COMPARATIVE STUDIES OF ZINC TOXICITY

IN TWO SPECIES OF FRESHWATER AMPHIPOD CRUSTACEANS

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Being a thesis submitted to the Faculty of Science, University of Leicester, in candidature for the degree of Doctor of Philosophy.

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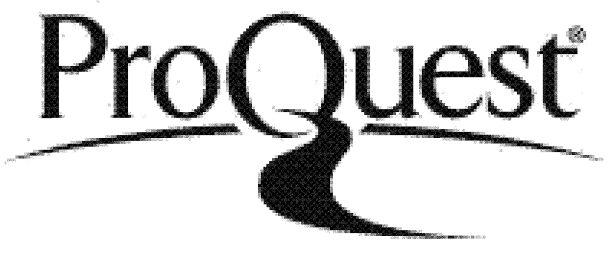

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TO MY MOTHER AND FATHER

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CONTENTS

CHAPTER 1.

GENERAL INTRODUCTION

A laboratory based toxicity study was conducted into the effects of dissolved zinc when present above essential trace levels, on two species of morphologically similar freshwater amphipod crustaceans, *Gammarus pulex* (L.) which is native to the British Isles, and *Crangonyx pseudogracilis* (Bousfield) which is a relatively recent introduction from eastern North America. The performance of the study, the results obtained, and their relevance to the field situation are described.

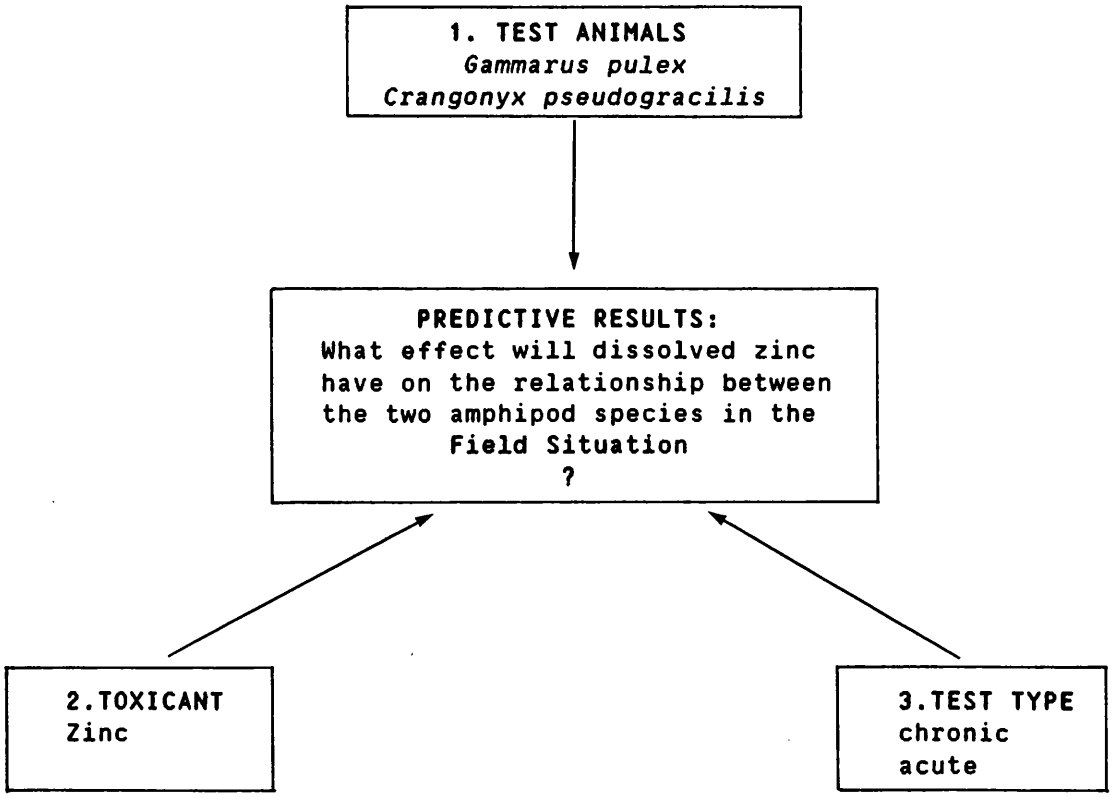
Studies of this nature are generally initiated in response to a specific environmental problem, and take the form of either a toxicity test or a bioassay. Whilst methodologically these two approaches are similar they can be distinguished by the relative emphasis given to animal and toxicant.

If the object of the study is to biologically quantify the strength of a toxicant using a pre-determined response of the test organism, the procedure adopted is a bioassay. The organism is essentially a tool and its response of no greater interest than its use as a standard with which to compare the toxic effects of different pollutants. Single celled organisms are popular for bioassay purposes, since they lack complex homeostatic systems for dealing with environmental change (Mills 1976). This approach has been comprehensively evaluated by Martin (1973). In a true toxicity test it is the organism and its response when exposed to different concentrations of a recognised toxicant which are of primary importance. Studies having this emphasis are less numerous within the literature than bioassays.

This laboratory study was initiated in response to a relatively transient but deleterious incidence of zinc pollution in Willow Brook,

a small Northamptonshire River. The main object of the study was to obtain consistent laboratory acute and chronic toxicity data for the two amphipod species, from which a greater understanding could be obtained of the way in which their distribution and abundance were affected by zinc pollution in the more complex field environment. This was therefore a true toxicity study since it was the animals and their comparative responses to the same pollutant which were of interest.

This approach is illustrated diagrammatically as follows;



The remainder of the introduction is dedicated to a more detailed consideration of these three components of the toxicity study.

1. ANIMALS

General features of the Amphipoda

The malacostracan super-order Peracarida contains an estimated 11500 species, 48% of which belong to the order Amphipoda. Peracarida is distinguished from other crustacean groups in having only one true thoracic segment fused to the head, and eggs which are borne in a thoracic brood pouch and which hatch into juveniles rather than a true larval stage. Amongst the peracardians the amphipods are distinguished by the following combination of characteristics: The body has no carapace and is laterally compressed with ventrally directed coxal plates. Coxal gills occur on the interior base of some thoracic limbs. There are seven pairs of uniramous limbs on the thorax, the first four pairs of walking legs being directed forwards, and the last three pairs backwards. The abdomen bears three anterior pairs of multi-jointed pleopods and three posterior pairs of few jointed uropods.

The order Amphipoda is divided into four sub-orders: Gammaridea, Caprellidea, Hyperiidea and Ingolfiellida. The Gammaridea, comprising 85% of known amphipod species are basically more primitive than hyperiids or caprellids. In contrast to the rest of the Amphipoda they are widespread with great diversity of habit and habitat. All major families are represented in the British fauna, those not recorded are mostly groups of 1-10 species having limited distributional ranges (Lincoln 1979). Both *Gammarus pulex* and *Crangonyx pseudogracilis* are members of the Gammaridea.

Classification

The classification of the Gammaridea is considered to be far from satisfactory (Lincoln op. cit.), and is presently undergoing considerable reorganisation of family groups. This includes a major restructuring of the

family Gammaridae which has long been recognised as a heterogeneous complex of species and genera that cannot be readily assigned to any of the other recognisable families of the Gammaridea (Bousfield 1977). Of interest in the context of this study is the effect of this reorganisation on the classificatory relationship between the two test species.

The Crangonyx section of the family Gammaridae was originally designated by Schellenberg in 1936 to comprise 15 genera of freshwater amphipod crustaceans which differed enough from other members of the family to warrant special recognition. There has since been considerable modification of Schellenberg's original definition (Holsinger 1977).

Bousfield (1958) classed Amphipoda under two headings: those of relatively recent marine origin and those of ancient freshwater lineage having no morphologically close relatives. The Crangonyx section of the Gammaridae was placed in the latter group and *Crangonyx pseudogracilis* defined as the new specific name for individuals previously described as *Crangonyx gracilis* (Forbes), *Melita parvimana* (Holmes), *Eucrangonyx gracilis* (Kunkel, Johansen, Hubricht & Mackin) and *Crangonyx gracilis gracilis* (Hubricht).

The erection of the family Crangonyctidae was formalised by Bousfield (1973). Whilst Holsinger (1974) was principally in agreement with the subdivision of the Gammaridae into smaller, more natural sub-units he objected to the new classification without further consideration of some disputable taxonomic criteria, and subsequently produced a revised diagnosis of the family Crangonyctidae and its component genera (Holsinger 1977). In the same journal Bousfield (1977) further revised his original scheme, proposing the creation of six new super-families from within the Gammaridae. Amongst the six were the Gammaroidea, containing almost all the epigean freshwater types endemic to the Northern hemisphere (including *Gammarus pulex*), and the Crangonyctoidea, which included the newly revised Crangonyctidae.

This classification has recently received criticism (Barnard & Karaman 1980), although the superfamily Crangonyctoidea is considered to be the most cohesive of the new proposals. Whilst *G. pulex* and *C. pseudogracilis* were therefore initially placed within the same family, with each subsequent revision of amphipod systematics clear classificatory differences have emerged.

Morphological differences between *G. pulex* and *C. pseudogracilis*

The obvious morphological differences between the two species which aid in their identification are shown in Fig. 1.1 & 1.2, and listed in an accompanying table (Table 1.1).

MORPHOLOGICAL DIFFERENCES

	CRANGONYX PSEUDOGRACILIS	GAMMARUS PULEX
1	RANGES IN COLOUR; WHITE TO SLATE BLUE. THINLY CUTICULIZED; OFTEN TRANSPARENT. CRAWFORD (1937)	ALMOST ALWAYS ORANGE-BROWN HEAVILY CUTICULIZED; OPAQUE. (PERS. OBS.)
2	MALE TYPICALLY SMALLER THAN FEMALE. BOUSFIELD (1958)	MALE TYPICALLY LARGER THAN FEMALE. BOUSFIELD (1958)
3	STERNAL GILLS PRESENT BOUSFIELD (1958)	STERNAL GILLS ABSENT BOUSFIELD (1958)
4	MALE ANT. 2 FLAGELLUM WITH CLUB-SHAPED SENSORY STRUCTURES - (ASTHETASKS ?) PINKSTER (1980)	MALE ANT. 2 FLAGELLUM WITH THICK BRUSH OF SENSORY SETAE INSERTED IN DOUBLE ROWS ON INNER MARGIN OF ALL BUT MOST DISTAL SEGS. GLEDHILL, SUTCLIFFE, AND WILLIAMS (1976)
5	ABDOMINAL SIDE PLATES 1-3 LATERAL CORNERS ACUTE, THAT OF 2 STRONGLY PRODUCED POSTERIORLY. FRYER (1952), BOUSFIELD (1958)	PLATE 2 HAS POSTERIOR CORNER OF VENTRAL MARGIN NOT AS ACUTELY PRODUCED AS 3. GLEDHILL, SUTCLIFFE, AND WILLIAMS (1976)
6	DORSAL SURFACE OF UROSOME WITHOUT SPINES OR SETAE OR VERY SPARSELY SETOSE. GLEDHILL, SUTCLIFFE, AND WILLIAMS (1976)	DORSAL SURFACE OF UROSOME WITH SPINES AND SETAE. GLEDHILL SUTCLIFFE AND WILLIAMS (1976)
7	OUTER RAMUS OF UROPOD 3 WITH MARGINAL SPINES, BUT SETAE RARE OR ABSENT. INNER RAMUS VERY SHORT; 1/5 LENGTH OF OUTER RAMUS. GLEDHILL SUTCLIFFE AND WILLIAMS (1976)	OUTER RAMUS OF UROPOD 3 ARMED WITH SPINES AND LONG SETAE. INNER RAMUS AT LEAST 1/2 LENGTH OF OUTER RAMUS GLEDHILL SUTCLIFFE AND WILLIAMS (1976)
8	TELSON CLEFT TO MIDDLE. GLEDHILL SUTCLIFFE AND WILLIAMS (1976)	TELSON CLEFT TO BASE. (PERS. OBS.)

TABLE 1.1
Morphological differences between *C. pseudogracilis* and *G. pulex*.

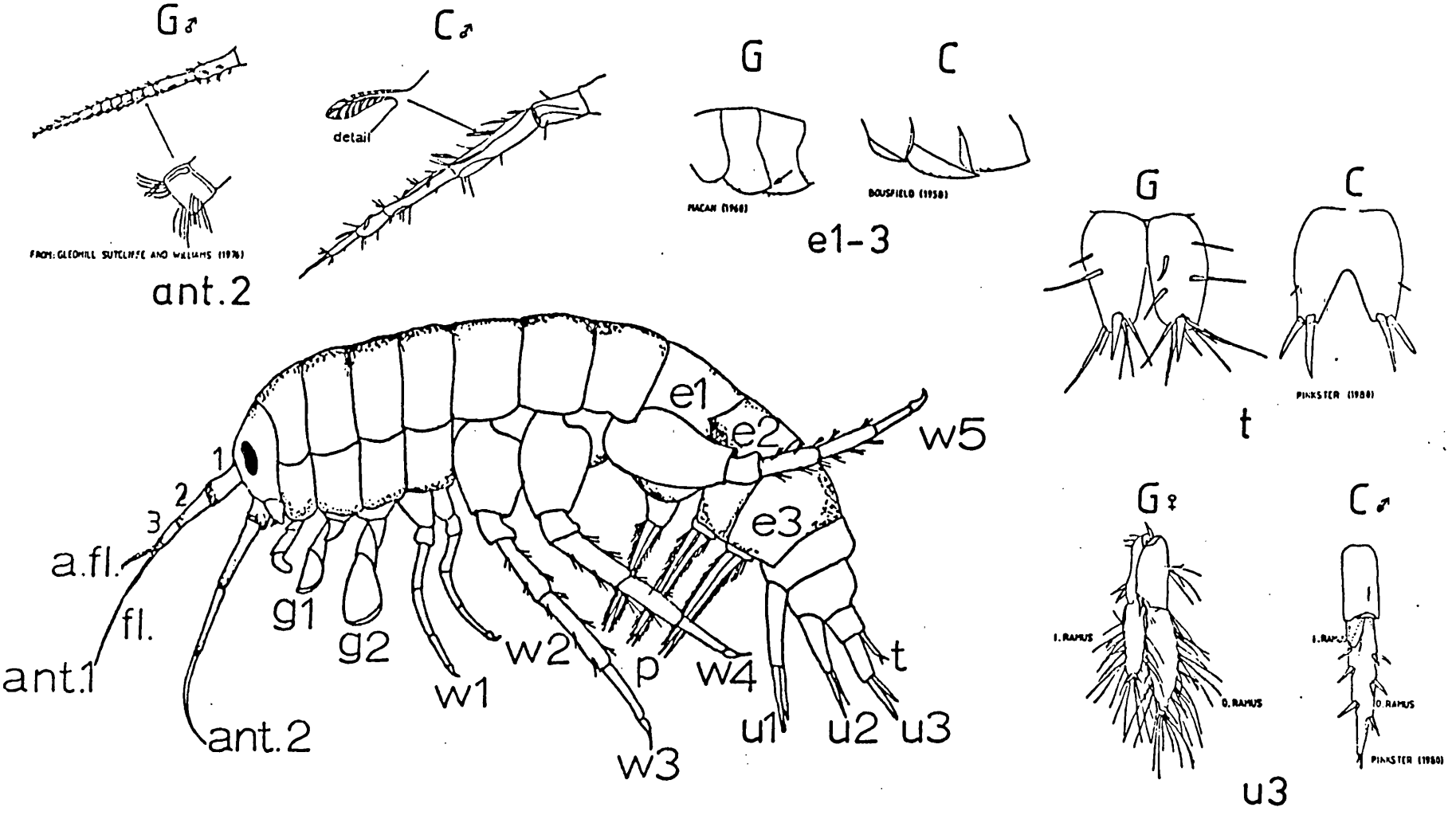


FIG. 1.1

Morphological differences between *C. pseudogracilis* and *G. pulex*. G = G. pulex, C = C. pseudogracilis. a.fl = accessory flagellum, ant = antennae, g = gnathopod, w = walking leg, p = pleopod, u = uropod, e = epimera, t = telson.

FIG. 1.2a
G. pulex ventral surface showing silver stained, quadrate, coxal gills.

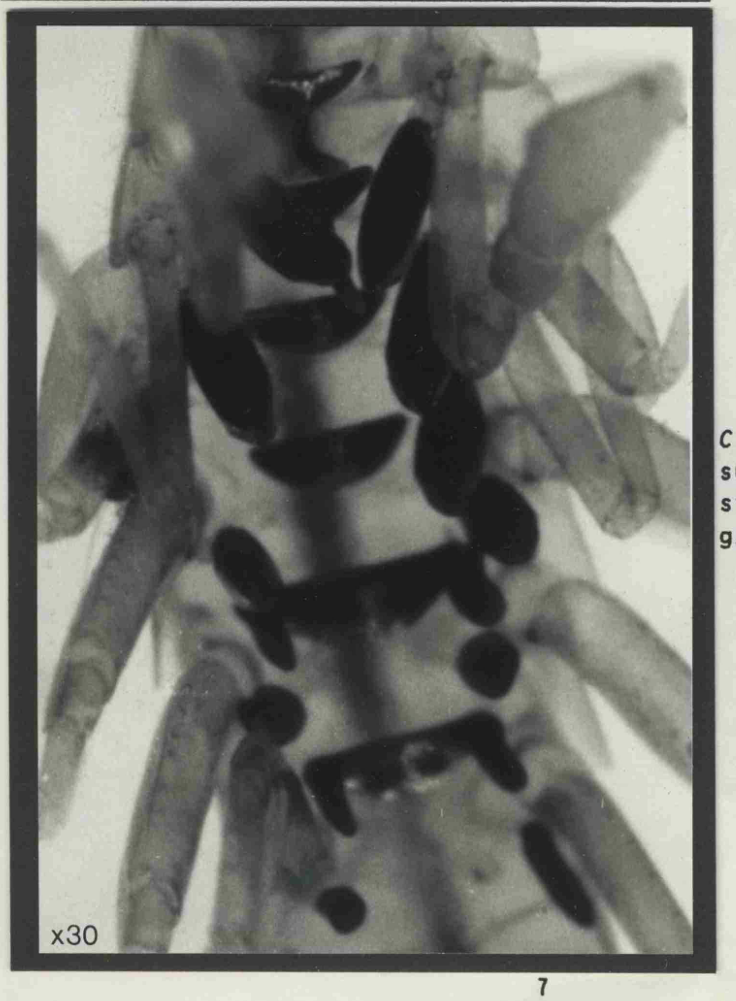


FIG. 1.2b
C. pseudogracilis ventral surface showing silver stained coxal and sternal gills.

In general *G. pulex* (Fig. 1.3), is a larger, morphologically more robust species than *C. pseudogracilis*, and invariably an opaque orange-brown in colour. In contrast *C. pseudogracilis* (Fig. 1.4 &1.5), exhibits a wide colour range from white to olive green and slate blue, with the internal organs frequently clearly visible through the more delicate, semi-transparent cuticle.

Whilst there is no clear sexual dimorphism in *G. pulex* the males tend to be larger since this is advantageous for pre-copulatory mating activity (Hynes 1955a). In *C. pseudogracilis* which has no pre-copulatory pairing there is marked sexual dimorphism with females (6-10mm) much larger than males (3-5mm) (Fig. 1.4 & 1.5). Locomotion in *G. pulex* is restricted almost exclusively to swimming on the side, whilst in *C. pseudogracilis* both crawling upright on the substratum similar to talitroid species, and swimming ventral side uppermost are common.

FIG. 1.3
Gammarus pulex ♂

FIG. 1.4
Crangonyx pseudogracilis ♀

FIG. 1.5
Crangonyx pseudogracilis ♂

Distribution and habitat

There are nine species of the genus *Gammarus* recorded from fresh and brackish water in and around the British Isles. *G. pulex* is a native eurytopic species found in streams and rivers of all types, as well as lakes and field ponds (Hynes 1955b). It occurs in waters of moderately high to low mineral content, but is not found where the pH consistently falls below 5.8 (Hynes, Macan & Williams 1960). Its distribution extends into mountain streams in Cumbria, but it is absent from some western peninsulas and the extreme north of Scotland, and mainly absent from islands including Orkney, Shetland and Faeroes, but present on the Isle of Man and Ireland.

G. pulex commonly lives below stones and loose leaves in well oxygenated waters. Agrawal (1965) studied gut contents of *G. pulex* and concluded that it fed mainly on algal filaments and vegetable matter although in laboratory conditions dead, dying or moulted animals may be eaten (pers. obs.; Clegg 1952; Willoughby & Sutcliffe 1976).

C. pseudogracilis is native to eastern North America, its distribution extending from South Ontario, southwestern Quebec, and Vermont to Missouri, throughout the Missippi drainage basin, the lower part of the St. Lawrence system and American Atlantic watersheds (Bousfield 1958; Smith 1977). It has also been recorded from the Great Lakes (Barton & Hynes 1976).

C. pseudogracilis was first recorded in Britain by Crawford (1937) in the filtration plant of the Lea Bridge Waterworks, Metropolitan Water Board. The inference that it had already been present in England for some time was made by Tattersall (1937) who stated that his identification of a specimen from the same area found some years earlier was now confirmed by Crawford's publication.

During the next twenty years reports of its occurrence in the Midlands (Bassingdale 1946), South of England (Reid 1948), Wales (Hynes 1951), and North of England (Fryer 1952) were made. Reid (1948) suggested that it was not the range of this species but the knowledge of its range which had increased so rapidly, but Hynes (1955b) who compiled a detailed distribution map concluded that it had spread rapidly throughout the canal system and navigable waterways since its introduction. This view was supported by Bousfield (1958). The first record of *C. pseudogracilis* from Scotland was by Warwick (1959) who suggested that it was likely to have been introduced directly from Canada to Grangemouth on timber. It was not taken in Ireland until 1969 where it was thought to have been carried on the feet and feathers of waterfowl (Holmes 1975).

C. pseudogracilis is characteristically thought to favour the slow flowing or still water environment of canals, canalised rivers, ponds and reservoirs (Bousfield 1958; Holland 1976). It is now common throughout the canal system and is still thought to be extending its range (Gledhill, Sutcliffe & Williams 1976). It has also been taken from brackish water (Hynes 1955b; Holland 1976). Holland (op. cit.) reported that *C. pseudogracilis* was unable to establish itself in any streams or rivers in the area of the Mersey & Weaver River Authority, attributing this to its inability to withstand fluviatile conditions. Whilst in nearly half the locations studied it was found to co-exist with *G. pulex*, Holland (op. cit.) concluded that there was no evidence for direct interspecific competition favouring the latter species. There have been several reports of *C. pseudogracilis* from organically polluted water (Hynes 1955b; Holmes op. cit.; Pinkster *et al.* 1980). However, Holland (op. cit.) was unable in his study to confirm that this species tolerated polluted conditions unacceptable to *G. pulex*.

