
Willowbrook	Wlb1/92	10
Willowbrook	Wlb6/91	7
Cam	Cam2/93	10
Willowbrook	Wlb2/92	8
Cam	Cam4/11	9
Willowbrook	Wlb9/92	10
Cam	Cam49/3	10
Blackwater	Blw3/92	10
Willowbrook	Wlb5/92	10
Tame	Tam8/91	10
Willowbrook	Wlb2/91	7
Soham lode	Soham1/93	10
Blackwater	Blw2/93	9
Foss	Foss6/93	9
Ray	Ray6/93	9
Willowbrook	Wbw2/92	7
Blackwater	Blw10/92	11
Selby Dam	Selb4/91	10
Selby Dam	Selb4/03	10
Willowbrook	Wlb8/92	10
TOTAL	25	231

The overall objective of the analysis of the laboratory data was to investigate relationships between the laboratory measurements and the measurements of water quality describing the river from which the fish were sampled. However, a subsidiary objective was to investigate whether there were genuine differences between the 25 sites for which there was individual fish data or whether the apparent differences could be accounted for by the extent of variation between fish within site. If this analysis revealed that a given variable did not vary significantly between sites, there would be little point trying to relate it to water quality later in the statistical analyses.

The 12 laboratory variables (listed in Table 4.7) were subjected separately to one-way analysis of variance in which the categorical factor was the sampling site. No transformations were used on any variables since it was beyond the scope of this statistical investigation. The results of the analyses of variance are summarised in Table 4.7 which gives, for each variable, estimates of the standard deviation between sites and between fish within a site.

Table 4.7 Summary of analyses between and within sites

Variable	Overall mean	SD between sites	SD between fish
Length	15.6	1.52	1.76
Weight	66.4	23.0	27.7
SSI	0.21	0.039	0.044
LSI	1.93	0.13	0.26
Total Count	2.21	NS*	0.26
Haematocrit	31.6	1.4	3.3
Mean Cell Volume	141.3	NS*	6.3
Haemoglobin	13.5	0.61	1.52
Glucose	8.3	1.65	4.0
Protein	6.5	0.94	2.3
MMC	1.19	NS*	0.75
Chemotaxis	1770	165	540

NS* no significant variation between the sampling sites

For the 3 variables, total count, mean cell volume and melano-macrophage centres, there were no significant differences between any of the 25 sites. For the other 9 variables the differences between the sampling sites was greater than could be accounted for by the variation between fish. Nevertheless, the variation between fish remained the dominant source of variation in every case. This variation includes laboratory error as well as the biological diversity between fish.

4.2.2 Relationships between laboratory data and river quality variables

The data consisted of the within-site averages of the measurements listed in Table 4.7, together with corresponding results from a further 10 sites for which site averages were obtained, rather than from individual fish. Alongside this data the following water quality variables were obtained: DO, Ammonia, BOD and NWC river class. For 12 of the sites there were additional single determinations of PAH in the sediment and of hepatic EROD activity based on individual fish. The objective of this analysis was to investigate whether there was any statistical association between the biological measurements and the five river quality variables.

The relationship between each of the laboratory variables and the NWC river class was assessed by a one-way analysis of variance of the relevant site averages, grouped into the three river water quality classes as described in 3.4.10. Equality of the 3 river class means was tested in the standard way using the F-ratio. The possible association between each variable was assessed by inspecting the bivariate correlation coefficient and comparing it with the percentage points in a standard statistical table.

Of the 12 laboratory variables examined, 3 showed significant differences between NWC river class. They were liver-somatic index, haematocrit and chemotaxis, and their class means are shown in Table 4.8. In addition, for 2 other measurements, haemoglobin and melanomacrophage centres there was weaker evidence of differences between NWC class (significant at 10% but not at 5% in the analysis of variance test). Whilst these means show gradients across the 3 river water quality classes, the differences could have arisen by chance.

Table 4.8 Means and their standard errors (SE) for LSI, Hct, Chetax, MMC and Hb

NWC Class	LSI		Hct		Chetax		MMC		Hb	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1	1.79	0.05	30.2	0.59	1910	120	0.80	0.14	12.6	0.32
2	1.97	0.04	31.7	0.46	1913	97	1.11	0.11	13.3	0.26
3	1.95	0.04	32.1	0.45	1620	80	1.21	0.10	13.6	0.25

It can be seen from Table 4.8 that for LSI and haematocrit the significant difference arises from the contrast between Class 1 and the other two classes, while for chemotaxis it was Class 3 that was the "odd man out".