Comparative value

The features which make these two species interesting for a comparative toxicity study can be summarised as follows;

1) They have both morphological and classificatory similarities.
2) *G. pulex* is a native species with a well documented range. *C. pseudogracilis* is a recent introduction which is presently still extending its range; the two species are known to co-exist but the conditions under which either is able to gain advantage have not been determined.
3) *G. pulex* is known to be sensitive to both organic and metal pollution (Abel 1980; Costa 1966, 1967a, 1967b, 1980; Davies 1940). *C. pseudogracilis* is assumed to be tolerant of organically polluted conditions but evidence of this is restricted to field observations. Its tolerance to metal pollution is unknown.

2. ZINC

Introduction

The name 'zinc' is of German derivation and literally translated means 'of unknown origin'. Zinc is one of the less common elements. It has been estimated to constitute 0.004% of the Earth's crust, is twenty fifth in order of abundance of elements, and is far scarcer than less familiar elements such as zirconium, vanadium, titanium or strontium (Rice 1963). Elemental zinc has atomic number 30, and atomic weight 65.38 and is chemically classed as a transition metal (Parkes & Mellor 1939; Sneed & Brasted 1955).

Zinc usually occurs in combination with sulphur or oxygen, generally as part of a poly-metallic ore of which lead-zinc ores, copper-zinc ores and lead-copper-zinc ores are the most common. These ores may also contain silver, cadmium, tin, bismuth, thallium, indium and germanium in economically important quantities (Rachamalla & Bell 1976).

Essential nature as a micro-nutrient

Zinc is a universal constituent of living matter, and its essential role as a micro-nutrient for the growth and development of micro-organisms, plants and animals has been concisely reviewed by Rice (1963). Unlike elements such as carbon and nitrogen which form structural components in the living cell, zinc is associated with enzymes which regulate the cellular metabolism. In metal proteins zinc is non-specifically bound and easily removed by dissociation, whilst in the metalo-enzymes it is specifically bound into the protein matrix (Bachmann 1963).

Toxic properties

In contrast to many organic pollutants, excess metals such as zinc cannot be microbially decomposed and are therefore potentially hazardous to living organisms. When the rate at which zinc is released into the environment, usually as a result of human activity, exceeds the natural rate of cycling it may be taken up in sufficient quantities by plants and animals to become toxic (Stumm & Bilinski 1972). If the human induced mobilisation of metals are compared with the global rate of mobilisation through natural geological processes, zinc joins iron, copper, manganese, lead, tin and antimony in having a markedly enhanced mobility (3930×10^3 tonnes per year as against 370×10^3 at the geological rate) (Weatherley, Lake & Rogers 1980).

A common characteristic of many publications concerning the impact of industralisation on the ecosystem is to utilise the term 'heavy metal' to describe a heterogenous group of metallic elements, including zinc, which have toxic properties to biological tissues. Chemically these elements are most commonly in the middle block of the periodic table, having atomic number 22-92 and specific gravity 4.5-22.5. However, the lanthanides and actinides which fit chemically into this category are not considered 'heavy'. As Waldechuck (1974) and Nieboer & Richardson (1980) point out, there is no really satisfactory chemical or biological grouping from which a clear definition can be constructed.

An alternative classification based on the atomic properties and solution chemistry of the metal ions is suggested by Nieboer & Richardson (op. cit.), and whilst this would provide a better basis for comparison between different metals, in the context of this study the replacement of 'Heavy metal pollution' with 'Pollution by excess quantity of trace metal' would seem most appropriate.

Zinc in the freshwater environment

In the freshwater environment zinc becomes partitioned between dissolved, sedimentary and biotic phases. Complex equilibria are established between the metal in the sediments and aqueous phases where soluble, colloidal and particulate forms are all present. This is illustrated diagrammatically in a figure reproduced from Coombs (1980) (Fig. 1.6). The zinc cycle may therefore be thought of as a flux of zinc between the different phases, superimposed on the movement of the phases themselves. For example, both the uptake of a zinc ion from solution by a clay particle and the movement of that particle as it settles to become part of the bottom sediments is included (Bachmann 1963).

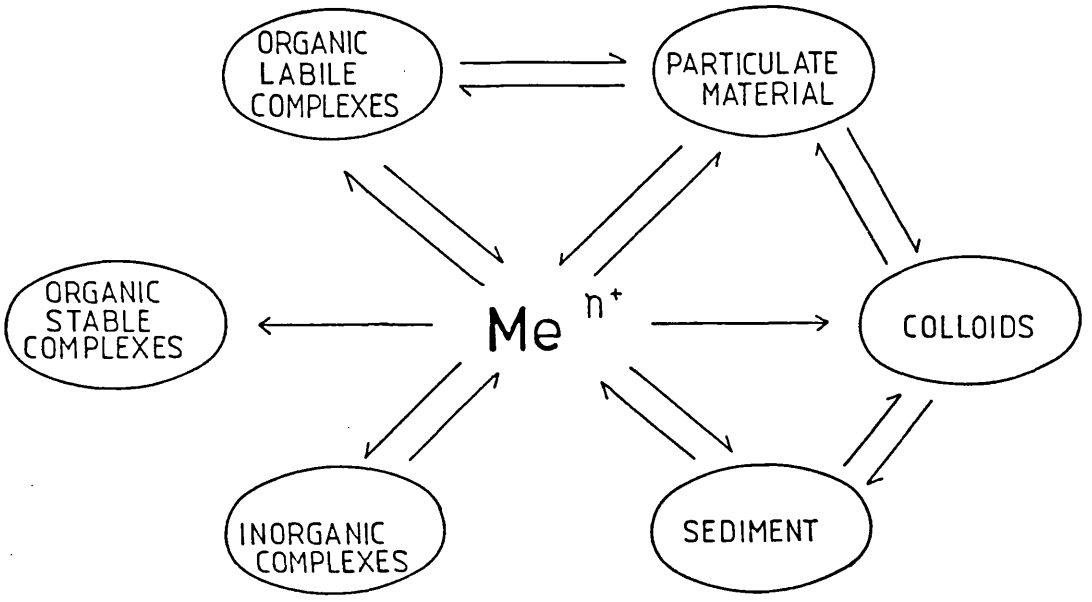


FIG. 1.6
Multi-phasic physico-chemical forms in which trace metals may occur in natural waters.
From Coombs (1980). In this example $Me^{n+} = Zn^{2+}$.

Zinc exists in the liquid phase as a hydrated ion, a complexed ion or associated with soluble organic compounds. The percentage of total zinc present which remains in solution will be determined by the pH, alkalinity and solute exposure. With increasing pH and alkalinity zinc is precipitated, whilst during flooding transient redissolution from sedimentary deposits may occur (Weatherley, Lake & Rogers 1980).

Zinc exists in the solid phase as part of an inorganic precipitate, occupying space in the crystal lattice of clays, as an exchangeable ion in organic or inorganic complexes, or as a functional component of the metabolic systems of living organisms. The relative insolubility of the salts zinc carbonate and zinc hydroxide place an upper limit on the quantities of dissolved zinc found in natural waters (O'Connor & Renn 1964).

The solubility of zinc carbonate is a function of the concentration of the carbonate ion, and is dependent upon the pH value and concentration of bicarbonate ion in solution. Either a 10-fold increase in hardness or unit increase in pH will bring about a 10-fold reduction in the concentration of dissolved zinc (Solbe 1974). Zinc hydroxide is formed from the following reaction;

$$Zn^{++} + 2H_2O \rightleftharpoons ZnOH^{+} + H_2O + H^{+} \rightleftharpoons Zn(OH)_2\downarrow + 2H^{+}$$

At pH 7 only 0.25% of the total dissolved zinc is $ZnOH^{+}$. As the pH rises the equilibrium shifts to the right forming zinc hydroxide. At pH 11, 97.2% of dissolved zinc is in the intermediate form. The only other inorganic precipitate of potential importance is zinc sulphide which is formed under reducing conditions such as the oxygen depleted hypolimnion, or within the sediments. Zinc may also be adsorbed or co-precipitated with other compounds (Bachmann op. cit.). A common co-precipitate in lakes is that of zinc with ferric oxide.

One of the major adsorbent species present in stream sediments is clay, and the fixation of zinc ions by clay minerals has formed the subject of a number of investigations (Farrah & Pickering 1977; Bourg & Filby 1974). As a cation, zinc may either occupy a position in the crystal lattice where it is unexchangeable, or attach to negatively charged sites on the surface where it is exchangeable and in dynamic equilibrium with ions in solution.

The ability to remove cations from solution is dependent on the cation exchange capacity (C.E.C.) of the clay mineral, the process being pH dependent. Clay minerals act as a buffer reservoir for the amount of zinc in solution and therefore if zinc ions are added to natural waters the suspended clays will tend to re-establish the original state by re-adsorbing some of the added zinc (Bourg & Filby op. cit.).

Determination of toxicity

Two mechanisms of zinc uptake by living materials have been defined as the specific utilisation of zinc in the synthesis of certain enzymes, and the non-specific adsorption or ion exchange reactions (Bachmann op. cit.).

The physico-chemical state of the metal is the key factor in the determination of its availability to the biota, whilst the metabolism of the individual organism will determine its toxicity. When complexed with clay or organic particles the availability of zinc for uptake, and therefore its toxicity, is considered to be relatively low (Weatherley *et al.* 1980). However, Wentsel, McIntosh & Anderson (1977) found that chironomid larvae avoided silt loam or loam sediments contaminated with zinc, chromium and cadmium. The major contribution of sedimented zinc towards toxicity may be in its potential for redissolution under appropriate environmental conditions such as reduced pH, elevated temperature and physical disturbance (Eyres & Pugh Thomas 1978).

It is generally in the dissociated ionic form Zn^{++} that zinc is considered most toxic as a freshwater pollutant (Lloyd 1960 ; Skidmore 1965). In recent years there has been a major attempt to identify more precisely the species of metals which exist in natural waters, since the common procedure of relating total metal concentration to toxicity has been found in some cases to provide misleading results (Florence 1977; Stumm & Bilinski 1972). Depending on the chemical form of the metal, a water with a high total metal concentration may be less toxic than one with a lower concentration.

According to Nuernberg (1982), voltametry constitutes obviously the most powerful and conclusive method for investigation on the speciation of dissolved metal traces in natural waters. Although its potentialities have yet to be fully exploited, there are difficulties with this technique.

Various metal classifications cannot be identified as exact chemical species, analytical measurement may destroy the natural equilibrium of metal forms present, and it is, at present, a long and technically complex procedure for large scale application.

Whilst information of the type of metal species encountered under different chemical conditions is advantageous for a better understanding of the distribution and function of trace elements in natural waters, these constraints necessarily make a consideration of metal speciation within this study impractical.

Sources of zinc pollution in the aquatic environment

Weatherley *et al.* (1980), identified several processes which cause the release of excess zinc into the freshwater environment at potentially toxic levels. These can be summarised as follows;

1) Weathering and washing of overburden zinc or other ore bodies and of corresponding ore tailings.
2) Leaching of smelter slags.
3) Spills, washdowns, and rinsings from electro-refining for zinc and industrial plants using zinc compounds or metals.
4) Disposal of materials containing zinc from chemical or industrial plants.
5) The scrubbing of smelter and incinerator fumes containing zinc.
6) Domestic and industrial sewage containing high zinc levels derived from the use of zinc containing products.
7) Radioactive isotope ^{65}Zn from nuclear power plant operations or fallout from nuclear weapon testing.

The major and most permanently hazardous sources of zinc pollution for freshwater environments come from mining of zinciferous ore bodies, and this topic has consequently received most attention in the literature (Carpenter 1936; Jones 1940, 1958; Brown 1977a; Scullion & Edwards 1980). The methods by which zinc is recovered from subsurface ores require both water and crushing equipment.

The fine wastes (tailings), produced in the extraction process contain insufficient quantities of zinc to be of economic importance and are consequently deposited as dumps. Even after centuries of disuse mine dumps may generate sufficient quantities of zinc by both physical transfer and bacterial activity to adversely affect the adjacent freshwater environment. The effects can be extensive, and are occasionally irreversible (Weatherley *et al.* 1980). Jones (1958) reported that at certain disused mines in the valley of the River Ystwyth, open adits continued to discharge water heavily polluted with zinc salts nearly 40 years after the mine had been closed.

As a consequence of the semi-permanent nature of this pollutant source it offers little opportunity for realistic short term studies in which both the effects of zinc pollution, and the subsequent recovery of the freshwater body, when the source is removed, can be determined. Harding, Say & Whitton (1981) studied the recovery of the River Ethrow from a mixture of organic pollution from sewage works, and zinc pollution from a paper processing factory. They reported a clear change in faunal community structure over 2 years as organic pollution decreased, but were unable to discern any clear beneficial consequences of the steady decrease in zinc concentration from 1.11 mM in 1975 to 0.27 mM in 1979, within the tributary carrying zinc effluent to the river.

3.THE MULTI-TEST APPROACH

This toxicity study comprises a series of tests of variable duration, having different individual objectives, but which collectively provide a comprehensive description of zinc toxicity to *G.pulex* and *C.pseudogracilis*.

The study is initiated with an analysis of acute zinc toxicity to mature adults of both species. The short test duration, and response criterion of death confers on it little field related value, but renders it a clear, unequivocal demonstration of toxicity and consequently a valuable starting point.

Of more applied interest are chronic toxicity tests which explore longer term effects of sub-acute toxicant levels. The ideal chronic test will determine the highest concentration of toxicant which causes no adverse effects on the distribution and abundance of the test species over at least 3-4 life cycles. The time consuming aspect of this approach makes it impractical, however, for a study of limited duration, and therefore a suitable compromise between ideal and practical objectives must be made. The period between fertilization of the egg within the female, and release of the young is generally considered to be the most vulnerable link in the life cycle of the test species (Sprague 1976). This period of approximately 21 days in both species has therefore been selected to explore the effects of zinc on brood size and development time, and on the condition of the adult female. The fate of the young over the first 21 days after release has also been monitored. Whilst this test is of longer duration than the acute approach, and the response criterion not so severe, the ultimate result is the same since an inability to produce viable young will eventually result in extinction of the population.

The third series of tests are concerned with behavioural responses to zinc and in contrast to the previous experiments it was not initially possible to categorise them as acute or chronic since both the effective concentrations, and the type of response which would be obtained was unknown. The value of this part of the study is not therefore to predict a zinc concentration for the extinction of the population, but to explore the application of behavioural techniques to indicate toxicity within the context of previously determined acute and chronic levels. The tests in this section are divided into two parts concerned with both avoidance, and activity changes in the presence of dissolved zinc. The demonstration of an avoidance response, especially below acutely lethal zinc levels, has obvious field related interest but changes in activity with no obvious survival value have also been considered.

The final series of tests concentrate exclusively on physiological aspects of zinc toxicity and, in common with the behavioural study, they do not automatically fall into acute or chronic categories. The criteria of toxicity are changes in oxygen consumption and gill ventilation rate, and the main aim of these tests is to assess their use as indicators of zinc toxicity, and to provide some insight into the mode of action of the toxicant.

Summary of study aims

A relatively transient incidence of zinc pollution in the upper reaches of Willow Brook, a small Northamptonshire river, has recently provided the opportunity to study the effects of zinc on freshwater flora and fauna during both polluted period (Solbe 1973, 1977), and subsequent recovery phase after changes in effluent treatment had effectively removed the source of pollution (Harper, Hancock & Davies 1979).

This study aims to determine the relative toxicity of zinc to two freshwater amphipod species found in Willow Brook, in order to assess the extent to which zinc pollution may have dictated their distribution and abundance during and after pollution. Since it is the animals, and their comparative response to the same pollutant which are of primary interest this is a true toxicity study rather than a bioassay series.

No single toxicity test used in this study can accurately describe the effects of zinc on the two tests species. However, when performed as a coherent unit under identical controlled conditions, the collective information obtained can both assist in predicting toxic effects of zinc within the field situation, and contribute towards current theories of toxicant action.

CHAPTER 2

WILLOW BROOK : A CASE OF ZINC POLLUTION

ENVIRONMENTAL BACKGROUND

Willow Brook is a small river which rises on Northamptonshire Sand Ironstone near the industrial town of Corby (SP 901.885) at an altitude of approximately 130 m O.S. It subsequently flows for 27 km through mainly arable land, with occasional deciduous and coniferous woodland, to join the River Nene near Elton Mills, Cambridgeshire (Fig. 2.1). The gradient decreases from approximately 8 m km^{-1} in the upper reaches to 2.2 m km^{-1} in the lower reaches at Fotheringhay.

Willow Brook drains a catchment area of 8962 ha. The land surrounding the upper half of the brook consists of grey-brown neutral to acid podsolic soils and includes some brown forest soils, acid brown soils, and gley soils. Drainage is good to imperfect. The land surrounding the lower half of the brook is acidic podsolized soil including iron podsols, humus podsols, acid brown soils and gley podsols with variable drainage. Much of the catchment area is arable farmland with the main crops being wheat, barley, oats, potatoes, sugar beet, fodder roots, green crops and grass. Occasional woodland is predominantly oak, ash, and other broad leaved species (Quinlan 1971).

The brook originates as three fast flowing small streams, only 3-5 m wide (Table 2.1), with an average gradient of 3.75 m km^{-1} (Solbe 1973). The Southern stream has its source in the south west corner of Corby (Fig. 2.2). The Central stream arises near the former Corby Steelworks and receives the town effluent before joining with the Southern stream near Weldon.

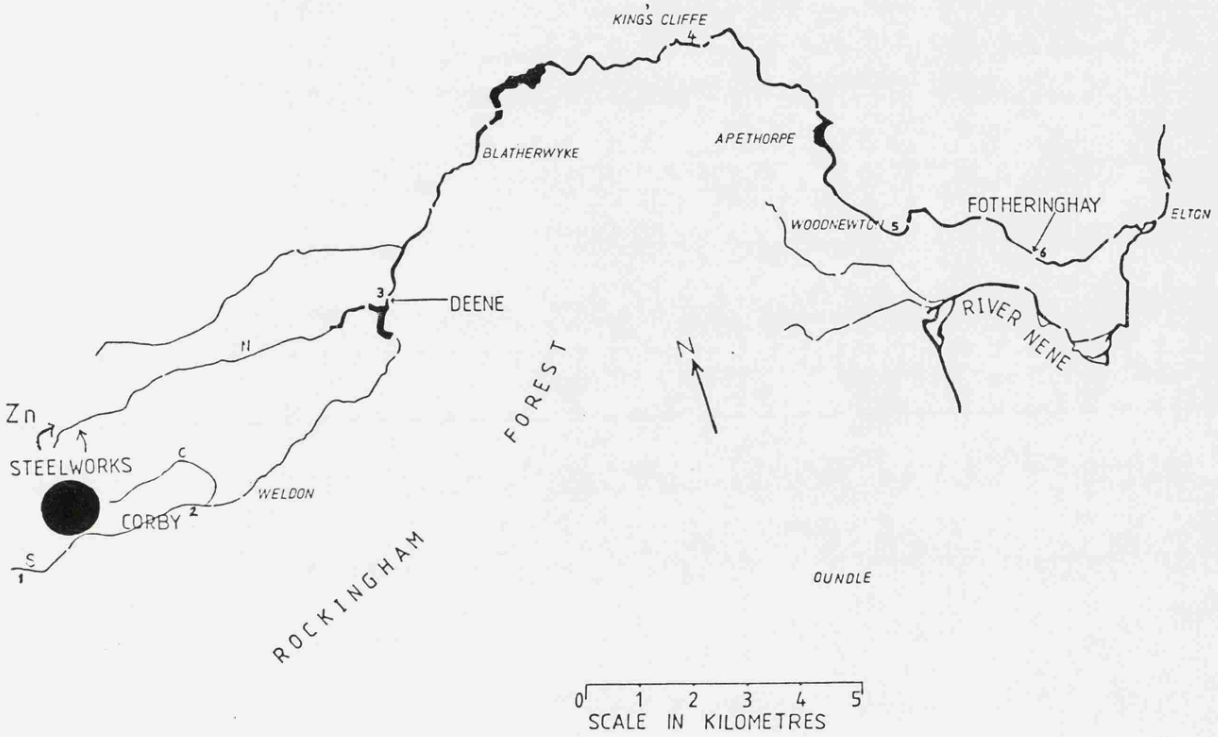


FIG. 2.1

Map of Willow Brook showing its rise near Corby as three small streams, and its confluence with the River Nene at Elton Mills.

S = Southern stream, C = Central stream
N = Northern stream

1)	Stanion road bridge	(SP 903.885)
2)	Weldon road bridge	(SP 929.894)
3)	Deene road bridge	(SP 955.928)
4)	King's Cliffe	(TL 002.970)
5)	Woodnewton	(TL 037.941)
6)	Fotheringhay	(TL 063.935)

The broader stream so formed continues north eastward, receiving village effluent before entering an 11.4 ha lake at Deene. The Northern stream arises to the north of Corby and flows east north eastwards to enter the north arm of Deene Lake.

Deene Lake is generally less than 2 m deep with a soft mud bottom. Water from the Northern stream initially follows the north bank of the lake since its flow rate is slower than the combined Southern and Central streams. Water leaving the north east side of the lake at the outfall weir is generally well mixed. Deene Lake therefore buffers variation in flow and chemical quality of upstream waters, before they are discharged downstream.

Below Deene Lake the brook flows in a north easterly direction over stony riffles and a hard clay bed (Fig. 2.3). Effluent from a village sewer is received at Bulwick before the brook passes into Blatherwyke Lake. This lake is the biggest in the system having a total area of 19.9 ha. It is approximately 3 m deep in the southern part and up to 6.5 m at the north eastern extremity.

Downstream of Blatherwyke Lake the flow changes to easterly and the brook receives effluent from a sewage treatment works at King's Cliffe. Here it is relatively fast flowing, over a stream bed of coarse gravel and weed. Approaching Apethorpe the Brook bends south and the velocity is reduced as it passes through a series of riffles and pools before entering a shallow (<0.5 m), muddy, 4.5 ha lake.