None of the other 8 laboratory variables examined showed any evidence of being related to NWC class. It is worth noting that neither length nor weight were significantly different between the 3 NWC classes although these variables had shown significant differences between sites.

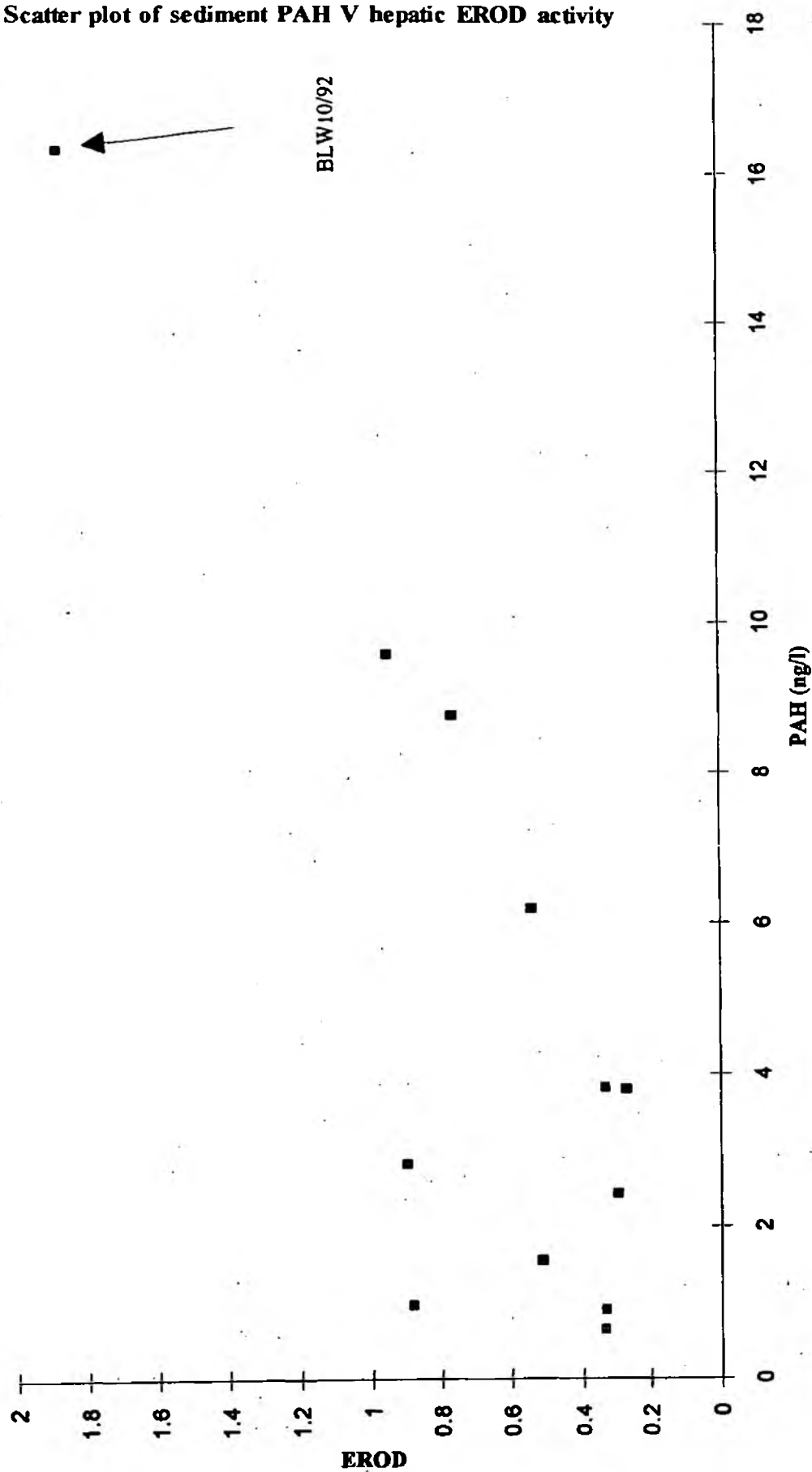
Of the possible 52 possible bivariate correlations between the 12 laboratory variables and the 4 river water quality variables only 2 stood out as being possibly indicative of an underlying relationship (when tested at the conventional 5% significance level). This number (2) is what might be expected to emerge by chance under the null hypothesis, and so these findings should be viewed with caution. The two possible significant "cause and effect" relationships were considered to be:

- 1) Between hepatic activity EROD and sediment PAH
- 2) Between SSI and ammonia

With the relationship between EROD and PAH the positive correlation was attributable to the single point representing site Blw10/92 (See Figure 4.7). Without this point the positive correlation would remain in doubt. Because the position of that point is dependant on single determinations of EROD and PAH it would be hasty to draw any conclusions from this scatter plot.

Figure 4.7 Scatter plot of sediment PAH v hepatic EROD activity

Scatter Plot of EROD v PAH



With the relationship between SSI and ammonia the positive correlation is similarly dependant on a single point representing the Tam 13891 (Figure 4.8). Here, however, the SSI value is the average of 10 mutually consistent results and the ammonia concentration should have taken account of several determinations. The point therefore may be indicative of a genuine relationship. Caution is, however, still required before inferring that the involvement of ammonia is as the "causative" agent.

4.3 Parasitological examination of fish

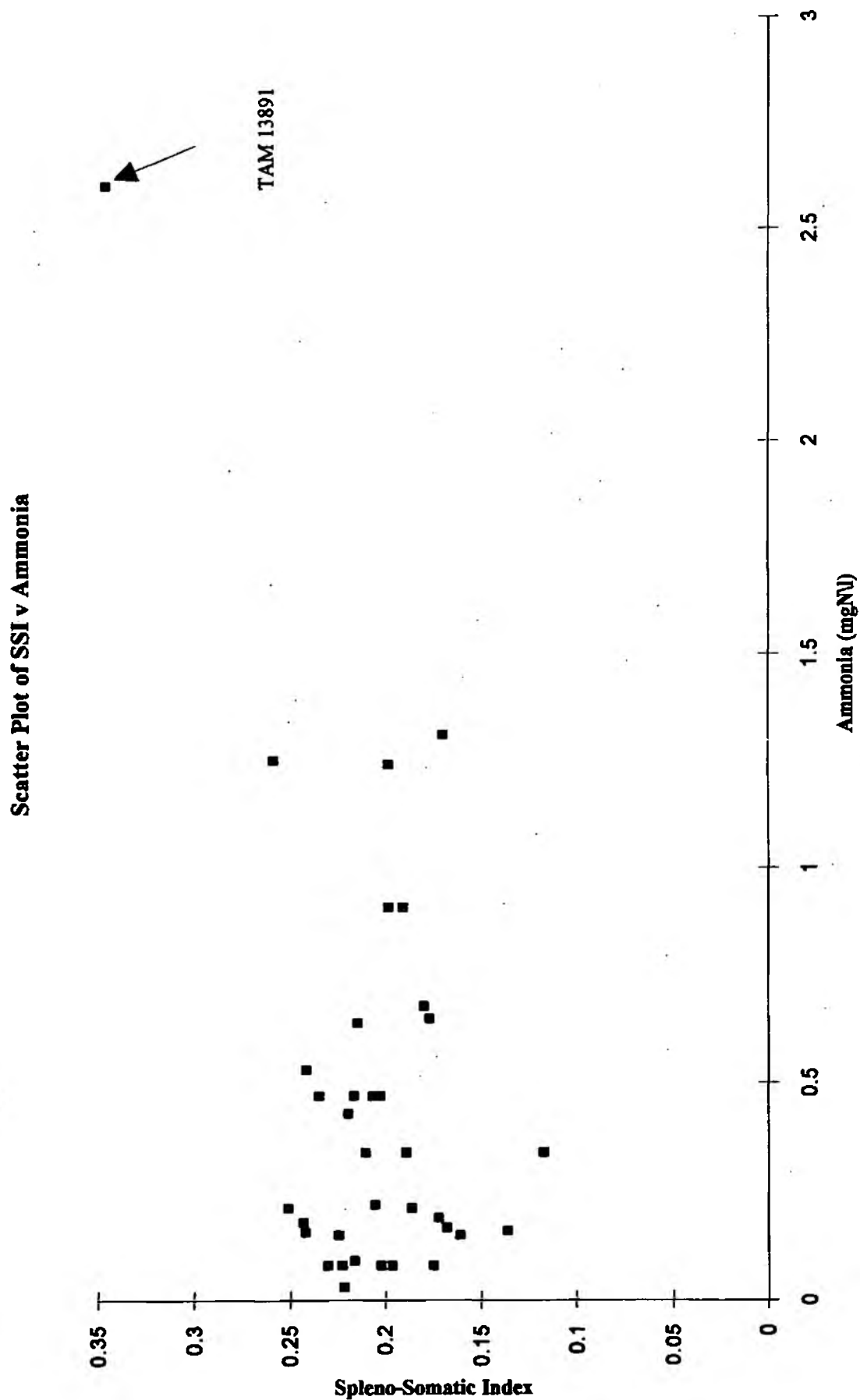
External examination of roach and chub in the field revealed a number of common external lesions due to range of parasites such as leeches (*Pisicola* sp.), black spot lesions caused by *Posthodiplostomum* sp. (See Figure 4.6) and cataracts of the lens due to *Diplostomum* sp.. Many of these parasites are common amongst fish population and are of a widespread. None of the 3613 fish examined at 35 sites were considered to have clinical infections of any of the above diseases.