Below the lake the Brook is slower and more weedy, entering a series of long riffles and pools with a gradient of 2.2 - 2.4 m km^{-1}. A south easterly flow is maintained to Woodnewton where it resumes an easterly course passing to the north of Fotheringhay (Fig. 2.4), to enter the River Nene at Elton Mills. The size of the brook is fairly uniform below Deene Lake at about 6.5 m wide and 0.3 m deep (Table 2.1). The underlying rock is for the greater part oolitic limestone and consequently the Brook maintains a pH of about 8 (Table 2.2).

FIG. 2.2

FIG. 2.2 STANION ROAD BRIDGE, CORBY (SP 903.885)
Southern stream site, upstream of both sewage works and steelworks. The steam is subject to considerable annual variation in depth. *G. pulex* is the dominant species.

FIG. 2.3 DEENE OUTFALL WEIR (SP 955.928)
Immediately downstream of Deene Lake this section of Willow Brook is a typical riffle habitat. The rocky stream bed is coated with sewage fungus.

FIG. 2.4 FOTHERINGHAY (TL 063.935)
Furthest sample site downstream, this site is dominated by *G. pulex*.

FIG. 2.3

FIG. 2.4

SITE	GRID REFERENCE	STREAM WIDTH (m)	MEAN VELOCITY OF FLOW ($m\ s^{-1}$)
STANION RD BRIDGE	SP 903.885	2.52	-
WELDON RD BRIDGE	SP 929.894	2.74	1.556
DEENE RD BRIDGE	SP 955.928	7.00	1.788
KING'S CLIFFE	TL 002.970	6.46	1.936
WOODNEWTON	TL 037.941	5.89	1.476
FOTHERINGHAY	TL 063.935	6.86	0.860

TABLE 2.1
Stream width and velocity of flow at six sites on Willow Brook, taken on 06.11.1980.

SITE	GRID REFERENCE	pH	TEMPERATURE (^{o}C)
STANION RD BRIDGE	SP 903.885	7.35	11.2
WELDON ROAD BRIDGE	SP 929.894	8.05	13.5
DEENE ROAD BRIDGE	SP 955.928	7.90	14.0
KING'S CLIFFE	TL 002.970	8.10	13.8
WOODNEWTON	TL 037.941	8.20	13.0
FOTHERINGHAY	TL 063.935	8.30	13.0

TABLE 2.2
pH and temperature at six sites on Willow Brook, taken on 02.10.80.

HISTORY OF STEELWORKS IN THE CORBY AREA

The history of ironworking in the Corby area dates back to Roman times. A small ironworks was built at Corby in 1910 but it was not until 1933 when an integrated Iron, Steel and Steel Tube works was built that the small village expanded into an industrial town.

Water for cooling and gas washing was derived from Eyebrook reservoir in the valley of the River Welland north of Corby, from two lakes on the Willow Brook, and a variety of groundwater and gravel pit sources in the area. Most of the effluent was discharged to the Central and Northern streams from which the Willow Brook originated.

Prior to 1965 iron was smelted in four blast furnaces, and steel produced by the Bessemer process. The resultant production of iron and steel and also effluent were relatively constant. Whilst there is no analytical data for this period, observation of Deene Lake and Blatherwyke Lake indicated only very periodic water quality problems, with no evidence for pollution of the brook below Deene Lake (Oliver 1975).

In 1965 international competition for iron and steel sales increased and operation of the blast furnace was altered to find more efficient and economical methods of iron production. The main changes were in the use of 100% sinter for charging the blast furnaces, and the use of less basic burden. This resulted in blast furnace gases with a greater acidity which when washed off particulate contaminants gave rise to an acidic solution which dissolved zinc from iron ore.

When the high levels of zinc within the blast furnace washings were detected, steps were taken to precipitate it in effluent treatment ponds, initially by the addition of lime. Since 1972 zinc has been precipitated by mixing blast furnace washings with waste water from the Basic Oxygen Steelmaking Plant which replaced the Bessemer process. In this process the refractory lining of the vessel is basic and therefore the washings alkaline. In September 1980 all production at the Steelworks ceased and no further effluent was discharged to the brook after this date.

BIOLOGICAL AND CHEMICAL EVIDENCE FOR ZINC POLLUTION

The first indication that changes in iron and steel processing had adversely affected the water quality of Willow Brook was obtained 2 - 3 years later in a series of complaints to the Welland & Nene River Authority concerning a marked deterioration in the quality of fishing. A subsequent survey of analytical data for Willow Brook collected between 1962 and 1968

showed no significant long term changes in pH, salinity, alkalinity, dissolved oxygen, B.O.D, or free and saline ammonia (Welland & Nene River Authority; unpublished data). A further survey of the ammonia concentration below Deene outfall weir between 1960 and 1968 concluded that if ammonia was the only toxicant in Willow Brook the water quality would be satisfactory for rainbow trout(Welland & Nene Authority unpublished data).

In the absence of any conclusive chemical data with which to characterise the pollutant, the River Authority carried out a hand net survey of invertebrates at Woodnewton in April 1968, for comparison with a similar survey from downstream of Deene Lake to Fotheringhay in May 1965. A comparison of the two Woodnewton samples revealed an absence in 1968 of 9 taxa including Ephemeroptera, Elminthidae, and *G. pulex*.

The importance of zinc as a pollutant was established after a full chemical test was performed on the brook in 1969 (Alabaster, Garland, Hart & Solbe 1972; Solbe 1973,1974,1977). In his investigations into the ecology of the brook during the period of heavy zinc pollution, Solbe(1973) concluded that dissolved zinc was the most predominant poison with concentrations over four times the experimentally determined 48 hr LC50 to rainbow trout. The only other pollutants capable of reaching lethal concentration in the upper streams were ammonia and phenol, although monohydric phenols were rarely encountered at high concentrations, and were generally absent. No other metal was present in sufficient quantity, and soft detergent levels were considered harmless.

Fishless zones were recorded for 4 km below the toxic discharge although 10 species were found 14 km downstream where toxicity was significantly reduced by metal precipitation. *Gasterosteus acuteatus* L. (stickleback) were found to be the most tolerant species and *Noemacheilus barbatulus* L. (stone loach) most sensitive (Solbe 1973).

Following the improvement of effluent treatment at the steelworks in 1972, levels of zinc fell and a subsequent biological survey by the River Authority in 1976 recorded several improvements in the benthic fauna. A detailed account of the recovery of Willow Brook from zinc pollution is given by Harper, Hancock & Davies (1979), concluding that benthic invertebrates had recovered to the state where they were now constrained by other pollutants. Fish populations had not, however, shown a complete recovery to pre-pollutant levels.

THE EFFECT OF ZINC POLLUTION ON THE DISTRIBUTION AND ABUNDANCE OF *G. PULEX* AND *C. PSEUDOGRACILIS*

In the River Authority survey of 1965, *G. pulex* was found to be a common inhabitant of the lower reaches of Willow Brook. In the subsequent survey of 1968 no *G. pulex* were recorded, and Harper *et al.* (1979) concluded that the main effect of the raised zinc levels was to totally eliminate *G. pulex* from the brook.

C. pseudogracilis was first recorded in Willow Brook in November 1970 where a small population existing below the outfall weir from Deene Lake during this highly polluted period were mistakenly reported as *G. pulex* (Harper pers. comm.). The lake was not sampled.

In the Anglian Water Authority survey of 1976 following improvement in effluent treatment, *C. pseudogracilis* was again found in the typical riffle habitat below Deene Lake, and also both immediately upstream and downstream of Blatherwyke Lake. *G. pulex* had reappeared at King's Cliffe and by the time the brook reached Woodnewton had become the numerically most abundant invertebrate species. A further invertebrate survey by the Water Authority in 1983 records the continued recolonisation of Willow Brook by *G. pulex* as far upstream as Bulwick Mill (Anglian Water Authority unpublished data).

THE UNSUITABILITY OF WILLOW BROOK AS A SOURCE OF TEST ANIMALS

In order to make a direct comparison of the effects of zinc toxicity on *G. pulex* and *C. pseudogracilis*, only individuals with no prior exposure to zinc must be tested. This totally excludes any tolerance which may be acquired by acclimation, since this is a variable factor dependent on the initial tolerance, period and concentration of exposure, and individual physiology of the animal.

Whilst all effluent discharge to Willow Brook containing zinc had ceased before the start of this study, and concentrations of zinc in the water were found to be negligible by Atomic Adsorption Spectrophotemetry (Hardwick 3rd year project), several considerations made it unsuitable as a source of experimental animals.

C. pseudogracilis had been present in the brook throughout the most polluted period, and therefore the possibility of genetically acquired tolerance being expressed in the present population made them unsuitable test animals. G. pulex was eliminated at the onset of pollution but recolonised before output of effluent from Corby Steelworks totally ceased. This made it necessary to test animals from an unpolluted source, to eliminate the possibility that recolonisation had required a concomittant increase in tolerance to zinc.

Finally, measurement of dissolved metal levels to the exclusion of other components of the system may not reflect the true potential for pollution if there are precipitated and sedimentary bound metals available for redissolution under the appropriate chemical conditions. In Fig. 2.5 the concentration of zinc within the sediments at seven sites on Willow Brook are shown for the post pollutant period November 1980 to February 1981.

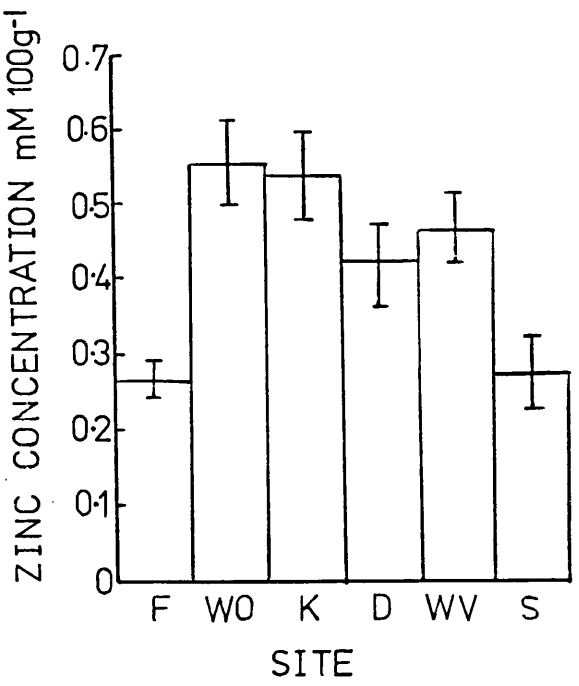


FIG. 2.5
Mean concentration of zinc in the sediments of Willow Brook for the period November 1980 to February 1981.
Results are presented with 95% confidence limits.

F = Fotheringhay
WO = Woodnewton
K = King's Cliffe
D = Deene road bridge
WV = Weldon village
S = Stanion road bridge

(Taken from 3rd Year BSc Hons. Project, M. D. Hardwick 1981)

It is clear that at four of the sites downstream from the steelworks, zinc concentrations within the sediments were approximately two times higher than in the upstream control site (S), or at the most distant point downstream from the discharge (F). The possibility that intermittent sub-lethal increases in zinc concentration could promote the acquisition of tolerance by both species further confirms their unsuitability as experimental animals.

CHAPTER 3.

RUTLAND WATER: SOURCE OF ANIMALS

Rutland Water is a 1260 ha reservoir in the county of Leicestershire, England (52^{o} 40'N, 0^{o} 37'W), located approximately 30 km east of Leicester. When full it has a volume of 130×10^{6} m^{3}, a shore line of 38.6 km, an average depth of 10.7 m, and a maximum depth of 35.5 m in the central basin. Rutland Water is principally a pump storage reservoir although there is some contribution from the natural catchment of the River Gwash. The soil in the catchment area is derived from Jurassic clays and limestone. Alluvial deposits and river gravels of pleistocene and recent origin occur along the stream bed of the River Gwash, with silt and marl deposits at the west end of both north and south arms of the reservoir (Daoud 1984). Whilst much of the area around the reservoir comprises pasture or arable land there are wooded areas to the north and south and on the penninsula.

The reservoir began to fill in February 1975 from the natural catchment. In January 1976 pumping of water from the River Nene at Tinwell, and Welland at Wansford was initiated but it was nearly nine months before this process became fully operational. *G. pulex* was abundant in North and South streams of the Gwash prior to flooding but colonisation of the reservoir floor was slow, and it was not until November 1977 that the estimated population density first exceeded 100 m^{-2}. In the late summer of 1978 there was a major peak of over 900 m^{-2} which was followed by a decline during the winter and a generally low population oscillating around 100 m^{-2}, for the next four years (Bullock, Clark & Ison 1981). After the summer of 1982 the mean population density dropped to below 50 m^{-2} and has thereafter shown no sign of recovery (Bullock unpublished data).

C. pseudogracilis was first recorded in the reservoir in March 1978. It was not present in the feeder streams, although Moon in a personal communication to Bullock *et al.*(1981), recorded this species in the northern stream some years previously. In 1980 the population increased markedly in the North and South arms and exceeded 100 m^{-2} in the summer of 1981 (Bullock *et al.* (1981). There was a major peak of over 500 m^{-2} in late summer of 1982 and since then the population appears to have stabilised at around 100 m^{-2} (Bullock unpublished data), significantly higher than *G. pulex.*[1]

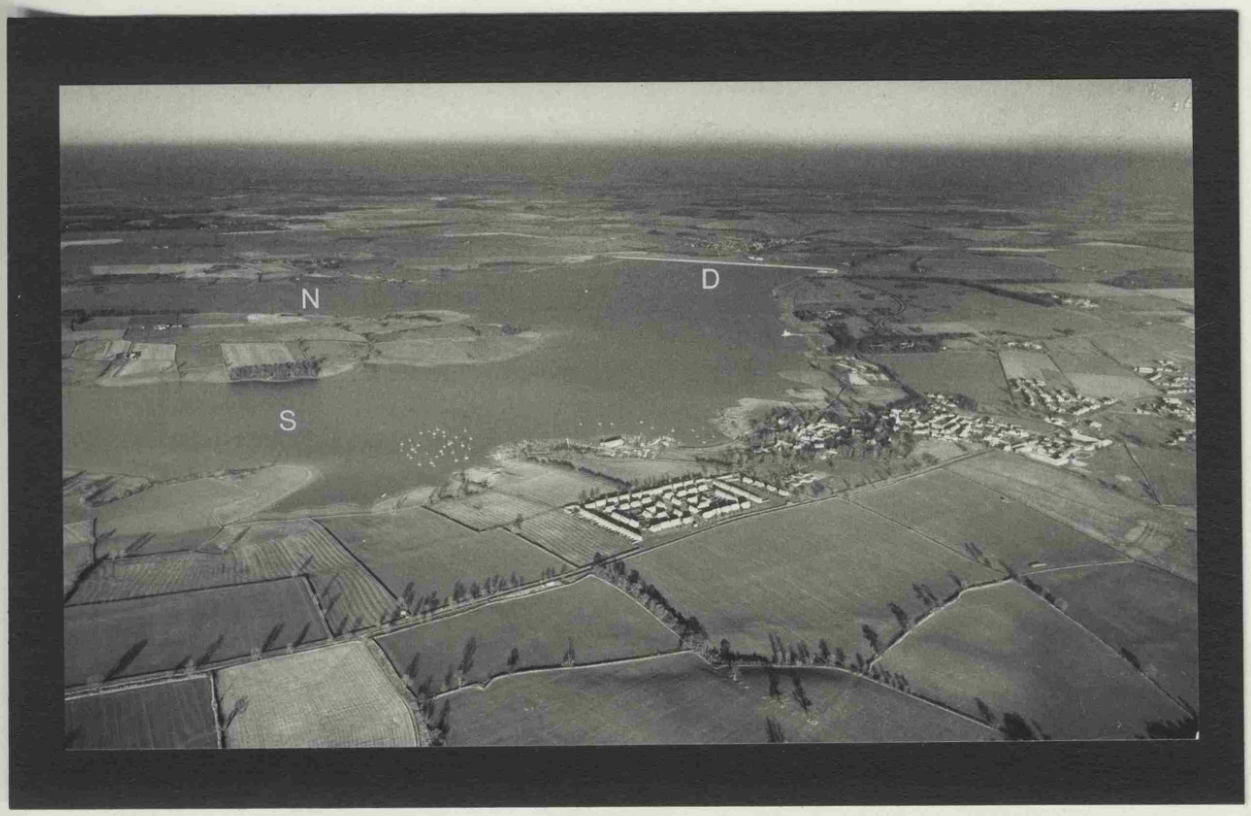


FIG. 3.1
Aerial view of Rutland Reservoir. N = North arm, S = South arm, D = Dam. (Reproduced with kind permission of Frank Clark)

1

Grab sampling throughout the Reservoir in July 1984 produced no *G. pulex* and only 3.3 individuals m^{-2} of *C. pseudogracilis.* It would therefore appear that both species are in rapid decline.

Rutland Water was an ideal source of animals for the purposes of this toxicity study since both species were available from a common source, similar in both chemical and physical characteristics to Willow Brook, but having no history of either trace metal, (Anglian Water Authority pers. comm.) or organic pollution (Table 3.1).

	DEENE LAKE			FOTHERINGHAY			RUTLAND WATER
	1978-1979	1979-1980	1980-1981	1978-1979	1979-1980	1980-1981	NOV 1979-OCT 1981
pH	8.15	8.00	7.85	7.96	7.81	8.25	8.27
Conductivity (µs/cm)	1570.00	1602.14	1280.00	1315.00	1379.37	1170.60	755.42
Susp solids - total (mg/l)	10.44	14.11	18.80	12.84	10.55	16.30	ND
Susp solids - ash (mg/l)	6.09	8.94	13.76	8.18	11.33	11.79	6.41
Temperature (^{o}C)	12.42	12.15	13.71	10.05	10.88	12.00	10.43
Dissolved O_2 (mg/l)	8.99	9.10	9.22	10.74	8.79	11.10	10.66
Dissolved O_2 (% satn)	83.23	83.59	88.44	95.20	78.33	103.77	95.00
B.O.D	7.24	6.56	6.00	6.30	5.03	2.90	ND
Ammonical N (mg/l)	9.46	13.07	1.31	1.77	4.34	0.30	0.08
Nitrite N (mg/l)	0.82	0.73	0.30	0.26	0.39	0.09	0.02
Nitrate N (mg/l)	11.01	10.62	10.89	10.40	11.94	8.99	1.76
Alkalinity ($CaCO_3$) (mg/l)	164.58	190.36	197.00	187.00	183.44	226.50	170.24
Tot Hardness ($CaCO_3$) (mg/l)	ND	ND	ND	ND	ND	ND	312.53
Dissolved P (mg/l)	1.65	1.74	2.39	ND	ND	ND	0.04
Chloride/chlorinity (mg/l)	200.25	152.93	89.40	136.80	117.75	75.40	47.98
Sulphate (SO_4) (mg/l)	410.33	406.47	312.00	338.20	350.59	265.10	150.55
Total Iron (mg/l)	ND	0.25	ND	ND	ND	ND	0.19
Manganese (mg/l)	ND	0.16	ND	ND	ND	ND	0.04

TABLE 3.1
A comparison between the mean physical and chemical characteristics of Willow Brook and Rutland Water. (Willow Brook; A.W.A Unpublished Annual Statistics) (Rutland Water; from Daoud 1984). ND = No data.

CHAPTER 4.

GENERAL METHODS

COLLECTION OF ANIMALS

G. pulex and *C. pseudogracilis* were collected from Rutland reservoir throughout the study period using both edge sampling and dredging techniques. The most productive area to obtain edge samples was on the north shore of the North Arm where an abundance of large stones and boulders less than 1 m below the surface were heavily colonised by both species (Fig. 4.1). Animals were collected by quickly lifting these stones into an adjacent hand net, sufficiently immersed to wash all the animals from the underside of the stone whilst retaining them in the net. In areas where the substratum was composed of smaller, more even sized particles, kick sampling with a hand net was the most effective collection technique.

When the level of the reservoir rose these permanent habitats became inaccessible for sampling, and the transiently flooded grassland areas yielded few animals. Under these conditions dredge samples were taken from the Penninsula where submerged weed beds supported populations of both species, often in discrete single species patches, for most months of the year (Fig. 4.1).

Dredge samples were partially sorted on site to reduce the volume of material for transportation. All samples were transported to the laboratory in large plastic buckets. On arrival they were immediately placed on aeration.

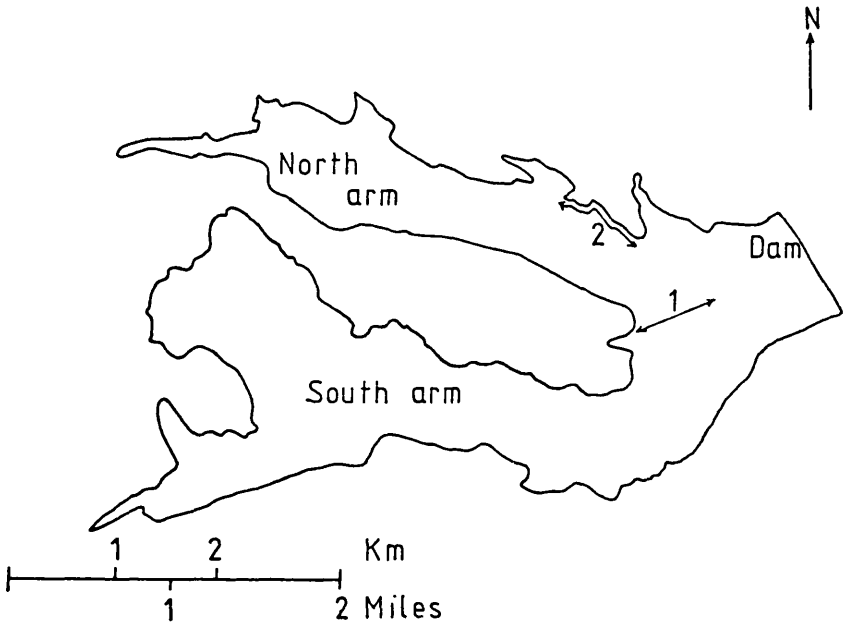


FIG. 4.1
Diagrammatic representation of Rutland Reservoir, showing sampling sites.
1) Penninsula (Dredge samples)
2) North arm (Edge samples)

SORTING AND MAINTENANCE OF CULTURES

All samples were initially sorted within one week of collection to give stock cultures of both species. These were maintained in plastic tanks (325 cm x 225 cm x 205 cm), at a temperature of $18\pm1^{o}C$, and regularly topped up with unfiltered reservoir water. Dead magnolia leaves were given as a food source. These were stored dry and pre-soaked in unfiltered reservoir water to allow fungal colonisation since this has been shown to be an essential part of their nutritional value (Kostalos & Seymour 1976; Wiloughby & Sutcliffe 1976).

Further sorting of the stock cultures was performed to obtain uniform groups of animals for experimental purposes. All juveniles from the initial sample were returned to stock culture. Gravid females of each species were isolated until all young had been released. The newly born amphipods were placed in stock culture and the females isolated for further sorting.

Mature adults of *G. pulex* were sorted by size and *C. pseudogracilis* by sex due to the marked sexual dimorphism in this species. Damaged animals and those showing visible signs of infection were rejected. The most common infections encountered in *G. pulex* were parasitic infections by acanthocephalans producing red tubules visible through the dorsal integument, and microsporidial infections producing white striations on the pleopodic segments. *C. pseudogracilis* did not appear susceptible to either infection.

Batches of 50 animals were maintained in plastic boxes (225 cm x 115 cm x 75 cm) in filtered (Whatman $N^{O}1$) reservoir water for at least one week prior to use. Temperature was kept constant at $18\pm1^{O}C$ and white fluorescent light supplied on a regime of 12 h light and 12 h dark. 'Bemax' was given daily as a food source. Cultures were inspected daily for dead or damaged individuals which were removed. Unused cultures were cleaned and re-established every 7 days.

PREPARATION OF ANIMALS FOR EXPERIMENTATION

In preparation for short term tests animals were starved for a 24 hr period prior to the test. This is 6 h longer than the gut clearance time calculated by Moore (1975) for *G. pulex* at $15^{O}C$, and the period was therefore assumed adequate to ensure that complete gut clearance had been achieved. Mixed sex cultures of *C. pseudogracilis* were established from mature males and females with ripe ovaries, in order to obtain gravid females for which brood size and development time could be recorded.

SELECTION OF ZINC SALT

The selection of a suitable zinc salt for this study was dictated by three important criteria;

1) The anion had to be a common component of freshwater systems.

2) Zinc solutions had to be prepared quickly and easily in large amounts to avoid precipitation in short term tests.

3) The form in which zinc was to be administered in laboratory tests had to resemble as closely as possible the form in which it enters natural waters as a mine or smelter work pollutant.

Three zinc salts commonly used in toxicity studies of this type are $Zn(NO_3)_2$ (Jones 1937), $ZnCl_2$ (Rehwolt, Lasko, Shaw & Wirhowski 1973; Patrick Cairns & Scheier 1969; Biesinger & Christensen 1972), and $ZnSO_4.7H_2O$ (Thorp & Lake 1974; Wurtz & Bridges 1961; Cairns, Buikema, Heath, & Parker 1978).

Zinc chloride was initially chosen since chloride in the form of Cl^- is one of the major inorganic anions in water (APHA 1975). It was found, however, that the molarities of solutions of this salt were always substantially lower than the notional values. This was partially due to precipitation but mainly the result of this salts' highly deliquescent nature which made accurate weighing impossible (Appendix 1A).

Hydrated zinc sulphate was an acceptable zinc salt since SO_4^{2-} is widely distributed in nature and may be present in natural waters in concentrations ranging from a few to several thousand milligrams per litre. Further, mine drainage wastes may contribute high sulphate levels by virtue of pyrite oxidation (APHA 1975).

CHOICE OF DILUENT

All zinc solutions were prepared by addition of non-deliquescent zinc sulphate (Analar grade) to millipore filtered reservoir water. It has been argued that a standard dilution medium would be desirable for obtaining truly comparative data for the toxicity of metal pollutants to both macroinvertebrates and fish (Murphy 1980). However, unless the chemical and physical properties of the chosen standard closely resemble those of the natural water from which the animals were taken, the resultant metal solution may differ markedly in toxicity giving results with little field related value. Buikema, Niederlehner & Cairns (1982) approve the use of natural dilution water providing it has low or undetectable pollutant levels, and the test organism survives through acclimation and test periods with no signs of stress, discolouration or unusual behaviour.

Florence (1977) also stipulates the need to ensure that the speciation of metal in the test medium is similar to that in the field for meaningful correlations to be made. This is best achieved by using the same dilution water. Finally, by placing animals in a different physical and chemical environment, compensatory stress may be induced in addition to the toxic effects of the metal pollutant.

The dilution medium was prepared by pumping reservoir water from a 40 litre sartorius stainless steel pressure vessel through a sartorius filtration unit loaded initially with a prefilter (type AP15) to remove gross particulate matter, and subsequently a 0.45µm millipore (type HAWP) to remove bacteria and remaining suspended materials (Steinnes 1982; Ramamoorthy & Kushner 1975).

PREPARATION OF ZINC SOLUTIONS

Preliminary experimentation indicated that at zinc sulphate concentrations > 1 mM a white flocculent precipitate formed which, while not subjected to analysis, was thought to be a basic zinc salt. Its slow rate of formation rendered attempts to remove it by filtration unsuccessful, with the gradual reappearance of more precipitate in the filtered solution. Redissolution could be achieved by addition of hydrochloric acid but the resultant pH was less than 5.7 and therefore unacceptable to gammarid crustaceans (Gledhill, Sutcliffe & Williams 1976), although this approach has been used in toxicity studies on the isopod *Asellus aquaticus* (Fraser, Parkin & Verspoor 1978).

In tests of greater than 5 h duration the following procedure was therefore used to prepare accurate zinc solutions. Quantities of zinc sulphate in excess of 1.0 g were weighed out on a sartorius balance with an accuracy of $\pm$ 0.1 mg, and added to 100 cm^3 distilled water. After vigorous shaking to obtain complete dissolution subsequent serial dilutions in 100 cm^3 distilled water were performed. 10 cm^3 aliquots were taken from the resultant solutions and made up to 5 litres of the required zinc concentration with filtered reservoir water.

After each zinc solution was prepared it was transferred immediately to a 5 litre aspirator and shaken vigorously daily for 3 days to enhance the rate of precipitate formation. After this time any precipitate formed was allowed to settle out for at least 1 week. Zinc solution was then drawn off as required from above the precipitate and notional concentrations above 0.1 mM were spectrophotometrically analysed (Varian Techtron AA-6) before use. Any samples containing precipitate which had been accidentally drawn off were discarded. The pH of each test solution was measured to ensure that it was greater than the required minimum indicated above for gammarid crustaceans (Appendix 1B).

Zinc solutions for use in tests of less than 5 h duration were prepared immediately before the test (by the serial dilution method described above) and used before precipitation occurred.

EXPERIMENTAL CONDITIONS

Acclimation of test animals and all subsequent toxicity tests were performed at a constant temperature of $18 \pm 1^{o}C$, The mean temperature was therefore within the range of $16\text{-}18^{o}C$, specified by the APHA (1975) for amphipods in general. White fluorescent light was supplied on a daily cycle of 12 h dark and 12 h light. This permitted short daily tests of less than 12 h duration in full light, to be performed alongside tests of longer duration requiring a fluctuating light source.

Polythene containers were used to hold stock zinc solutions, since it was shown by Florence (1977) that samples stored in polythene containers for over 3 weeks at $25^{o}C$ showed little change in either total metal concentration or metal species. Where possible apparatus was constructed of plastic or perspex, and glass containers were used only in short term tests of less than 8 h duration. Before each test all apparatus was thoroughly washed, rinsed in dilute hydrochloric acid and distilled water and dried in a heated cabinet.

CHAPTER 5.

ACUTE TOXICITY TESTING

INTRODUCTION

The acute toxicity test has received justifiable criticism for its use as a method of determining 'safe' levels for sustained input of toxicants into natural waters, because of its short duration and consequent severe criterion of toxicity (Martin 1973; Murphy 1980). In addition no insight is gained into the actual mode of action of the toxicant (Sindermann 1979).

In spite of these restrictions an acute test forms an important precursor to a more comprehensive chronic toxicity study since it is quick, reproducible, and defines the upper concentration limit for subsequent long term tests (LaRoche *et al.* 1973). Thus it is a vital starting point for a more comprehensive analysis if, like *C.pseudogracilis*, the test species is new to toxicant studies.

Furthermore, whilst an acute test alone provides little more than a comparative measure of toxicity, when performed in conjunction with chronic tests it becomes possible to determine the concentration difference between that which can be temporarily tolerated, and that which will eventually prove fatal. Thus, if two species have a common chronic threshold they will be equally susceptible to sustained toxicant input, but the species with the higher acute tolerance may be less affected by short intermittent toxic discharges. The acute toxicity test is therefore more accurately defined exclusively as a measure of short term tolerance.

Four distinct methods for performing acute toxicity tests have been distinguished by Hunter (1949: pages 113-114); "The first type of toxicity standard is based on the average time required to kill the organism when it is kept continuously in a solution having a definite concentration

of the metal. The second is based on the average concentration of the poison required to kill the organism when it is only immersed for a definite period of time in the poison solution and then returned to its normal non-toxic environment. A third, which combines these two to some extent, is the toxicity assessment based on the average number of animals surviving a definite period of contact with a definite concentration of poison. Finally, there is the commonly used assessment - median lethal concentration - the concentration required to kill 50% of the animals within a definite period of time."

The first method outlined above requires constant surveillance of the test animals and is consequently prone to error if exact measurement is not possible. The second approach has been used by Abel (1980) in a study of the effects of γ-hexachlorocyclohexane (lindane) on *G. pulex*. The rationale behind this approach is that the duration of exposure to a poison required to cause death is frequently less than the length of time taken to die. By removing the animal from the toxicant before death occurs, both the effect of poison concentration and duration of exposure can be observed independently. The last approach outlined above was chosen for this study. It is quick and easy to perform and is consequently the most commonly used of the four methods outlined above. This is advantageous when making comparisons with toxicity literature on other macro-invertebrates.

MATERIALS AND METHODS

Apparatus design and construction

The apparatus was designed with the aim of maintaining a number of animals in a fully oxygenated, uniform zinc concentration whilst ensuring sufficient individual isolation to prevent aggressive intra-specific interactions.

Each unit comprised a perspex base with a detachable compartmentalised chamber, and a plastic lid to reduce evaporation. The composition of a single unit is illustrated diagrammatically in Fig. 5.1a-d. The principle of construction was to maintain complete oxygen saturation of the medium within the chamber by supporting it on a cushion of air within the base.

The compartmentalised chambers were constructed from two 100 mm^2 repli-dishes (Gallenkamp), sealed about their top outside edges to form twenty five retangular compartments. A 1 mm wide gap between the internal divisions of each half of the chamber permitted the medium to flow throughout the chamber, whilst restricting each animal to a single, 14 cm^3 compartment (Fig. 5.1d). Each compartment had a central 7 mm diameter hole in the top for insertion and removal of animals, and a perforated bottom (Fig. 5.1b).

The base was a square open top perspex box of total depth 25 mm. A lightly greased cylindrical rubber seal partially inset into the sides of the base 2 mm below the top edge, held the chamber bottom tightly against a supportive lip 5 mm below the seal. Two hollow 30 mm long plastic pipes of internal diameter 3.5 mm inserted centrally into opposite sides of the base were inlet and outlet for pumped air (Fig. 5.1a). A complete assembled unit is shown individually in Fig 5.2a, and the experimental layout of ten identical units, supplied from a common Rena 301 air pump in Fig. 5.2b.

The compartmentalised chamber of each unit (350 cm^3) was 70% filled with the test medium through the top holes. When a balance point had been established for each unit by adjustment of air flow through the base, any unit could be removed at the inlet, and later re-introduced into the system without affecting the other units, provided the air supply to that unit was not adjusted. Test solutions were changed as necessary by fully opening the outlet to release the air cushion, causing the medium within the chamber to drain into the base. On closing the outlet, the air cushion was re-established and the chamber quickly refilled from above.

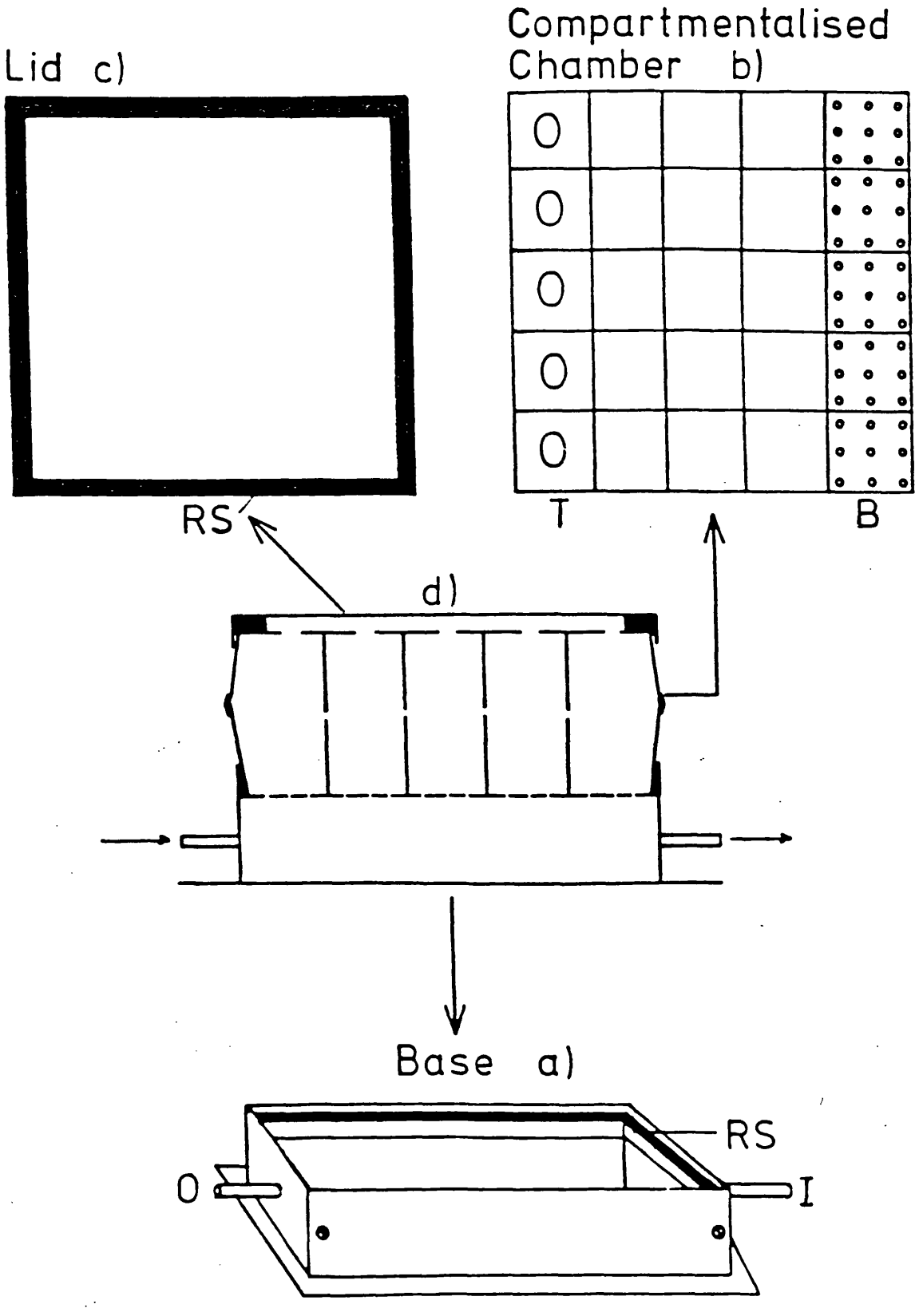
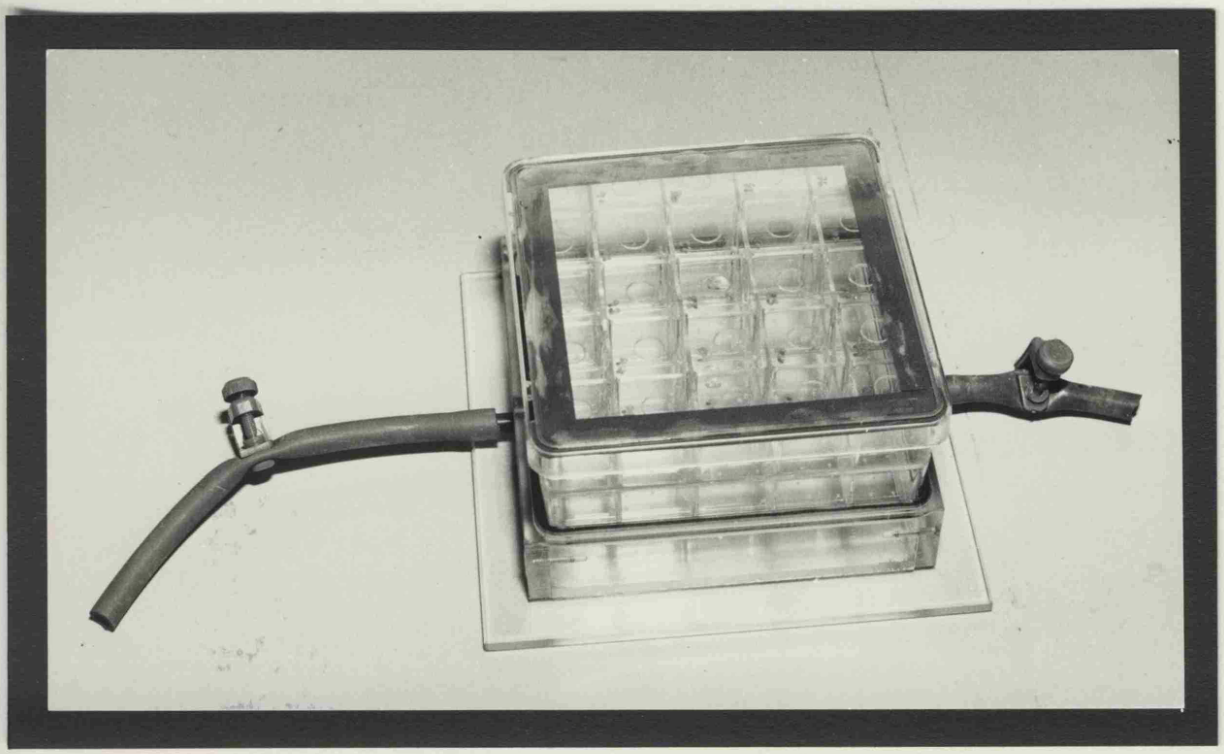


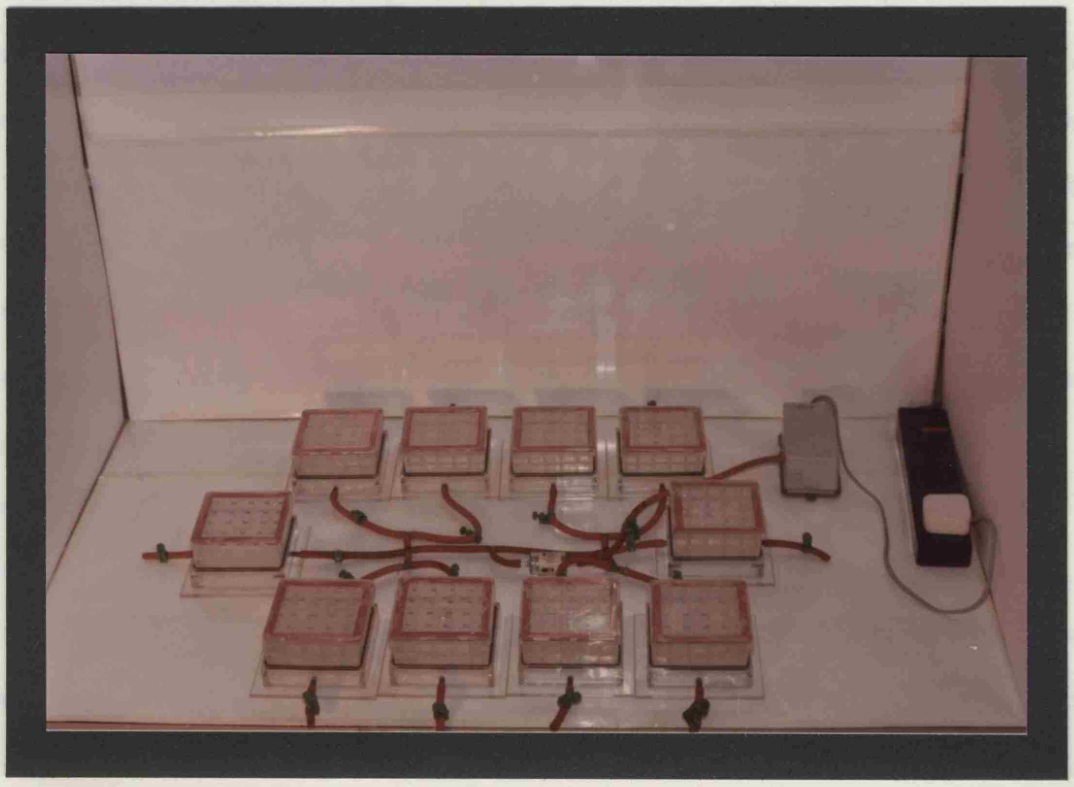
FIG. 5.1

Diagrammatic representation of a single unit for acute toxicity testing.

a) perspex base (O=outlet, I=inlet, RS=rubber seal)
b) Compartmentalised chamber showing top (T) and bottom (B) surfaces
c) Plastic lid with rubber seal (RS)
d) Cross section through an assembled unit



a)



b)

FIG. 5.2
Toxicity testing apparatus. a)Single assembled unit
b)Ten units in operation from a common air supply

Experimental methods

1) *Exploratory tests*

A series of exploratory tests were conducted with the dual purpose of defining the critical zinc concentration range for each species, and measuring the actual concentration of the experimental solutions throughout the test period.

Groups of 10-20 animals of each species were subjected to zinc concentrations in a range from 1 M to 1 µM. From these exploratory tests, a notional concentration range of 50 mM to 0.1 mM for *C. pseudogracilis* (♀), 20 mM to 0.1 mM for *C. pseudogracilis* (♂), and 100 µM to 1 µM for *G. pulex* (5-10mm long) were selected.