A detailed internal examination of the fish was possible on the 231 fish returned to the FL for biochemical analyses. Again a wide range of common and widely distributed parasites were observed such as Microsporidian and Myxosporidean spores in gills, liver, spleen and kidney, monogenean flukes such as *Dactylogyrus* sp. on the gills, digeneans such as *Tylodelphys clavata* in the aqueous humour of the eyes and cestodes such as *Ligula intestinalis* (immature, plerocercoid stages in the body cavity of roach) and adult stages of *Caryophyllaeus laticeps* in the gut of roach. Chub from certain sites were found to harbour the acanthocephalan (Spiny-headed worm) *Pomphorynchus laevis* in their guts.

4.4 Bacteriological examination of fish

Bacterial examination of ulcerative lesions of roach and chub were performed. In the majority of cases atypical *Aeromonas salmonicida* subspecies *achromogenes* (the causative agent of roach ulcer disease (Owen, 1988)), *Aeromonas hydrophila* and *A. sobria* were isolated in pure culture on Tryptone Soya Agar (TSA) and TSA + 5% (v/v) horse blood. Bacterial isolates were identified using API 20E and 20NE identification kits. In addition, in a number of cases, a number of opportunistic bacterial isolates such as *Pseudomonas fluorescens* and *Shewanella* (*Pseudomonas*) *putrefaciens* were isolated in mixed culture. Both motile and non-motile members of Aeromonads have been demonstrated as the causative agents of ulcerative lesions in a variety of freshwater species including roach, perch and chub.

Figure 4.8 Scatter plot of spleno-somatic index vrs ammonia concentration in water



4.5 Barbel study

Following reports by anglers catching barbel with tumours in the River Lee, two sampling trips were arranged to examine these reports in detail. On both sampling visits, 2 types of lesion, based on their relative appearance, size and distribution on the body were found. Histological examination showed these to be epidermal papillomas and epidermal hyperplasia. The barbel also had a variety of external disease abnormalities and their prevalence are listed in Table 4.9. Of the 76 barbel examined, 22% show one or both of these skin abnormalities with only 6.5% suffering from fully developed papillomas. These lesions were not found on any other species examined.

Table 4.9 Prevalence of epidermal hyperplasia and papilloma in barbel

	Sample I (5/91)	Sample II (2/92)
No. of fish examined	14	62
No. of fish affected	3 (21.4%)	14 (22.5%)
No. of fish with hyperplasia	2 (14.3)	12 (19.3%)
No. of fish with papillomas	1 (7.1%)	4 (6.4%)
No. of fish with both lesions	0	2 (3.2%)

The hyperplasias appeared as a thickening of the epithelium over individual scales on the flanks of the fish posterior to the dorsal fin (Figure 4.9). The growths did not extend beyond the edge of individual scales, the number affected varying from 5-10 scales in most, to 50 in one extreme case. they appeared white to pink in colour and varied from 1-3mm in thickness.

Papillomas were larger, varying in size from 1-2.5cm in diameter, pink and fleshy in appearance with numerous folds giving a typical cauliflower like appearance, and were typically found near the base of the pelvic and pectoral fins (Figure 4.10). A similar growth was also found at the base of the operculum on one fish.

Figure 4.9 Gross morphology of an epidermal hyperplasia in barbel



Figure 4.10 Gross morphology of an epidermal papilloma around the base of the ventral fin in barbel



Figure 4.11 Histological appearance of an epidermal papilloma arising from fin epithelium and supported by strands of connective tissue (CT). Mucous cells appear as vacuoles (V). Haematoxylin & eosin stain (H&E) x30 magnification.