Samples of zinc solution were taken from the experimental chambers prior to the start of the exploratory tests, and at subsequent 24 h time intervals. These were analysed by Atomic Adsorption Spectrophotometry to determine the actual initial zinc concentration, and detect any decrease over the test period. If the average concentration of any zinc solution over the 96 h duration of the test differed from the initial concentration by greater than 5%, it was changed daily in further exploratory tests and re-analysed to ensure that the required error limit would not be exceeded during subsequent testing. (Results of the analysis are given in Appendix 1C).

2) *LC50 determination*

100 animals (4 replicate experimental chambers) from each group were tested in 8-10 zinc concentrations over the previously determined critical range. 100 *G. pulex* and 325 *C. pseudogracilis* of each sex, were tested in a zinc free control. The larger number of the latter species was due to the ready availability of suitable individuals at the time of the tests.

Each animal was rapidly surface dried on absorbent paper before introduction into the test chamber to prevent dilution of the zinc solution.

Whilst this was not thought to cause any mechanical damage to the animals some of the smaller individuals had to be pushed gently under the surface of the test medium to counteract the surface tension. Dead animals and exuviae were recorded at 24 h intervals for 96 h, and were then removed from the chamber. Death was assumed in the absence of antennal or pleopodal movement after gentle prodding for 1 min. No animal surviving the test was re-used.

RESULTS

The raw data consists of a series of test concentrations with corresponding mortalities at 24, 48, 72 & 96 h time intervals (Table 5.1). Plotting these data in an unmodified state would result in a sigmoid dose-response curve. Therefore, in order to accurately determine an LC50 value it is necessary to statistically transform this curve into a straight line.

Several methods to achieve this transformation are reviewed by Armitage & Allen (1950), including a simple calculative method described by Litchfield & Wilcoxon (1948). The approach chosen for this study was a probit transformation (Finney 1971). Whilst this method is procedurally the most complex, it is more accurate than graphical methods and is therefore the most commonly used parametric technique. A computerised probit analysis was carried out on a Cyber 77 mainframe computer, using a modification of a fortran program in Davies (1971), (Appendix 1$).

Percentage mortality at each concentration was first corrected for control mortality using Abbots formula. Control mortality was never greater than 3%.

CRANGONYX PSEUDOGRACILIS

Zinc concentration (mM)	Total number of dead individuals out of 100							
	24 h		48 h		72 h		96 h	
	F	M	F	M	F	M	F	M
46.708	95		100					
18.425	34	100	94		100			
8.691	20	60	71	94	93	99	98	100
6.074	19	33	56	94	80	98	90	100
3.974	16	23	42	85	66	96	89	100
3.026	4	14	29	51	47	75	71	89
2.081	2	5	14	42	34	65	46	77
0.998	1	4	10	18	17	38	28	59
0.454	0	0	2	1	4	12	5	31
0.097	0	0	0	0	1	0	1	1
* 0.000	1	1	1	1	3	1	4	4

GAMMARUS PULEX

Zinc concentration (µM)	Total number of dead individuals out of 100			
	24 h	48 h	72 h	96 h
500	100			
100	90	99	99	100
75	97	99	100	
60	42	84	98	99
50	32	80	98	100
25	14	46	67	86
20	5	13	36	52
15	2	9	27	44
10	3	7	18	29
5	1	2	4	12
1	0	1	3	4
0	0	0	1	1

TABLE 5.1

Total number of dead individuals in each zinc concentration after 24, 48, 72 & 96 h time intervals.

F = *C. pseudogracilis* (♀)

M = *C. pseudogracilis* (♂)

* Total number of control individuals = 325.

Weighted regression lines of probit - transformed percentage mortality against log concentration were plotted for both species at each time interval (Fig. 5.3a-c). The log of the LC50 value corresponding to a probit value of 5 (= 50% mortality), is show on each graph by a broken line.

In order to make a direct comparison between the LC50 values for the two species they have been plotted as a function of time on a vertical scale of zinc concentration (Fig. 5.4), and tabulated with fiducial limits at 90, 95 and 99% confidence levels (Table 5.2). As the slope of the line increases there is a tendancy for the fiducial limits to decrease but they are also affected by the number of test animals, responses between 0-100% mortality, distance between the LC50 value, and the mean test concentration. According to Buikema, Niederlehner & Cairns (1982) the width of the fiducial limits is least if the response slope is steep, if there are 3 or more deaths, if more than 10 organisms are exposed at each concentration and if there is a minimum of three replicates. All these conditions have been satisfied in this analysis.

In both species the LC50 value decreased with increasing time of exposure, but the tests were of insufficient duration for a lower asymptote to be reached, when increasing exposure time produces no further mortality. Both sexes of *C. pseudogracilis* had much higher LC50 values than *G. pulex*. A marked tolerance difference was found between the sexes of *C. pseudogracilis* which was greatest at 24 h, when the female LC50 value was nearly three times that of the male. At 48 h it had fallen to approximately twice the male value, a difference which was maintained for the duration of the test.

FIG. 5.3a-c (Over page)
Plot of probit values against zinc concentration for test durations of 24, 48, 72 and 96 h. The best fit regression line is indicated with a solid line. (Note difference in scales for the two species)

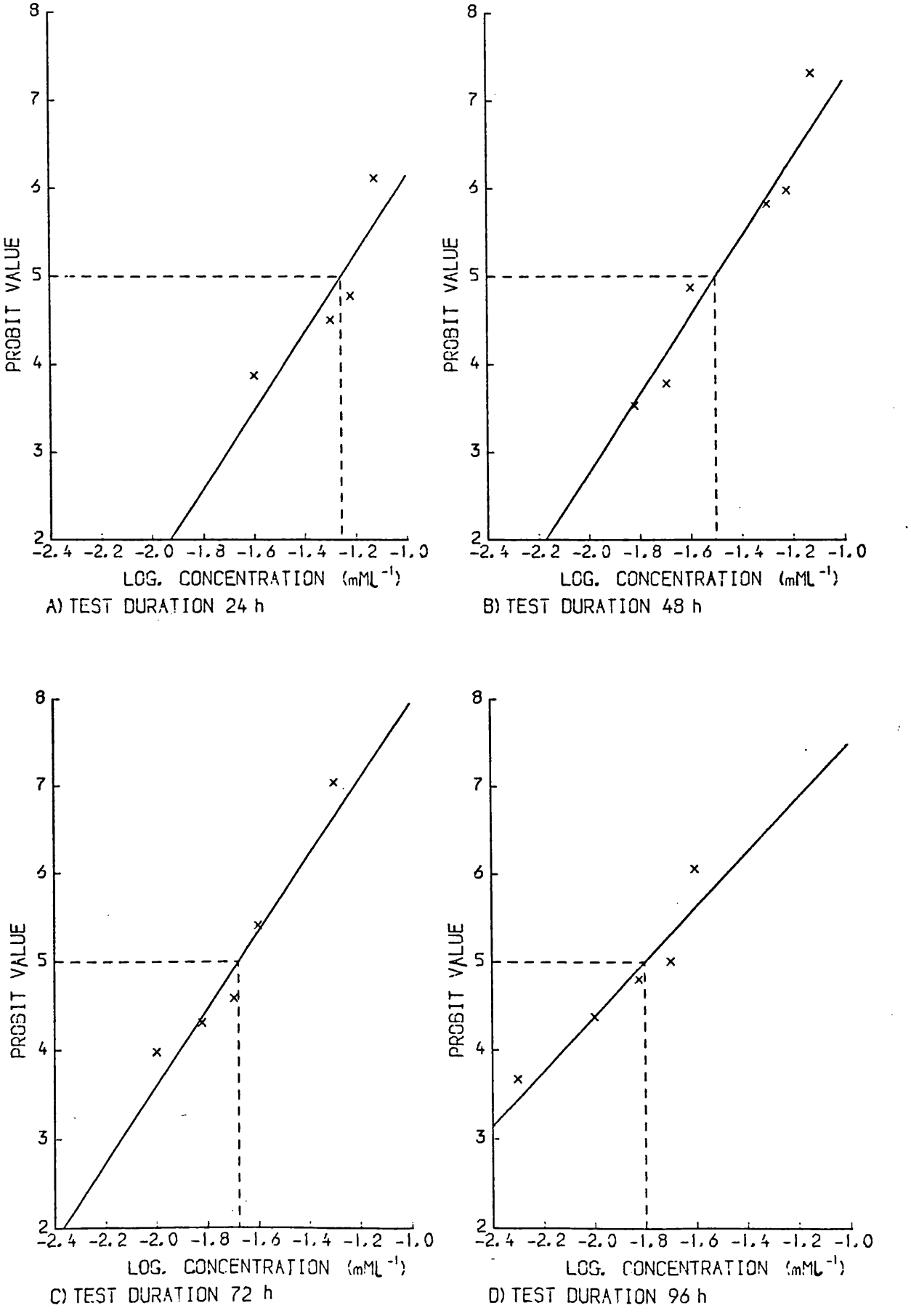


FIG. 5.3a - *Gammarus pulex*

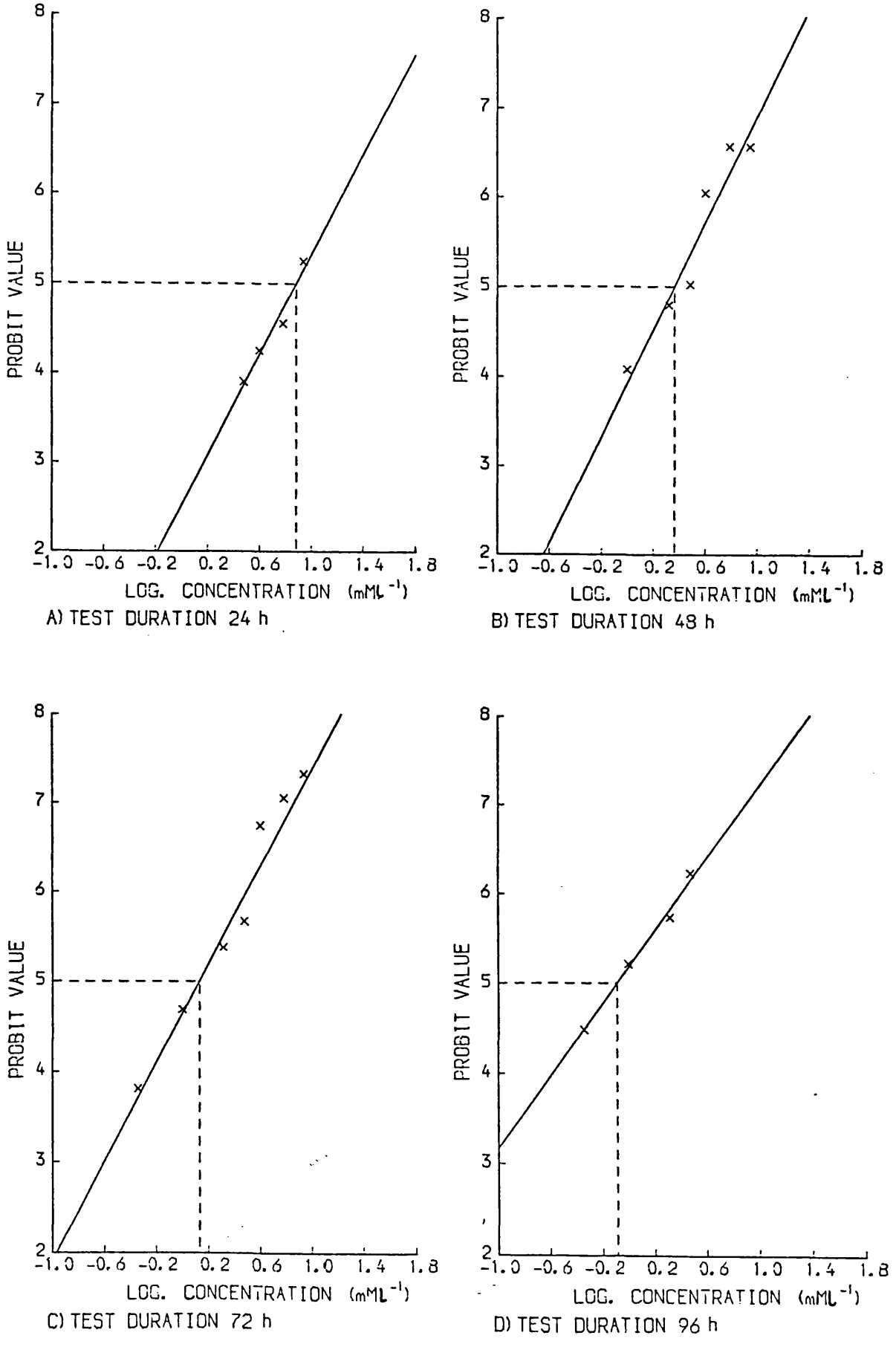
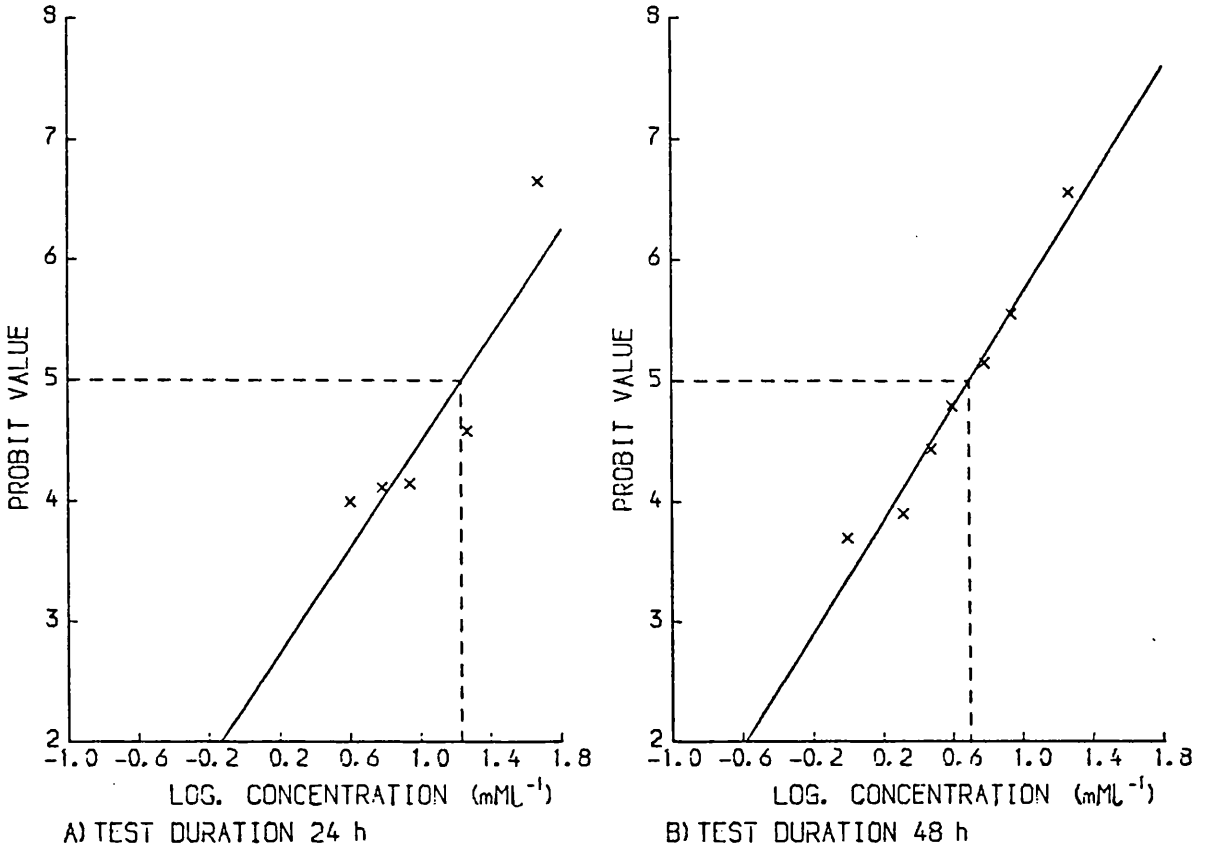


FIG. 5.3b - *Crangonyx pseudogracilis* (♂)



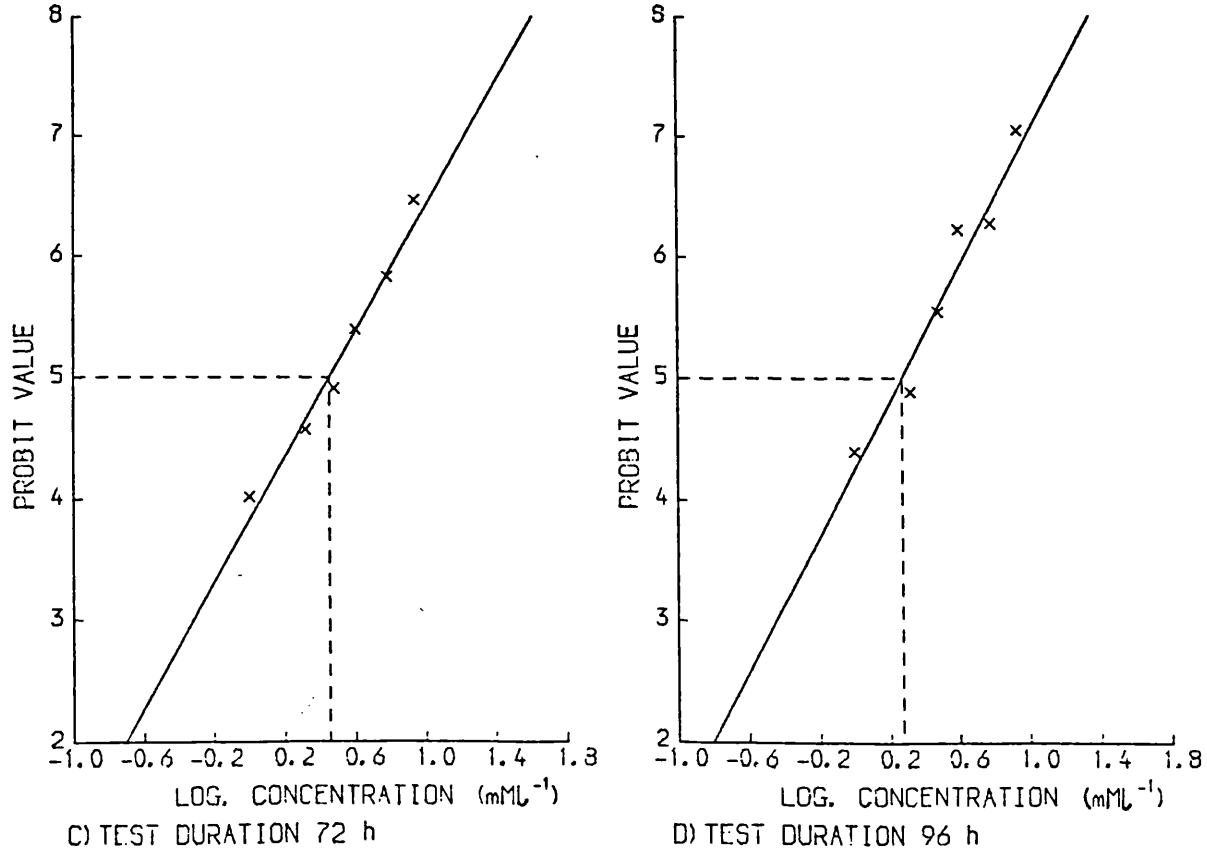


FIG. 5.3c - *Crangonyx pesudogracilis* (♀)

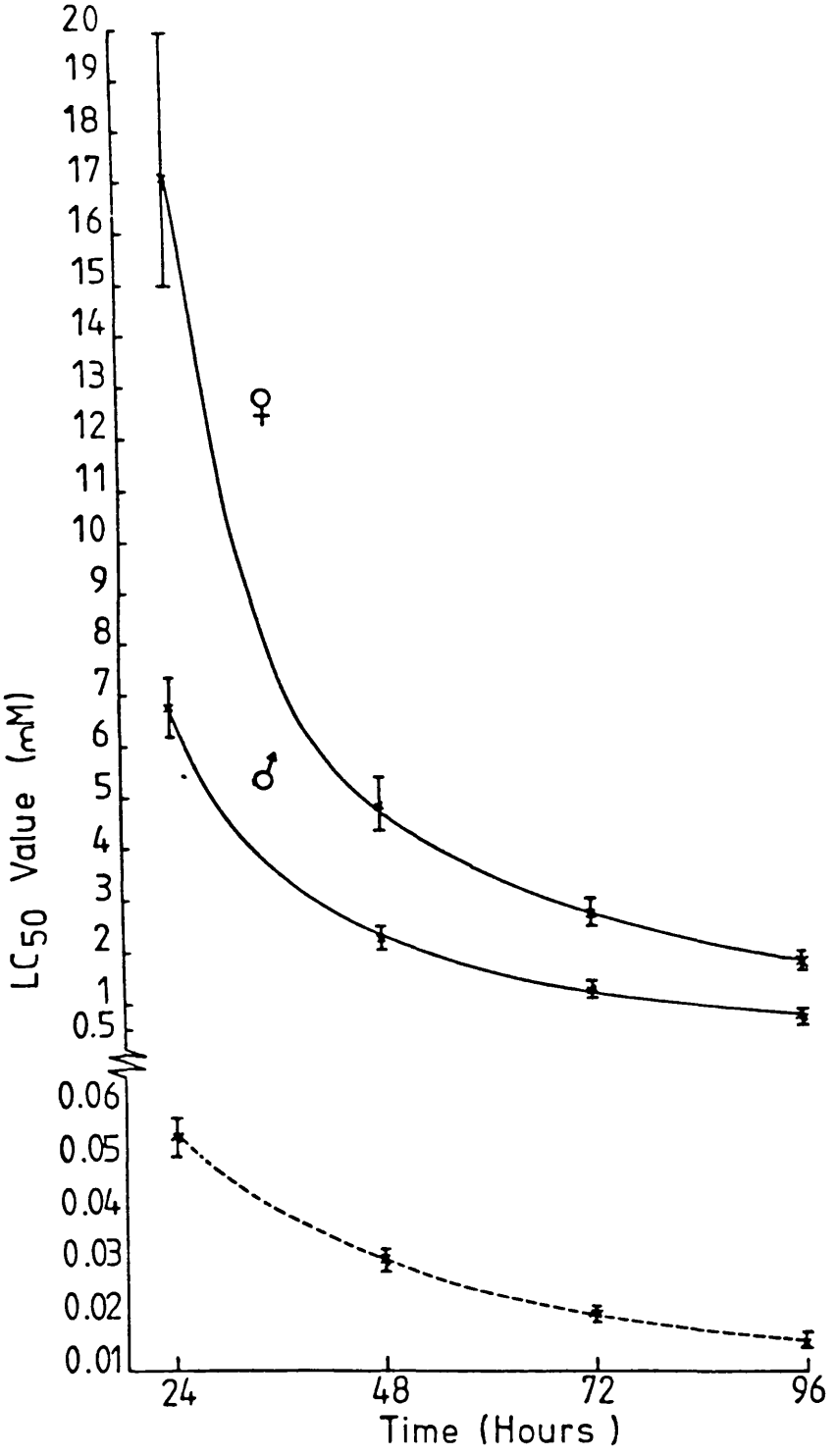


FIG. 5.4

The relationship between LC50 value and Time for *G. pulex* (-----), and *C. pseudogracilis* (———). Values are given with 95% confidence limits, and curves fitted by eye. The vertical scale is broken to allow presentation of both species on the same graph.