The hyperplasia and papillomas were similar in structure, composed principally of proliferating epidermal basal cells. The large growths seen on the fins and operculum were well developed papillomas with finger-like outgrowths of epidermal cells supported by connective tissue with a well developed vascular system (Figure 4.11). The cells comprising both the hyperplasia and papilloma were cuboidal in shape becoming flattened in the outermost layers. Mucous cells were prominent and often present in the centre of papillomatous folds. Mitotic figures and infiltrating white blood cells were occasionally observed. there was no evidence of invasion into the dermal layers or of cytoplasmic viral inclusion bodies. Transmission electron microscopy revealed two distinct regions within the lesions. These were composed of light and dark staining cells, the latter with strongly osmiophilic cytoplasm (Figure 4.11). The dark cells originated from the basement membrane with the light cells continuing to the outer layers of the lesion.

5. DISCUSSION

Fish in their natural environment are subjected to a number of stressors including unfavourable or fluctuating temperatures, high water flow and turbidity, low dissolved oxygen, limited food availability and other variables. In addition, pollution stress can add to the insults that fish may already experience in many systems. All of these factors, individually or together, can impose considerable stress on fish at the molecular and sub-cellular level, through to the tissue level and ultimately impair their health and affect population and community structures of fish populations (Passino, 1984; Möller, 1985; Wedemeyer *et al.*, 1984, 1990; Adams *et al.*, 1993).

There have been many field-based and laboratory-based studies to assess the potential use of a variety of biological markers or "bioindicators" which may potentially be used to predict the health of fish in wild populations without need to examine a large sample of fish (Adams *et al.*, 1989, 1990, 1993; Karr, 1991). These studies examining the effects of pollution stress on fish health have concentrated heavily on marine and estuarine environments examining cause and effect relationship particularly between pollutants and tumours in fish (Malins *et al.*, 1984, 1985, 1987, 1988; McVicar *et al.*, 1988; Mix, 1986; Möller, 1990; Murchelano and Wolke, 1991; Weeks *et al.*, 1986a,b). Once an ideal bioindicator has been identified it may be applied to fisheries of differing qualities and used to examine what factors affect fish health. In addition, "bioindicators" may be used as an early warning marker to predict the onset of disease, so that remedial actions may be taken (Karr, 1991).

Many of the potential bioindicators examined to date differ widely in their properties, ranging from short term, rapid biochemical and molecular indicators which respond quickly to the various pollutants (Adams *et al.*, 1989). These biochemical and molecular changes at the cellular level may then affect fish at a tissue or organ level which may ultimately result in long term changes or damage to the fish or to populations of fish at the population or community level, resulting in changes in fish numbers, fish biomass and species composition and diversity (Karr, 1991). Once these long term bioindicators are observed in the wild populations, potentially irreversible damage may have been done, and as such these indicators of fish health are generally considered to be retrospective indicators (Kerr and Dickie, 1984; Munkittrick and Dixon, 1989).

An ideal bioindicator is considered to be one that is a true measure of the health status of fish in wild populations. The bioindicator should respond rapidly to changes in the environment, reaching high levels quickly and maintaining these levels for a relatively long period after the insult; it should be demonstrated that this rapid change leads to long term damage, ultimately in population structure. The bioindicator should not be affected by capture stress, ideally measured using non-invasive techniques; it should be cheap and simple to perform in the field and must have a well established "cause and effect" relationship with no interference from other external parameters, that is it should be a narrow spectrum, rather than a broad spectrum bioindicator (Adams *et al.*, 1989, 1993).

Laboratory studies in the past have generally provided a high precision for predicting effects of individual contaminants under controlled conditions, but these studies fail to duplicate the many interacting variables present in the environment. Field studies have generally addressed real environmental problems, often in response to major pollution incidents, for example the "Braer" oil tanker incident in the Shetlands, but often fail to establish "cause and effect" relationships and are usually not sensitive enough to detect adverse effects before they reach crisis point.

Many laboratories now consider a variety of approaches to examine pollution stress in fish and effects on fish health. Many have adopted multidisciplinary or integrated approaches using an array of potential bioindicators from molecular and biochemical indicators through to the more traditional population and community indicators. An integrated approach was successfully used to assess the health of fish populations by Adams *et al.* (1993) using a system described as a Health Assessment Index (HAI). This model compared favourably with more traditional methods, and was quicker and simpler to use.