SPECIES	TIME (h)	SLOPE	INTERCEPT	MEDIAN LETHAL DOSE WITH FIDUCIAL LIMITS						
				LC50 ESTIMATE (mML^{-1})	0.90		0.95		0.99	
					U	L	U	L	U	L
G. PULEX	24	4.452	10.600	0.055	0.058	0.052	0.059	0.051	0.050	0.060
	48	4.461	11.697	0.032	0.033	0.030	0.034	0.029	0.034	0.029
	72	4.348	12.298	0.021	0.022	0.020	0.023	0.019	0.023	0.019
	96	3.104	10.584	0.016	0.017	0.015	0.018	0.014	0.018	0.014
C. PSEUDOGRACILIS ♂	24	2.805	2.522	7.646	8.885	6.858	9.221	6.734	10.037	6.509
	48	2.986	3.922	2.296	2.487	2.104	2.524	2.066	2.597	1.991
	72	2.730	4.647	1.347	1.475	1.220	1.500	1.196	1.549	1.148
	96	2.013	5.178	0.815	0.937	0.691	0.960	0.666	1.008	0.616
C. PSEUDOGRACILIS ♀	24	2.196	2.292	17.106	19.445	15.234	19.971	14.912	21.098	14.307
	48	2.351	3.365	4.962	5.431	4.564	5.530	4.471	5.733	4.327
	72	2.590	3.825	2.841	3.093	2.596	3.144	2.549	3.245	2.456
	96	2.783	4.244	1.869	2.051	1.679	2.086	1.641	2.154	1.566

TABLE 5.2

LC50 estimates at 24, 48, 72 & 96 h intervals with upper and lower fiducial limits for 0.90, 0.95 & 0.99 confidence levels.

Behavioural effects of zinc.

In control conditions both species settled quickly on the bottom or sides of the compartment and very little activity was observed throughout the test period. Within the higher test zinc concentrations *G. pulex* and females of *C. pseudogracilis* were hyperactive for about 30 min after introduction into the chamber. A similar initial hyperactivity was observed in male *C. pseudogracilis* which resulted in several animals breaking the surface and becoming trapped in the surface tension. This effect was enhanced by a tendency to crawl out of the toxicant solution and cling to the top of the compartment within only a thin film of moisture.

Moulting

A comparison was made between mortality in moulted and non-moulted animals of *G. pulex* and *C. pseudogracilis* over their 96 h critical zinc concentration ranges (Fig. 5.5).

G. pulex was found to show a much greater tendency to moult under both test and control conditions, than either sex of *C. pseudogracilis*. In the lowest test concentration of 1 μM moulting frequency in *G. pulex* was significantly higher than in the control (χ^2 =4.50; p<0.05), but survival was not appreciably impaired. With increasing zinc concentraton a decline in Ormoulting frequency was accompanied by a higher mortality rate amongst those animals which did moult. In a 20 μM solution 43% of animals survived the test, but all moulted animals (15% of the total), died. The lowest moulting frequency of 9% was obtained in a 50 μM solution where both moulted and unmoulted animals died within 96 h (Fig.5.5a).

C. pseudogracilis (♀) showed a very similar trend in the relationship between mortality and moulting over its 96 h critical zinc concentration range but the overall moulting frequency was much lower than for *G. pulex* (Fig.5.5b). *C. pseudogracilis* (♂) moulted very infrequently under test conditions, and not at all in the control (Fig.5.5c).

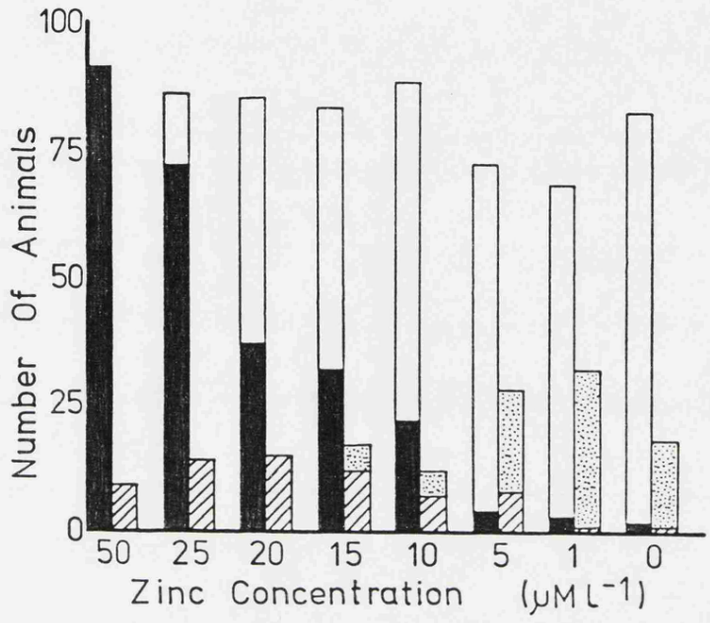


FIG. 5.5a - *Gammarus pulex*

FIG. 5.5a-c

A comparison between mortality in moulted and non-moulted animals after 96 h within zinc solution and in a zinc free control.

- Not moulting + surviving
- Not moulting + dying
- Moulting + surviving
- Moulting + dying

Zinc concentrations in which 100% mortality occurred in less than 96 h have been omitted.

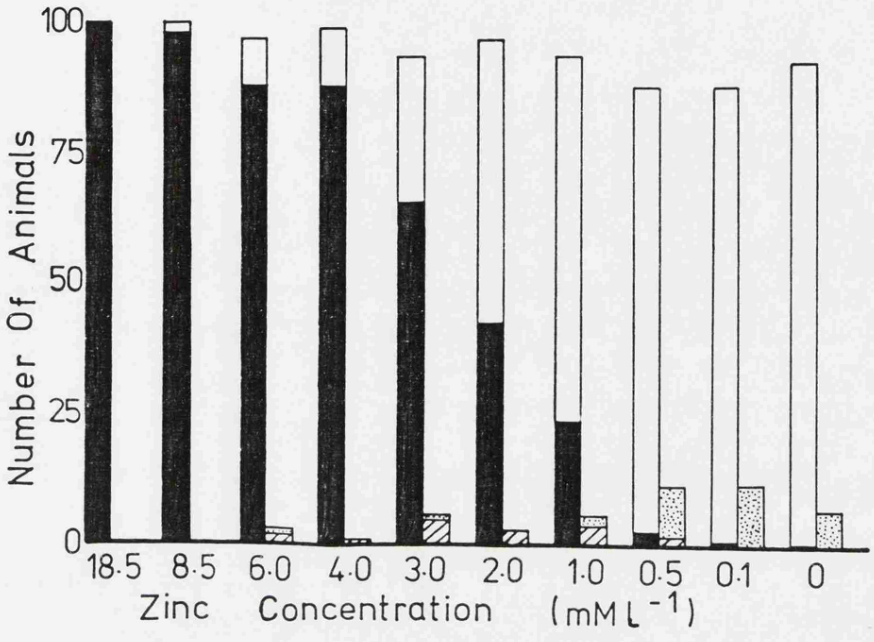


FIG. 5.5b - *Crangonyx pseudogracilis* (♀)

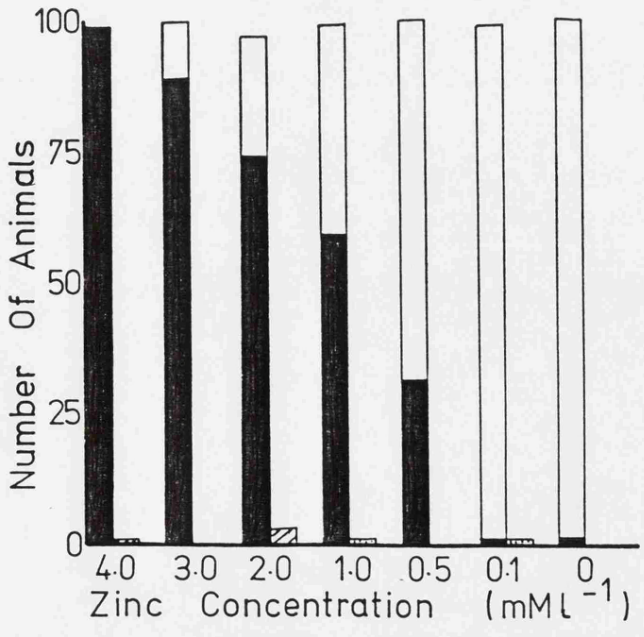


FIG. 5.5c - *Crangonyx pseudogracilis* (♂)

DISCUSSION

Experimental method

Static acute toxicity tests in which groups of animals are exposed to a toxic solution in non-adsorbent containers are economic in that relatively small volumes of dilution water are required, and the design is inexpensive in construction and easy to operate. Despite these advantages this approach has often been rejected in favour of more complex and costly continuous flow tests, since the concentration of static test solutions may be significantly altered by adsorption, micro-organism activity, and volatilisation, the last process being further enhanced by the requirement for constant aeration within the test chamber. In addition unremoved metabolic waste (CO_2 + ammonia) from the animals will become toxic if the test is prolonged (Martin 1973; Brungs 1973a).

These problems can mostly be overcome by regular changing of the test solution, but the associated handling of the animals is undesirable (Ahsannsullah 1976). The static test apparatus used in this study was inexpensive and easy to operate whilst eliminating most of the disadvantages characteristically associated with this type of test. Since oxygenation of the medium within the chamber was by forced diffusion no air stone was required, decreasing zinc loss by adsorption and volatilization. In addition sampling of the medium throughout the chamber confirmed that aeration was uniform, with no localised mechanical disturbance. The test medium was changed without the necessity to stress the animals by handling or to remove them from the chamber, a facility essential to the maintenance of a constant test concentration.

A remaining disadvantage of the static system is that solids cannot be kept in suspension, thus contrasting the 'real environment' in which it is very likely that the toxicant will be a mixture of precipitated and ionic zinc. Mount (1966) using a continuous flow apparatus showed that when

precipitated zinc was kept in suspension its toxicity to minnows increased with pH instead of decreasing. However, in a toxicity study confined exclusively to dissolved forms of zinc, static testing is more appropriate. Subsequent studies on a solution suspension mixture would be useful to determine the contribution made by suspended zinc solid to overall toxicity.

The common observation made in macro-invertebrate toxicity experiments using starved animals is that aggressive and cannibalistic behaviour, especially towards weakened individuals, may increase mortality in both control and test media (Thorp & Lake 1974: Ahsannsullah op. cit.; Besch 1977; Price & Uglow 1979). In their natural environment small or weakened animals, eg. those moulting, can avoid predation by seeking refuge in the substratum. An artificial hide is easily supplied in laboratory tests but will increase surface area for metal adsorption, and unless identical in all containers will constitute a further variable (Murphy 1980). By individual compartmentalisation of animals within a common test medium deaths resulting from intra-specific interactions are eliminated. An additional advantage of this feature is that moulted animals can be identified without the need for a potentially toxic or stressful marking procedure.

Effects of zinc on behaviour.

A hypersensitive reaction during invertebrate toxicity testing, similar to that of male *C. pseudogracilis* was observed by Ferguson, Culley & Cotton (1965). Specimens of *Palaemonetes kadiakensis* (freshwater shrimp), in solutions of chlorinated hydrocarbon insecticides, jumped up the sides of the test vessel and became trapped in the surface tension. *C. pseudogracilis* may survive several days in only a thin film of water, however, (pers.obs.) and so this response, which would initially appear detrimental to the animal, has potential survival value from short term input of toxic substances, since it greatly reduces the amount of available metal for uptake.

Mortality and moulting in *G. pulex* and *C. pseudogracilis*

The marked acute tolerance difference to zinc between the sexes of *C. pseudogracilis* may be a direct consequence of their difference in size. Fraser, Parkin & Verspoor (1978) suggested that, since lead uptake in *Asellus aquaticus* probably involved adsorption through the gills in addition to direct ingestion, uptake would depend on surface area, whilst toxicity was a function of volume. Animals with a smaller volume to surface area ratio would therefore acquire a lethal dose faster than larger animals. Since zinc uptake is also thought to occur across the gill surface in *C. pseudogracilis* (Hardwick 3rd Year BSc Hons project 1981), this hypothesis would adequately explain the greater tolerance in the female in view of the much greater body size of this sex. In *G. pulex* the initial selection of test animals with respect to sex was random. The absence of a significant sexually related tolerance difference in similar sized individuals of this species is therefore not susceptible to test, but is indicated by the narrow 95% confidence limits of the mixed sexes 24-96 h LC50 values.

The initially high female tolerance of *C. pseudogracilis* followed by rapid mortality may be explained in terms of a short term detoxification mechanism which fails on continuous exposure to a highly toxic solution. Price & Uglow (1979) proposed that the decapod crustacean *Crangon crangon* (L.) had a cellular tolerance or detoxification mechanism which was initially highly efficient. Death was inferred to follow rapidly upon breakdown of this mechanism. Ahsannullah (1976) found that invertebrate mortality in cadmium solutions followed a similar pattern.

G. pulex and *C. pseudogracilis* (♂) showed a very similar rate of decrease of LC50 value with time, although the former species was over 100 times more susceptible to zinc toxicity. This requires explanation since *G. pulex* is a more robust, heavily cuticularised species than

C. pseudogracilis and might therefore be expected to have a higher tolerance. An immediately obvious distinction between the species under test conditions was the propensity of *G. pulex* to moult. Anderson (1948) has suggested three ways in which moulting in the presence of a toxic metal may make *Daphnia sp.* more susceptible than during the intermoult period. These factors may be of equal importance in amphipod crustaceans;

1) The new cuticle is more permeable and will allow substances to enter at a faster rate.

2) The animals size increases and therefore the uptake rate on a surface area to volume ratio will also increase.

3) The new integument may develop abnormally.

Lockwood & Inman (1973) demonstrated that at moult the permeability of the the cuticle of *Gammarus duebeni* to water increased by a factor of two. Lockwood & Andrews (1969), however, reported that there was no increase in the silver staining areas of *G. pulex* at moult, making it doubtful that areas other than the gills would become significantly permeable for uptake of zinc ions.

This increase in moulting frequency is probably due in some part to stress of captivity. Increased levels of moulting in control conditions were recorded by Ahsannullah (1976) for *Palaemon* sp. and Wright (1980) for *G. pulex*. However, since the selection of *G. pulex* with respect to stage in the normal moult cycle was assumed to be random, the significant increase in moulting frequency in a 1 µM zinc solution, above that in the control strongly suggests that at this sub-acute concentration, zinc is in some way stimulating the onset of moulting.

Ahsannullah (op. cit.) states that moulting in the presence of a toxicant has important ecological significance in that the moulted cuticle effectively releases metal to be recycled. In addition, the animal is

inactive at the time of moult and unless what he terms the 'hide response' is also activated the animal will become easy prey. Any possible stimulatory effect of zinc above this concentration was obscured by the reduced time of survival during which moulting could have occurred.

To summarise, there is clearly a higher level of mortality amongst moulted animals of *G. pulex* in zinc concentrations > 5 μM, and this is therefore contributing to its lower tolerance compared with *C. pseudogracilis*. It is obviously insufficient to explain the whole tolerance difference since mortality levels in non-moulted individuals of *G. pulex* was also much higher.

Walker (pers. comm.), in studies of gill parasites on amphipods from Rutland Water, observed that *G. pulex* characteristically carries several protozoan gill parasites, whilst the gill surfaces of *C. pseudogracilis* are clean. In addition, *G. pulex* is susceptible to both microsporidial and acanthocephalan infections which do not affect *C. pseudogracilis* (pers. obs.). It is therefore possible that parasitism reduces the resistance of *G. pulex* to zinc but further investigation is needed to clarify the importance of this effect. From the results and observations of this experiment it must therefore be concluded that *C. pseudogracilis* can restrict the entry of, tolerate, or detoxify ionic zinc at much higher concentrations than *G. pulex*.

Comparison with other invertebrate species.

The necessity for test conditions to have specific field relevance makes it difficult to make comparisons between the toxicity data for invertebrate species determined under different test conditions. With these constraints in mind a summary of available toxicity data for freshwater invertebrates is given in Table 5.3, where all tests were performed under static conditions and results presented as LC50 values.

TABLE 5.3 (Over page)

Summary of some toxicity data for freshwater invertebrates.

Abbreviations

ZINC SALTS	C	Zinc chloride ($ZnCl_2$)
	S	Zinc sulphate ($ZnSO_4.7H_2O$ - hydrated)
	SO	Zinc sulphate ($ZnSO_4$)
SPECIES	(A)	Crustacea, Amphipoda
	(CL)	Crustacea, Cladocera
	(CO)	Crustacea, Copepoda
	(D)	Insecta, Diptera
	(G)	Mollusca, Gastropoda
	(I)	Crustacea, Isopoda
	(M)	Crustacea, Malacostraca
	(N)	Annelida, Naididae
	(O)	Annelida, Aeolosomatidae
	(OD)	Insecta, Odonata
	(OS)	Crustacea. Ostracoda
	(OT)	Annelida, Tubificidae
	(R)	Aschelminthes, Rotifera
	(T)	Insecta, Trichoptera (species unspecified)
LIFE STAGE	L/N	Larval or nymphal forms
WATER	CM	Culture medium
	NW	Natural water
	SM	Synthetic medium
	TW	Tap water
TEST CONDITIONS	Al	Alkalinity (mgl^{-1} $CaCO_3$)
	FE	Fed during the test
	Hd	Hardness (mgl^{-1} $CaCO_3$)
	pH	pH units
	T(^oC)	Temperature

SALT	SPECIES	LIFE STAGE	WATER	T(°C)	Hd	Al	pH	TIME(h)	LC50 CONC(µM)	REFERENCE
S	Philodina acuticornis(R)		CM	5.0	45.0		7.5	48	5.39	Cairns et al. (1978)
S	P. acuticornis		CM	25.0	45.0		7.5	48	1.74	Cairns et al. (1978)
C	Physa heterostropha(G)		FE	30.0	171-180			96	8.21-22.12	Cairns & Scheier (1958)
C	P. heterostropha		FE	20.0	141-190			96	9.25-19.37	Cairns & Scheier (1958)
S	P. heterostropha	3-6mm		32.2	100.0		7.8	96	17.04	Wurtz (1962)
S	P. heterostropha	Adult		21.1	100.0		7.8	96	10.99	Wurtz & Bridges (1961)
S	P. heterostropha	3-6mm		10.6	100.0		7.8	96	6.68	Wurtz (1962)
S	P. heterostropha	3-6mm		32.2	20.0		7.3	96	5.39	Wurtz (1962)
S	P. heterostropha	3-6mm		10.6	20.0		7.3	96	4.46	Wurtz (1962)
C	P. heterostropha		FE	20.0	38-61			96	2.75-4.42	Cairns & Scheier (1958)
C	P. heterostropha			20.0	43.0			96	2.75-4.42	Patrick et al. (1969)
S	P. heterostropha	Adult		21.1	20.0		7.3	96	3.86	Wurtz & Bridges (1961)
C	P. heterostropha		FE	30.0	35-47			96	2.16-2.17	Cairns & Scheier (1958)
S	Helisoma campanulatum(G)	Adult		12.8	100.0		7.8	96	46.60	Wurtz (1962)
S	H. campanulatum	Adult		22.8	20.0		7.3	96	19.48	Wurtz (1962)
S	H. campanulatum			22.8	100.0		7.8	96	19.48	Wurtz (1962)
S	H. campanulatum	Adult		12.8	20.0		7.3	96	13.39	Wurtz (1962)
C	Amnicola sp.(G)	Eggs		17.0	50.0		7.6	24	97.73	Rehwoldt et al. (1973)
C	Amnicola sp.	Eggs		17.0	50.0		7.6	96	70.25	Rehwoldt et al. (1973)
C	Amnicola sp.	Adult		17.0	50.0		7.6	24	58.43	Rehwoldt et al. (1973)
C	Amnicola sp.	Adult		17.0	50.0		7.6	96	48.69	Rehwoldt et al. (1973)
S	Nitocris sp.(G)		TW	5.0	45.0		7.5	48	16.69	Cairns et al. (1978)
S	Aeolosoma headlyei(O)		TW	5.0	45.0		7.5	48	62.95	Cairns et al. (1978)
S	A. headlyei		TW	25.0	45.0		7.5	48	46.95	Cairns et al. (1978)
S	Limnodrilus hoffmeisteri(OT)		SM	21.1	100.0		7.8	96	8.0	Wurtz & Bridges (1961)
SO	L. hoffmeisteri/T. tubifex		SM	20.0			7.5	24	159.98	Whitely (1968)
S	Tubifex tubifex(OT)			20.0	261.0	234.0	7.3	48	209.36	Brkovic-Popovic & Popovic (1977a)
S	T. tubifex			20.0	34.2	7.5	6.8	48	10.36	Brkovic-Popovic & Popovic (1977a)
S	T. tubifex			20.0	34.2	22.5	7.2	48	8.94	Brkovic-Popovic & Popovic (1977a)
S	T. tubifex			20.0	0.1	0.1	6.3	48	0.38	Brkovic-Popovic & Popovic (1977a)
C	Nais sp.(N)			17.0	50.0		7.6	24	73.73	Rehwoldt et al. (1973)
C	Nais sp.			17.0	50.0		7.6	96	63.99	Rehwoldt et al. (1973)
S	Cyclops abyssorum(CO)	0.62mm	NW	10.0		29.0		48	19.13	Baudouin & Scoppa (1974)
S	Eudiaptomus padanus(CO)	0.43mm	NW	10.0		29.0		48	1.74	Baudouin & Scoppa (1974)
S	Daphnia hyalina(CL)	1.27mm	NW	10.0		29.0		48	0.14	Baudouin & Scoppa (1974)
C	Daphnia sp.			30.0	114.0	82.0	8.5	24	15.5	Qureshi et al. (1980)
C	Daphnia sp.			30.0	114.0	82.0	8.5	48	11.0	Qureshi et al. (1980)
S	Daphnia pulex(CL)		TW	5.0	45.0		7.5	48	5.56	Cairns et al. (1978)
S	D. pulex		TW	25.0	45.0		7.5	48	0.97	Cairns et al. (1978)
S	Daphnia magna(CL)		TW	5.0	45.0		7.5	48	8.00	Cairns et al. (1978)
S	D. magna		TW	25.0	45.0		7.5	48	1.95	Cairns et al. (1978)
C	D. magna	12+12h	FE	18.0	45.3			48	0.97	Biesinger & Christensen (1972)
C	D. magna	12+12h		18.0	45.3			48	0.35	Biesinger & Christensen (1972)
C	Cypris sp.(OS)			30.0	114.0	82.0	8.5	24	12.75	Qureshi et al. (1980)
C	Cypris sp.			30.0	114.0	82.0	8.5	48	10.4	Qureshi et al. (1980)
S	Paratya tasmaniensis(M)	22.4mm		15.0	10.0			96	4.17	Thorp & Lake (1974)
S	Asellus communis(I)			21.1	100.0		7.8	96	44.17	Wurtz & Bridges (1961)
S	A. commmunis			21.1	20.0		7.3	96	30.26	Wurtz & Bridges (1961)
C	Gammarus pulex(A)			17.0	50.0		7.6	24	35.47	Rehwoldt et al. (1973)
C	G. pulex			17.0	50.0		7.6	96	28.17	Rehwoldt et al. (1973)
S	G. pulex	Adult	NW	18.0	312.5		7.3	24	55.00	THIS STUDY
S	G. pulex	Adult	NW	18.0	312.5		7.3	48	32.00	THIS STUDY
S	G. pulex	Adult	NW	18.0	312.5		7.3	72	21.00	THIS STUDY
S	G. pulex	Adult	NW	18.0	312.5		7.3	96	16.00	THIS STUDY
S	Crangonyx pseudogracilis(A)	Adult(♂)	NW	18.0	312.5		7.2	24	7646.00	THIS STUDY
S	C. pseudogracilis	Adult(♂)	NW	18.0	312.5		7.3	48	2296.00	THIS STUDY
S	C. pseudogracilis	Adult(♂)	NW	18.0	312.5		7.3	72	1346.00	THIS STUDY
S	C. pseudogracilis	Adult(♂)	NW	18.0	312.5		7.3	96	815.00	THIS STUDY
S	C. pseudogracilis	Adult(♀)	NW	18.0	312.5		6.8	24	17106.00	THIS STUDY
S	C. pseudogracilis	Adult(♀)	NW	18.0	312.5		7.3	48	4962.00	THIS STUDY
S	C. pseudogracilis	Adult(♀)	NW	18.0	312.5		7.3	72	2841.00	THIS STUDY
S	C. pseudogracilis	Adult(♀)	NW	18.0	312.5		7.3	96	1869.00	THIS STUDY
C	Tricoptera(T)	L/N		17.0	50.0		7.6	24	217.71	Rehwoldt et al. (1973)
C	Tricoptera	L/N		17.0	50.0		7.6	96	202.06	Rehwoldt et al. (1973)
C	Zygoptera(OD)	L/N		17.0	50.0		7.6	24	111.29	Rehwoldt et al. (1973)
C	Zygoptera	L/N		17.0	50.0		7.6	96	91.12	Rehwoldt et al. (1973)
S	Argia sp.(OD)	L/N		21.1	20.0		7.3	96	141.55	Wurtz & Bridges (1961)
C	Chironomous sp.(D)	L/N		17.0	50.0		7.6	24	74.77	Rehwoldt et al. (1973)
C	Chironomous sp.	L/N		17.0	50.0		7.6	96	63.30	Rehwoldt et al. (1973)

TABLE 5.3
See preceeding page for legend and symbol explanation.

The most striking feature of this table is the wide range of reported LC50 values both between and within several invertebrate groups (eg. 48 h LC50 values of 209.36 µM & 0.14 µM for *Tubifex tubifex* and *Daphnia hyalina* respectively). Whilst this clearly emphasises the lack of a standard method for obtaining data, a definite trend relating mortality to test conditions can be distinguished. The LC50 value is mainly lowered by an increase in temperature (eg. the 48 h LC50 values for *Daphnia pulex* determined by Cairns *et al.* 1978), or a decrease in hardness and pH value (eg. the 96 h LC50 values for *Physa heterostropha* determined by Wurtz 1962). However, without a more comprehensive set of results for any one species the relative contribution of each of these factors towards overall toxicity is unclear. In general the Insecta appear to be the most zinc tolerant group and micro-crustacea the least tolerant, with the Isopoda and Amphipoda falling somewhere in between.

The results given for *G. pulex* by Rehwoldt *et al.* (1973) are close to those obtained in the present study although the hardness of the water used was about four times lower. It is also noticeable that the LC50 values obtained by Rehwoldt *et al.* (1973) for 24 h and 96 h do not show a high short term tolerance followed by rapid mortality, as reported earlier in this study. This suggests that any short term tolerance may be related to higher water hardness.

C. pseudogracilis has much higher 24-96 h LC50 values than any of the other species listed in the table, and unless this is purely an effect of the high water hardness it is exceptionally tolerant to zinc in comparison with other invertebrate species. Further tests on this species at water hardnesses between 20 mgl^{-1} ($CaCO_3$), and 100 mgl^{-1} ($CaCO_3$) are necessary to clarify the extent of this apparent anomaly.

CHAPTER 6.

CHRONIC REPRODUCTIVE IMPAIRMENT

INTRODUCTION

If a species is to survive in an environment where there is a sustained source of heavy metal pollution such as zinc, a high short term tolerance level is of little advantage if cumulative longer term stress prevents normal maturation and reproductive success. When testing toxicity it is consequently ideal to monitor the survival of the test species in sub-acute metal concentrations over at least 3-4 life cycles, in order to determine the maximum concentration having no deleterious effects on survival of the species.

The micro-crustacean genus *Daphnia* is ideally suited for this type of test since it has an average life span of only one month and is readily amenable to laboratory culture (Murphy 1980). Biesinger & Christensen (1972), in a study of the effects of various metals on survival, growth, reproduction and metabolism of *Daphnia magna*, showed that whilst 48 h and 3 week LC50 values ranged between 1.53-4.28 µM and 2.23-2.60 µM respectively, a lower zinc concentration of 1.07µM was sufficient to cause a 16% reduction in reproductive success.

The feasibility of such a comprehensive test schedule is therefore limited by the length and complexity of the life cycle of the test animal. If the lifespan exceeds 2-3 months it becomes necessary to superimpose seasonal effects over general test conditions (APHA 1975). Assuming that this can be reliably and accurately achieved a further consideration must be the length of time available for testing. In most cases where the general biology of the animal is known, this factor will be the limiting practical consideration.

Finally, if both sufficient time and facilities are available for the test, its potential use must be balanced against that of attempting several different techniques within the same time period.

If these principles are applied to the use of *G. pulex* and *C. pseudogracilis* as test animals, the disadvantage of performing complete life cycle studies are apparent. Both species have life spans in excess of one year (Hynes 1955a; Welton & Clarke 1980) thereby making it impractical to culture more than one or two generations in the time available for the study. Even then, without accurate reproduction of seasonal conditions, the test would lack any realistic field value. It was therefore concluded that a chronic test concentrating exclusively on the most vulnerable period in the life cycle would provide the best compromise between high test sensitivity and practical application.

Whilst tests which do not cover the whole life cycle cannot necessarily include toxic effects which may result from prolonged exposure to zinc, the most sensitive shorter term toxicity experiments are generally considered to be those which examine chronic effects on the reproductive cycle or on the juvenile stages of an organism (Sprague 1976; Sindermann 1979). This approach has consequently been favoured by most authors researching higher macro-invertebrates and fish. Jorgensen & Jensen (1977) examined the effects of zinc chloride on the hatching rate of dried *Artemia salina* eggs, and concluded that this was a much more sensitive measure of zinc pollution than adult mortality, since impairment was detectable at concentrations similar to those found in natural sea water.

The failure of isolated *Artemia* eggs to hatch at low zinc concentrations was presumably caused by damage to the embryo, but there is evidence to show that in another macro-invertebrate, *Amnicola sp.* (Gastropoda), the eggs are the most zinc resistant stage in the life cycle (Rehwoldt *et al.* 1973). This tolerance was attributed to a relative insensitivity of the eggs to water quality, as a result of their independent internal environment.

Another study of this type was performed by Skidmore (1965) on the resistance of the zebrafish (*Brachydanis rerio* Hamilton Buchanan), to zinc sulphate solution. He showed that the egg was the most resistant stage in the life history, although the newly hatched young were the most sensitive stage. The collective results of these studies strongly indicate that the sensitivity of the egg stage to zinc toxicity is largely dependent on the extent of their communication with the external environment.

In *G. pulex* and *C. pseudogracilis* the eggs are transferred after fertilization into the female brood pouch where the young are retained throughout the development period until hatching. Two consecutive test periods were therefore chosen for this chronic study. The time between fertilization of the egg and hatching of the young animal, was designated as TEST 1. This was to investigate the survival rates of ova and juveniles in the brood pouch at sub-acute zinc concentrations. TEST 2 was designed to measure the resistance of the newly hatched young over the first few days after release.

MATERIALS AND METHODS

Test Rationale

Gravid females of both species were kept in individual test chambers within a known zinc concentration for periods of up to 28 days. Brood size and developmental stage were recorded daily in order to obtain a normal record of development, from which any change resulting from exposure to sub-lethal zinc solutions could be detected. All successfully released young were subsequently transferred to chambers within a continuous-flow system at the same zinc concentration, and survival recorded at approximately four day intervals over a period of 21 days.

TEST 1 : Selection of test animals

This test was performed between the months of July and September when a sufficient supply of actively reproducing females of both species could be obtained from Rutland Water. The average number of eggs per female is known to increase with increasing size (Hynes 1955a), In order to reduce variability in initial brood size, only female *G. pulex* between 9-10 mm long and *C. pseudogracilis* between 7-8 mm long were tested, these being the most commonly encountered size classes over the test period for the respective species. There is no indication that in any one size group the number of eggs per female shows any seasonal variation (Hynes op. cit.).

Initial Test Procedure

The procedure for obtaining newly gravid females was dictated by pre-copulatory behaviour and therefore differed slightly between the two species.

In *G. pulex* there is a sustained period of pre-copulatory activity which begins with the male seizing the female between the head and first thoracic segment with one of his primary gnathopods, and between the fifth and sixth segment with the other. The phase ends after 3-4 days with ovulation and copulation. Immediately copulatory pairing was observed between suitably sized individuals they were transferred from secondary stock culture into individual test chambers containing control medium. These were subsequently checked twice daily and day 1 recorded as the release of the moulted female and resultant transfer of fertilized eggs to the brood pouch. The male was removed to prevent cannibalism of the female.

In *C.pseudogracilis* there is no precopulatory period as the male is too small (Hynes op. cit.). The female becomes attractive just before moulting and several males may jostle for position. The successful male lies across the female and holds on with his gnathopods between the third - fourth and fifth - sixth thoracic intersegmental membranes (Fig. 6.1).

FIG. 6.1
Female *C. pseudogracilis* in pre-moult condition, with attached male.

This position lasts for approximately 10 min, and the female lays the fertilized eggs about 4 h later. A culture containing mature males and females with ripe ovaries was established and checked twice daily for suitable sized gravid females with full brood pouches, which were then transferred to individual chambers and recorded as Day 1.

Apparatus

The test chambers were a modification of those used by Welton & Clarke (1980) (Fig. 6.2), consisting essentially of a plastic container 90 mm high with basal diameter 45 mm and top diameter 60 mm, and a plastic lid. A fine mesh platform was constructed by making a central hole of diameter 25 mm in the base of a 50 mm diameter plastic petri-dish base, and replacing it with monofilament mesh of hole size 1 mm x 1.5 mm. This platform fitted securely into the chamber 25 mm above the base, producing a lower compartment into which newly emerged juveniles could enter to escape cannibalism by the adult.

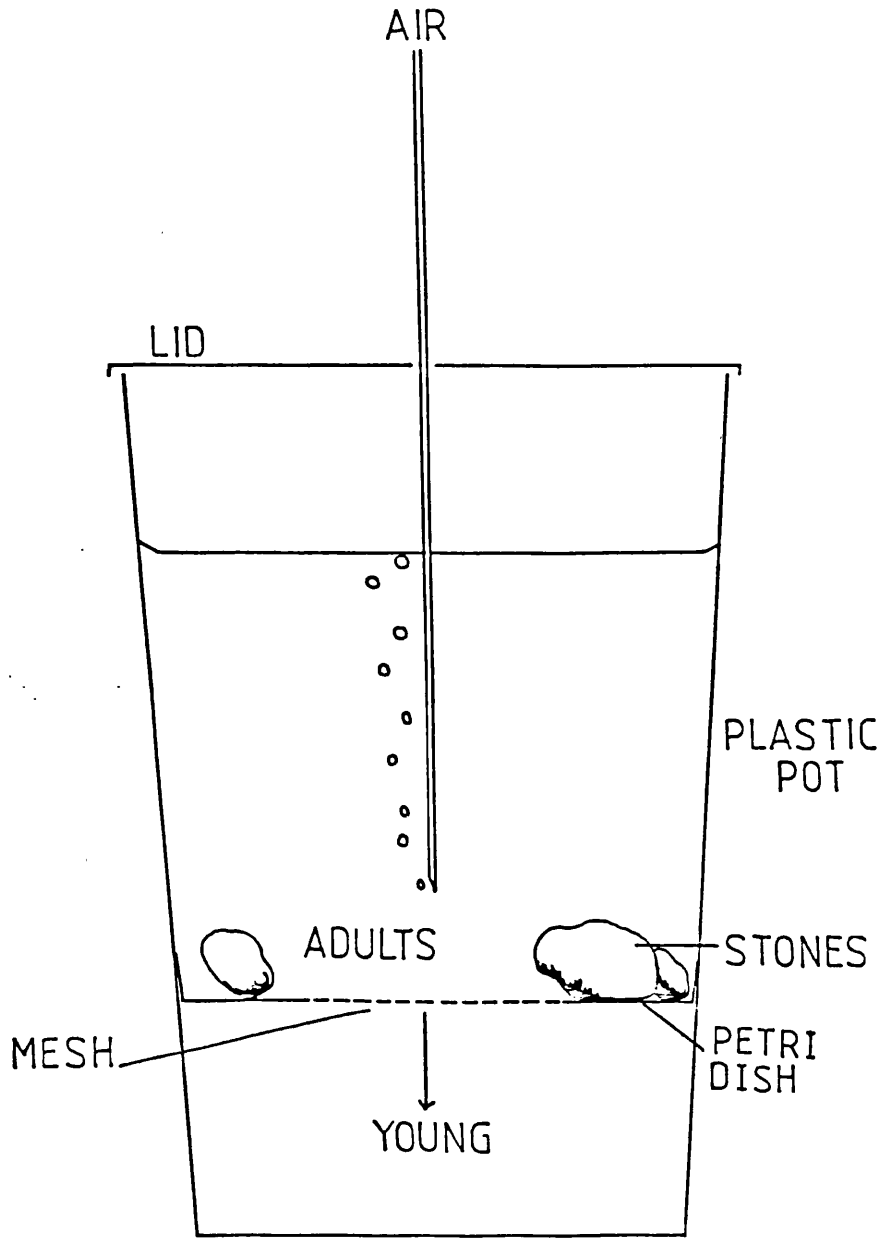


FIG. 6.2
Diagram of a single test chamber (not to scale).

Each chamber was filled with media to a height of 75 mm, and continuously aerated through stainless steel syringes, inserted centrally through the lid. Two washed stones from Rutland reservoir were placed in each pot to provide cover for the adult. The test medium within the chamber was changed every 2 days, and the female fed a small amount of Bemax (wheat germ).

A zinc concentration of 50.0 µM, approximately equal to the 24 h LC50 of G. pulex was initially chosen as the highest test concentration. Preliminary experimentation subsequently indicated that zinc concentrations of 1 mM, 0.5 mM & 1.0 mM were more suitable to investigate the effects of sub-acute toxicity. Ten different females of each species were tested at each zinc concentration, and in a control of untreated reservoir water (Fig. 6.3). Animals dying within the first 24 h of the test were replaced and not recorded, except at a zinc concentration of 50 µM where the expected mean survival time did not exceed 24 h.

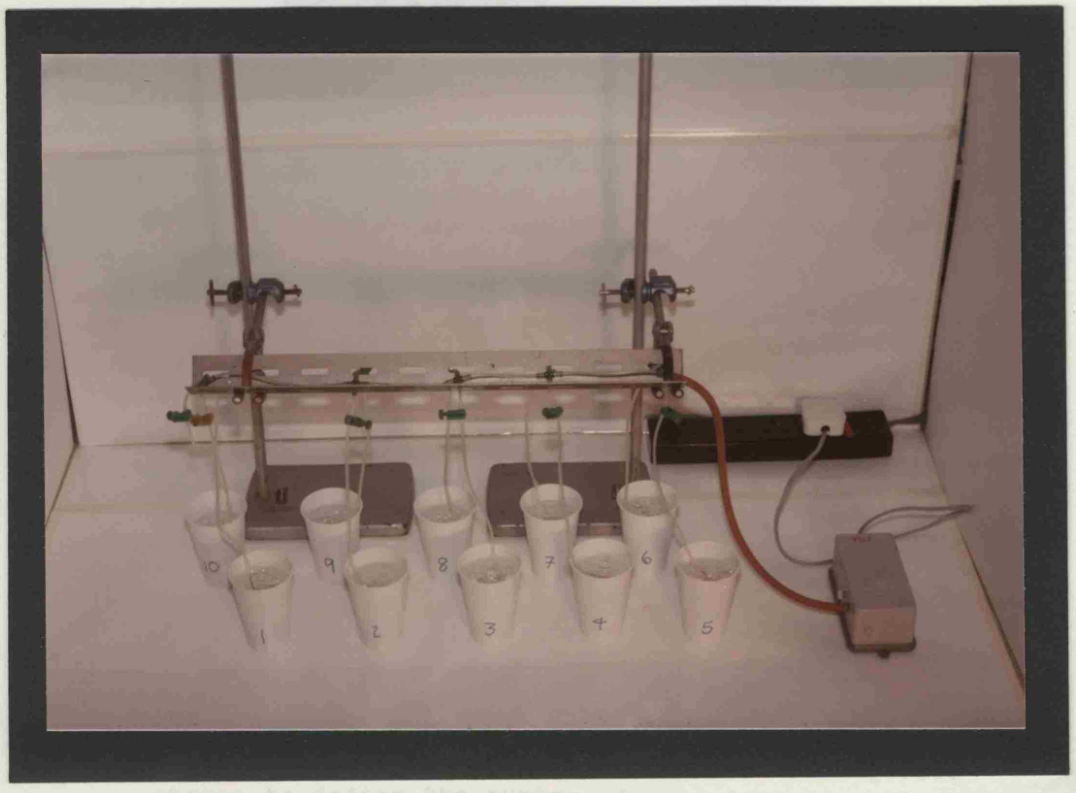


FIG. 6.3
Ten test chambers in operation from a common pumped air supply, (lids not shown).

Recording brood size

As it proved impossible to count the number of eggs within the brood pouch accurately without stressing the female, an approximate scoring method was used which did not require microscopic examination. On close visual examination the following three categories were readily defined (Fig. 6.4);

LARGE :Brood pouch totally full of eggs

MEDIUM :Eggs lost peripherally leaving a layer lining the body

SMALL :Few eggs present incompletely lining the body

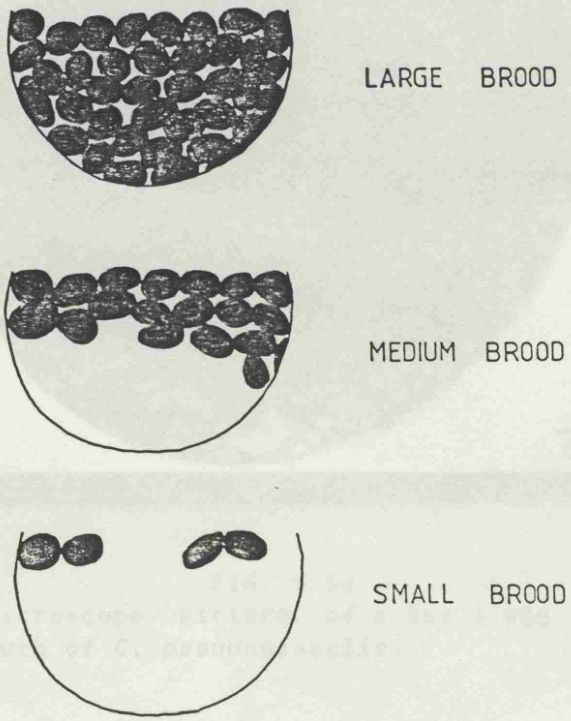


FIG. 6.4
Diagrammatic representation of the categories chosen to define the number of eggs (shown as black ovals) within the brood pouch (shown as a semi-circle).

Recording developmental state

Three obvious colour phases occur in both species, between fertilization of the egg and release of the young animal. Preliminary investigations showed that at $18\pm1^{o}C$ each phase could be closely identified with a distinct level of development.

From 1-9 days in *G.pulex*, and 1-5 days in *C.pseudogracilis* a normally developing egg appears black to the naked eye. This corresponds to the period when little or no cleavage of the embryo has occurred and is illustrated with a light microscope picture of a day 1 egg from *C.pseudogracilis* (Fig. 6.5a).