Current research by many leading laboratories involves an integrated approach, concentrating chiefly on molecular and sub-cellular bioindicators such as examining genetic damage to cancer genes and oncoproteins, assessing lysosomal integrity etc. At present, no one bioindicator has been identified that fulfils all the characteristics of an ideal bioindicator and there are no established or standardised methodologies to guide workers in this area of research, although the International Council for the Exploration in the Sea (ICES) have gone some way to standardise procedures, especially amongst member countries participating in the North Sea Task Group (Vethaak and Rheinalt, 1990) (See Introduction).

In this study, an array of potential "bioindicators", both field-based and laboratory-based, were examined in this three year study assessing the health of fish in wild populations in relation to water quality. Collaboration with other organisations was encouraged throughout the study and links between MAFF scientists at the Fish Disease Laboratory, Weymouth, IFE scientists at Windermere and the University of Derby were established.

Although both chub and roach were examined in this study, efforts were concentrated on roach, a well-studied species which has a ubiquitous distribution within England and Wales (which avoids dependence on species and sampling sites with limited geographical distribution). In addition, roach are non-migratory species and although they are known to shoal in certain waters in the winter months, the avoidance of sampling during this period overcame this problem. Roach are also known to be affected by several readily recognizable diseases such as ulcer disease (Hotchin and Williams, 1981; Owen, 1988).

Thirty five sampling sites of differing water qualities were selected in this study following discussions with local NRA Fisheries Officers. Water quality was assessed using the National Water Council (NWC) classification scheme. All of the sites had well established water quality and fisheries data, which were supplied by the relevant NRA regions.

The study comprised of both field-based and laboratory-based studies. In the field studies, the presence or absence of 8 well recognised external disease symptoms including fin rot, ulceration, petechiae, haemorrhaging, mouth damage, skeletal deformities, healed ulcerative lesions and epidermal hyperplasia were examined as potential "bioindicators" of fish health

in waters of differing water quality. The prevalence of these external disease "bioindicators" was considered to be low compared with many European and American-based studies where more extremes of polluted environments were studied (Malins *et al.*, 1985, 1987). The overall prevalence rate for all external disease symptoms was found to be 8.2% in the 3372 fish sampled from the 35 sites. Although many of the external disease symptoms observed were considered to be of an unknown aetiology, ulcerative and haemorrhagic lesions on the body surface of the fish were considered to be predominantly of bacterial aetiology. The well established bacteria, *Aeromonas salmonicida* subspecies *achromogenes*, the causative agent of roach ulcer disease (Owen, 1988) was isolated from ulcerative and haemorrhagic lesions. The prevalence of ulcerative and haemorrhagic lesions contributed over 50% to the total external disease prevalence, with prevalences of 2.8% and 2.7% respectively.

Independent statistical analysis of the study data was performed by WRc statisticians. Their analyses indicated that the observed prevalence rates of the 8 external disease markers were not related to river water quality, as measured by NWC classification scheme. Although the prevalence rates were generally considered to be higher in NWC 2 and 3 waters ie those of poorer water quality, they were not considered to be statistically separable from NWC 1 rivers. Variation between fish (which was greater than variation of fish between sites) was considered to be an over-riding factor in this study. This is considered to be a common problem when examining wild fish populations (Owen, 1988).

In addition, prevalence rates were not considered to be related to the time of sampling or related to several of the components of the NWC classification score such as dissolved oxygen levels (DO), ammonia concentration and biological oxygen demand (BOD) when examined at the 5% level of significance. However, one component of the NWC classification score, BOD, was found which had an effect on the overall prevalence rate, but only at a 7% level of significance. For an epidemiological study such as this, only a significance level of <0.1% is nearer to being acceptable, before any conclusions can be reached about its association with the observed prevalence rates. In addition, there are no studies reported in the scientific literature associating BOD levels and fish health apart from asphyxiation of fish resulting in significant mortalities ("fish kills") following certain pollution incidents involving pollutants with high organic contents such as farm silage run-off and dairy products such as milk.

A statistical model based on BOD was, however, assessed in the statistical analysis of the data by WRc statisticians. The model considered was based on a "generalised linear model" (GLM) designed for coping with a "binomial" response, r , the number of abnormal fish out of a total n examined at a particular site. r is assumed to be a binomial distributed variable with site-specific prevalence rate p , whose independence on the possible explanatory variables is modelled the following equation of the form;

$$\log_e (p/(1-p)) = b_0 + b_1x_1 + b_2x_2 + \dots + b_kx_k$$

where x_1, \dots, x_k are explanatory variables and b_0, \dots, b_k are parameters available for estimation.

The proposed statistical model was considered to be unsuccessful in that its predictions were below the threshold of delectability for such an epidemiological study.

It was apparent from the statistical analyses of the field-based "bioindicators" that their prevalences were not related to water quality as defined by the NWC classification scheme. This may be a reflection of the fact the prevalence of external diseases in roach are low due to the good and improving water quality of the rivers in England and Wales (*The Quality of Rivers, Canals and Estuaries in England and Wales - NRA Report of the 1990 Survey*, Water Quality Series No 4, December 1991). Similarly, the low prevalence rates may be due to the sampling of waters which did not exhibit extremes of water quality and pollution stress, as observed in many estuarine studies in the USA (Malins *et al.*, 1984, 1985). In addition, the many components which contribute to the overall NWC classification score which may be related to disease prevalence may not be present in significantly different quantities between waters of differing score. Many disease-based "bioindicators" are considered to be relatively long term responses which result from chronic rather than acute exposure to pollutant stresses. NWC scores are a reflection of the water quality over a period of time which may not take into account of "spikes" of pollution.

In laboratory-based studies examining 12 "bioindicator" responses, 231 roach from 35 sampling sites were sub-sampled during the field-based studies and returned to the NRA Fisheries Laboratory to assess a range of potential biochemical, immunological and physiological "bioindicators". In view of the possibility that disease-based "bioindicators" were induced by possible long term, chronic exposure to pollution stress, a selection of laboratory-based "bioindicators" were assessed since they are thought to be more sensitive short-term responses to pollution stress (Adams *et al.*, 1989). From the sampling sites, approximately 10 fish per sampling site were examined in this manner. Initial statistical analysis of this data indicated that sample sizes of 10 fish per site, was in fact a satisfactory sample size in that the variation in the "bioindicator" responses between fish was lower than the variation in the responses between sites.

For three out of twelve "bioindicators", that is, liver-somatic index, haematocrit and chemotactic responses of roach macrophages, these were considered to be associated with water quality at the 5% level of significance in an analysis of variance test. This number was however considered to be the number that may have arisen by chance. A further 2 parameters, haemoglobin concentration and the presence of melano-macrophage centres in liver, spleen and kidney material was loosely associated with water quality at the 10% level of significance. Interestingly, melano-macrophage centres have been proposed as suitable "bioindicators" of pollution stress by Wolke *et al.* (1985) and Macchi *et al.* (1992).

Many workers considered immunological "bioindicators" such as the chemotactic response of fish macrophage to be ideal "bioindicators" since several studies have demonstrated impairment of immune system by exposure to pollution stress (Anderson, 1990; Secombes *et al.*, 1991). Any impairment of this system may the lead fish open to infectious agents such as bacterial and viral infections and diseases such as tumours and neoplasia. The chemotactic and phagocytic response of spot and hogchoker were found to be severely affected by exposure to pollutants such as PAHs, PCBs and tributyl tin (TBT) in Chesapeake Bay in the USA, considered by many to be the most contaminated water worldwide (Weeks and Warriner, 1984; Warriner *et al.*, 1988).

The laboratory-based data was then using in an attempt to establish any possible "cause and effect" relationships between 52 possible bivariate correlations arising from the 12 laboratory-based "bioindicator" responses and the 4 river water quality variables. Two potential relationships were revealed in this analysis, although it was considered possible that these two may have arisen by chance. Of the two possible "cause and effect" relationships between spleno-somatic indices and ammonia and between sediment PAH levels and hepatic EROD activity, the latter, although not proven statistically in this study, has been demonstrated in other several other studies (Addison, 1984; Addison *et al.*, 1981, 1988; Spies *et al.*, 1984, Van Veld *et al.*, 1990; Varanasi *et al.*, 1992) and is considered to be suitable "bioindicator" in that EROD is only induced in presence of PAH pollutant in order for its degradation or detoxification (Burns, 1976).

During the course of this study a number of reports were received from anglers fishing on the River Lee, of fish, particularly barbel, suffering from neoplastic tumours. In collaboration with MAFF scientists at the Fish Disease Laboratory, Weymouth, 2 sampling visits were arranged to investigate the reports. On both sampling visits, 2 types of lesions, based on their relative appearance, size and distribution on the body were found. These lesions were identified histologically as being epidermal papillomas and epidermal hyperplasias. The two lesions differed in prevalence, with 18.4% having hyperplasia and 6.6% having papilloma. No cause for the two lesions could be determined by either NRA or MAFF scientists. At the time of investigation there were no reports of barbel in other river systems being affected and generally in the UK there have been few studies on the incidence of tumours in wild fish populations in the freshwater environment.

Recently, Tyler and Everett (1993) have examined the incidence of these lesions in barbel in the river Lee and also in another two rivers of differing water quality. The authors found the prevalence of tumours in barbel in the river Lee to be 25%, which was significantly higher than the two other rivers. They suggested there was a loose link between water quality and fish health since marine studies have concluded that the presence of tumours in bottom dwelling species are most specifically correlated with chemical contaminants and pollutants (Bowser *et al.*, 1990; Harshbarger and Clark, 1990; Malins *et al.*, 1984, 1985, 1987, 1988, 1991; Sonstegard, 1977; Sonstegard and Leatherhead, 1984). In the UK studies, the aetiology of the hyperplasias and papillomas has not been determined as with previous tumours (Mawdesley-Thomas, 1975; Bucke and Feist, 1985) although electron microscopy studies ruled out the possibility of viral aetiology in the barbel study (Bucke, pers. comm.)

To conclude, a number of external disease "bioindicators" and a number of sensitive laboratory-based "bioindicators" were examined in fish from waters of differing water qualities. Overall, the study failed to establish the potential of any of the "bioindicators" examined. This was chiefly due to the "bioindicators" not being sensitive enough to differentiate between waters of differing water quality, the variability of the "bioindicator" responses of fish examined from wild populations and waters of differing water quality, as judged by the NWC river classification scheme did not offer extremes of pollution stresses which may have triggered many of the "bioindicators" examined during the course of the study.

6. CONCLUSIONS

This study describes an epidemiological study carried out to assess the effects of differing water quality (determined by NWC score) on the health of wild fish populations. A total of 3613 fish from 35 sites were examined for the presence of disease markers and "bioindicators".

The following conclusions were reached:-

- Overall disease prevalence was low at 8.2%. This may be a reflection of the fact that river water quality is improving and that pollution levels in our rivers are in fact low compared with other studies.
- the prevalence of disease was not related to water quality as determined by NWC score. NWC score is an average value of water quality measured over the year. It does not record "spikes" of pollution which may result in rapid "fish kills" or from which fish may flee.
- Three "bioindicators" were related to water quality at 5% level of significance although no "cause and effect" relationships could be established. This level of significance is considered to be too low for an epidemiological study such as this.
- The sensitivity of disease markers and "bioindicators" was generally insufficient to differentiate between waters of differing NWC classification.
- No one "bioindicator" was a reliable as a marker of the health of fish. Generally "bioindicators" were considered to be potential markers of specific pollutants.

7. RECOMMENDATIONS FOR FURTHER WORK

A number of areas for further work in the future have been highlighted during the course of this study. These are listed below:

- It was concluded in this study that the sensitivities of disease markers and bioindicators used were insufficient to differentiate between waters of differing water quality. Future studies should concentrate on waters of extreme water quality.
- There is a general feeling amongst workers in this field that there should be more frequent sampling visits to fewer sampling sites. This would enable effects such as seasonality, temperature, water flow etc to be examined in more detail.
- Future studies should examine a wider range of "bioindicators", possibly by collaboration with other organisations which have an interest in this field.
- Disease marker and "bioindicator" responses to differing water qualities may be more apparent in young fish and fry. Future studies may examine "bioindicator" responses in smaller size ranges of fish.
- Many disease markers and "bioindicators" are too broad spectrum in their response. There is a future need to concentrate on one or two specific "bioindicators". Recent developments concentrate on molecular biomarkers and ecotoxicological studies where "cause and effect" relationships are better understood.
- There is a future need for field studies to be performed in concert with laboratory studies so that "cause and effect" relationships determined in the laboratory may be examined in the field.
- Future studies should avoid the use of disease markers where the diseases are of unknown aetiology eg skeletal deformities and "bioindicators" in which there no established "cause and effect relationships established.

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