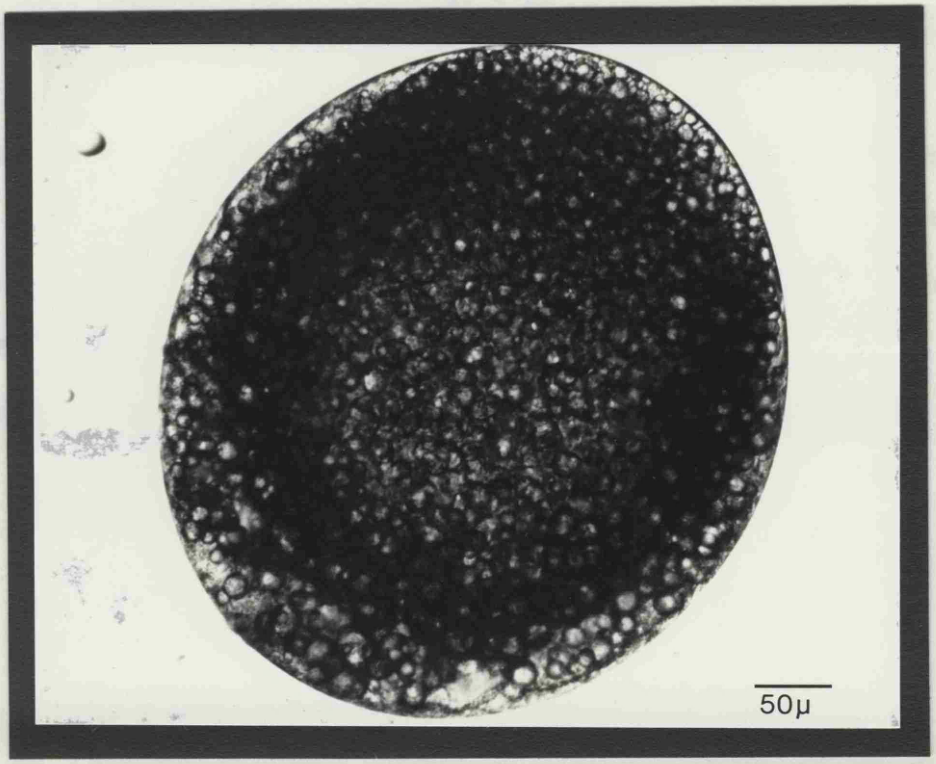


FIG. 6.5a
Light microscope picture of a Day 1 egg from the brood pouch of *C. pseudogracilis*.

From 10-15 days in *G.pulex*, and 6-11 days in *C.pseudogracilis* the developing embryo is clearly visible as a pink comma shaped streak within the egg (Fig. 6.5b).

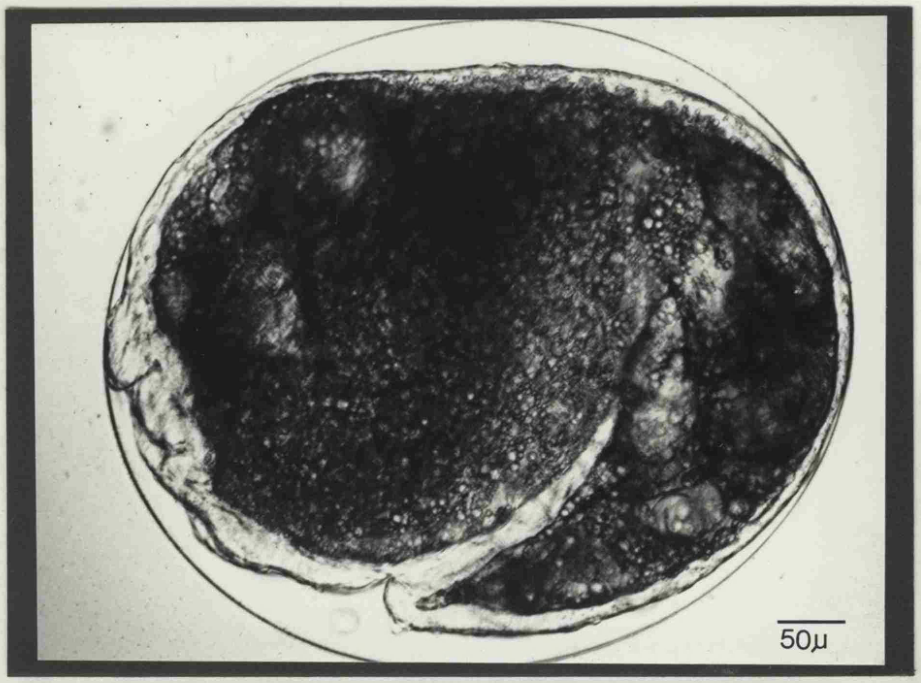


FIG. 6.5b
Light microscope picture of a Day 6 egg from the brood pouch of *C. pseudogracilis*.

For the last 5 days before hatching in both species the developing young totally occupies the egg-case and appears pink to the naked eye. By 10 days the young individual of *C.pseudogracilis* has taken on a recognizable form (Fig. 6.5c), with heart beat and gut peristalsis clearly visible under the microscope, but limb and eye development still incomplete.

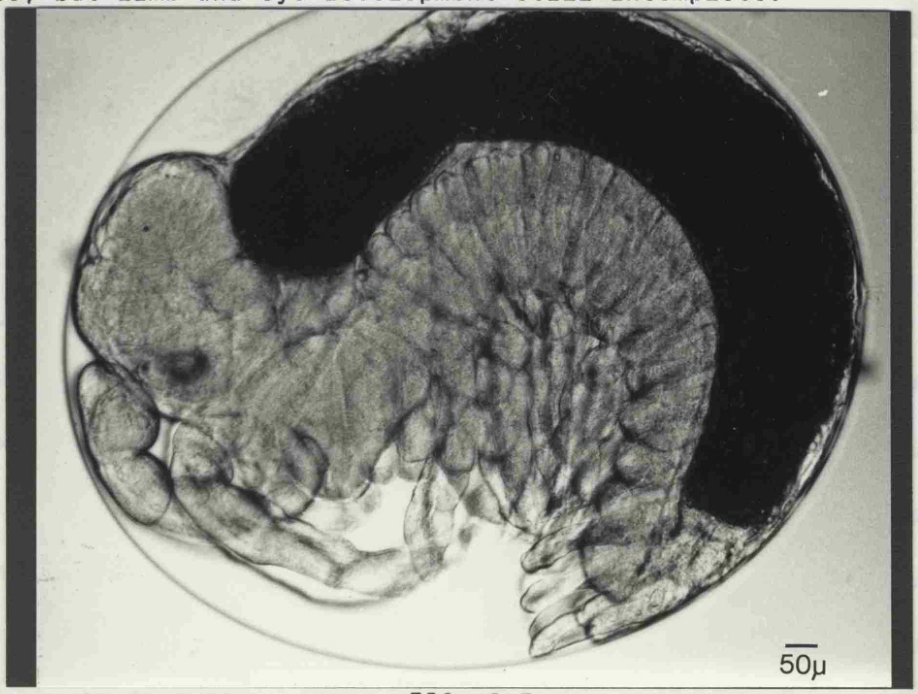


FIG. 6.5c
Light microscope picture of a Day 12 egg from the brood pouch of *C. pseudogracilis*.

Four days later the juvenile has hatched from the egg-case and is ready to leave the brood pouch (Fig. 6.5d). In order to cause minimal disturbance to both female and developing young the developmental stage for both species was consequently recorded in terms of colour stage; black, black-pink, or pink.

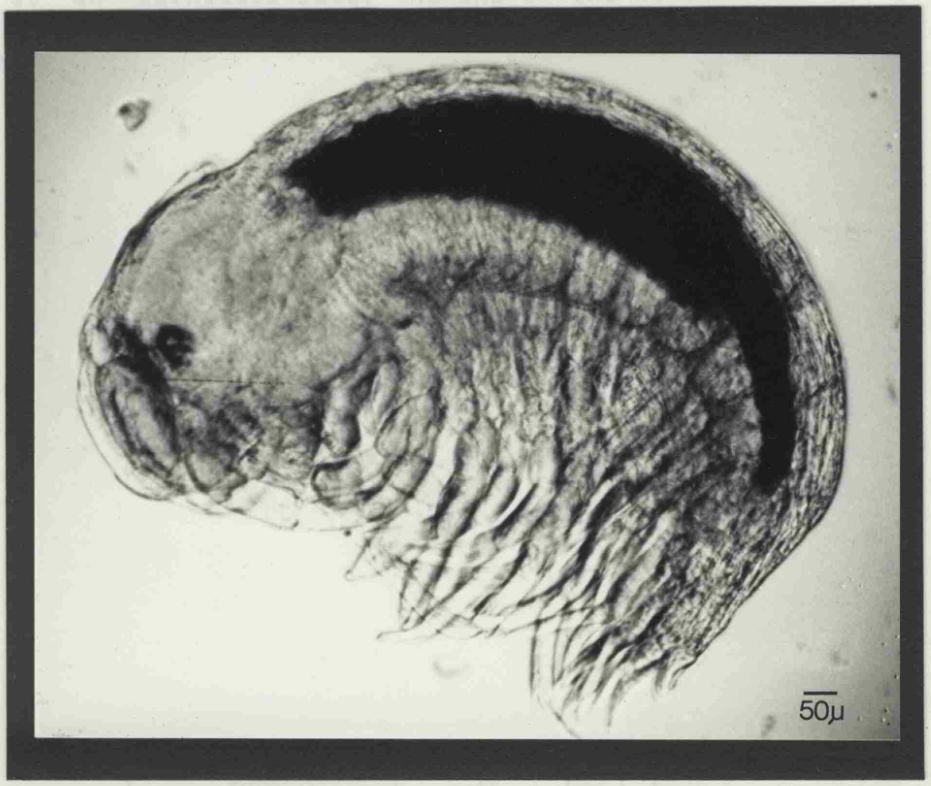


FIG. 6.5d
Light microscope picture of the newly hatched *C. pseudogracilis*.

Test 2 : Procedure

Hatched young of both species were counted, and transferred in a teat-pipette with a fine bore tube, into single species continuous flow cultures at the same test concentration. Cumulative additions to each culture was recorded, and the total number of live individuals counted every 4-7 days. After 21 days all cultures were discontinued and the total number of live animals in each recorded.

The continuous flow system is shown in Fig. 6.6a. Each test tank had dimensions 225 cm x 115 cm x 75 cm and held approximately 1 litre of water. Fine gravel was provided as a substrate and small pieces of elm leaf were given as a food source. Test medium was pumped into one side of the chamber at a rate of 2 litres per day and drawn from the opposite side of the chamber at an identical rate. The end of the outlet tube was covered with fine mesh to prevent animals from being sucked out (Fig. 6.6b).

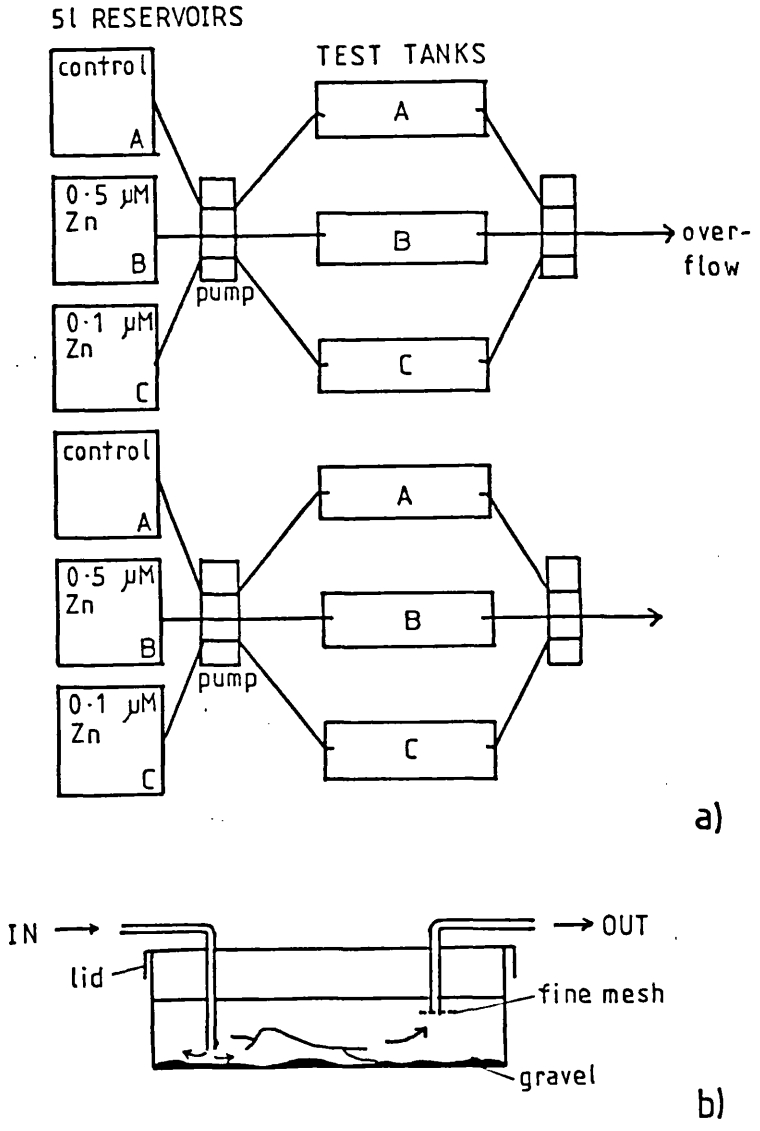


FIG. 6.6
Continuous flow system for exposing juvenile animals to a known zinc concentration.
a) General layout
b) Individual tank showing through flow of medium

RESULTS

Test 1 : Production of young

The numbers of young successfully produced in zinc solutions and in a zinc free control, by separate groups of 10 females of *C. pseudogracilis* and of *G. pulex* are shown in Table 6.1a.

In filtered, untreated reservoir water only one female *C. pseudogracilis* completely failed to produce young. The largest brood size of 33 was attained by three individuals with the remaining females producing between 3 - 31 young, giving a mean brood size of 21.1. Under identical test conditions, *G. pulex* produced 204 young from 10 females but the distribution of young/female was even less uniform than in *C. pseudogracilis*. Three individuals failed to produce any young, and three broods of 40 or more young were produced. The largest brood size successfully released by this species was 48.

In a 0.1 µM zinc sulphate solution *C. pseudogracilis* showed little difference from the control both in the distribution of young/female and total young produced. In contrast *G. pulex* suffered a reduction of nearly 50% in total young, with a largest brood size of only 17.

In a 0.5 µM zinc sulphate solution *C. pseudogracilis* again showed little departure from control results, with only one female totally failing to produce young. This clearly contrasts with the increased reproductive impairment of G. *pulex* at this concentration. Only five females of this species produced broods, the largest of which only comprised 11 juveniles. A total of 29 young represented less than 15% of the control success.

In 1.0 µM, and 50.0 µM zinc sulphate solutions no female of either species produced any young.

SPECIES	[ZINC]	NUMBER OF YOUNG / ANIMAL										TOTAL	MEAN SE
	(μM)	1	2	3	4	5	6	7	8	9	10		
CP	0.0	33	33	33	31	29	18	16	15	3	0	211	21.1 ± 3.99
	0.1	37	34	33	25	23	21	19	15	13	0	220	22.0 ± 3.53
	0.5	35	35	33	27	26	24	20	19	19	0	238	23.8 ± 3.29
	1.0	0	0	0	0	0	0	0	0	0	0	0	0.0
	50.0	0	0	0	0	0	0	0	0	0	0	0	0.0
GP	0.0	48	47	40	32	22	14	1	0	0	0	204	20.4 ± 6.37
	0.1	17	15	14	14	13	12	11	10	0	0	106	10.6 ± 1.87
	0.5	11	8	7	2	1	0	0	0	0	0	29	2.9 ± 1.31
	1.0	0	0	0	0	0	0	0	0	0	0	0	0.0
	50.0	0	0	0	0	0	0	0	0	0	0	0	0.0

a)

SPECIES	[ZINC] (μM)	NUMBER OF YOUNG RELEASED BETWEEN DAYS 10-20											RT50 (Days)
		10	11	12	13	14	15	16	17	18	19	20	
CP	0.0		17	0	38	123	19	14					13.80
	0.1				8	21	11	73	82	25			16.25
	0.5					3	128	35	63	9			15.78
GP	0.0						9	10	99	46	24	16	17.56
	0.1							13	4	35	32	22	18.43
	0.5							1	0	10	8	10	18.90

b)

TABLE 6.1

a) Total number of young hatched per female in control and zinc solutions. Females are labelled 1-10 and are entered from left to right in order of decreasing brood size.

b) Combined number of young released daily from all 10 females between days 10-20 of incubation. RT50=Mean time for 50% of the total young to be released.

CP = *C. pseudogracilis* GP = *G. pulex*

Development time

Brood development time was defined following Welton and Clarke (1980), as the time between copulation and release of the young from the brood pouch. In both *G. pulex* and *C. pseudogracilis* young were gradually released from the brood pouch, characteristically over a period of 2-3 days. This is shown for both species in zinc solutions and zinc free control in Table 6.1b. A 50% average release time for each test condition was calculated by multiplying the number of young born on each day by that day, and dividing the result by the total number of young.

In the control medium over 75% of the total young *C. pseudogracilis* were released on days 13 and 14 with shortest and longest individual incubation times of 11 and 16 days respectively. In a 0.1 µM zinc solution development was clearly retarded with only 13% of the total young released before day 15. The average time for 50% of the total young to be released was consequently 2.45 days longer than in the zinc free control. In a 0.5 µM zinc solution brood development time was similarly retarded, with no young released before day 14, and a 50% release time of 15.78 days.

In the control medium *G. pulex* required approximately four days longer than *C. pseudogracilis* to reach maturity within the brood pouch, with 48.5% of the total young released on day 17. As with *C. pseudogracilis* a clearly longer development time of the young within the brood pouch was observed for those females held in 0.1 µM and 0.5 µM zinc solutions. At the latter concentration, only 1 of a total 29 young was released before day 18.

Developmental trend

Those results pertaining to final brood size and individual development times are incorporated into a more comprehensive diagrammatic record, in which both the fate of the female, and progressive development of the brood are presented (Fig. 6.7 & 6.8).

FIGS. 6.7 - 6.8 (over page)
Developmental record of ten broods from *G. pulex* and *C. pseudogracilis* in four zinc concentrations and in a zinc free control.

Brood size: LARGE MEDIUM SMALL NO BROOD

Stage of egg development within the female:

1 BLACK
2 BLACK-PINK
3 PINK

Young: Cumulative release of juveniles

Post Gravid: Successful release of a small, medium or large brood by the female

Total egg loss: Indicates a female losing all her brood before development was complete. The size of brood carried immediately prior to total loss is recorded

Adult Death: Indicates death of the female before her small, medium or large brood was released

Females retaining undeveloped eggs for the duration of the experiment (28 days), have been recorded. The number of post-gravid females, total egg loss and adult death has been recorded cumulatively until Stages 1-3 finished, or the broods in these stages were no longer viable. This enables the fate of all ten females to be determined on any day up to completion of the experiment.

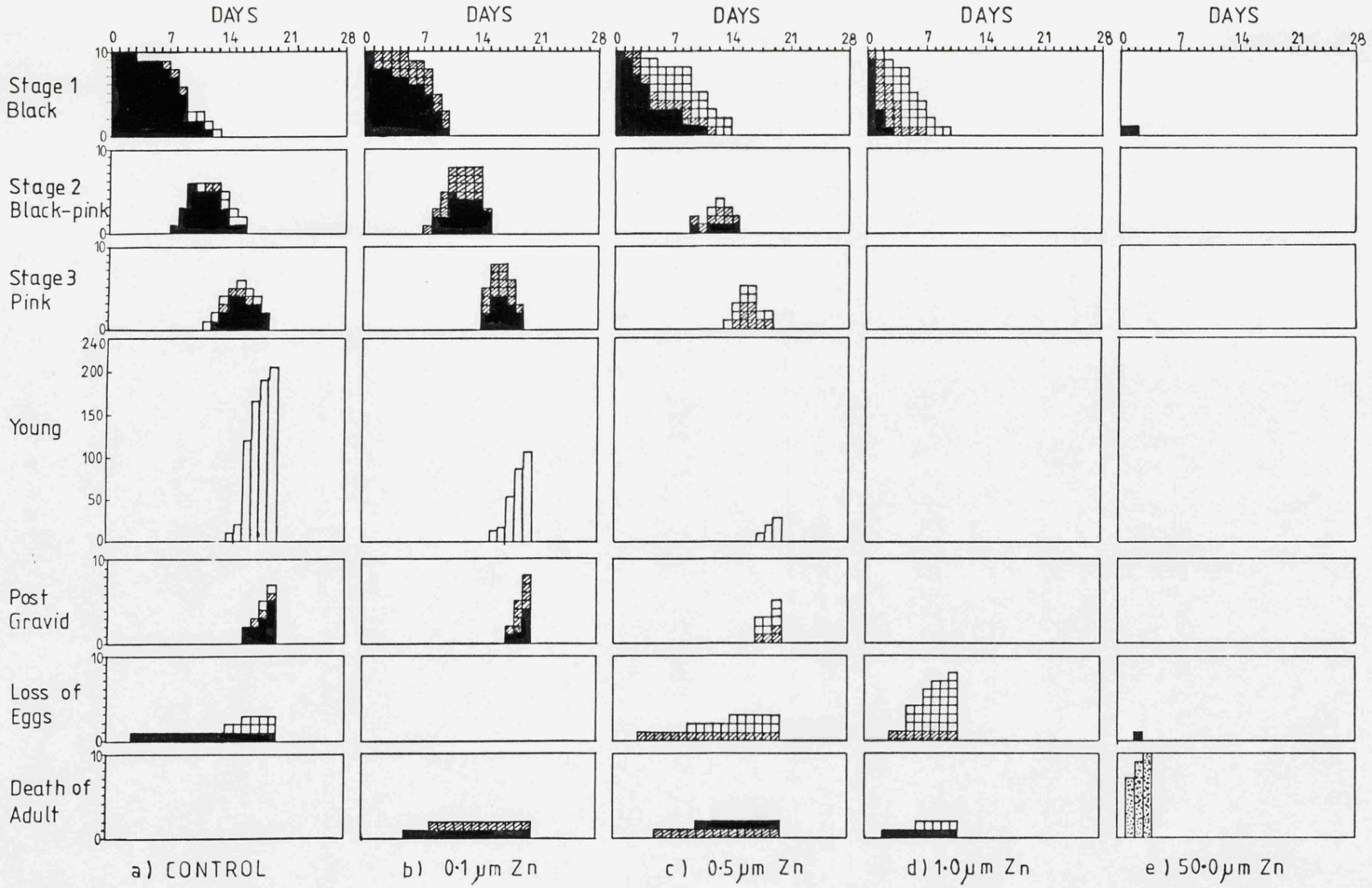


FIG. 6.7 *G. pulex.*

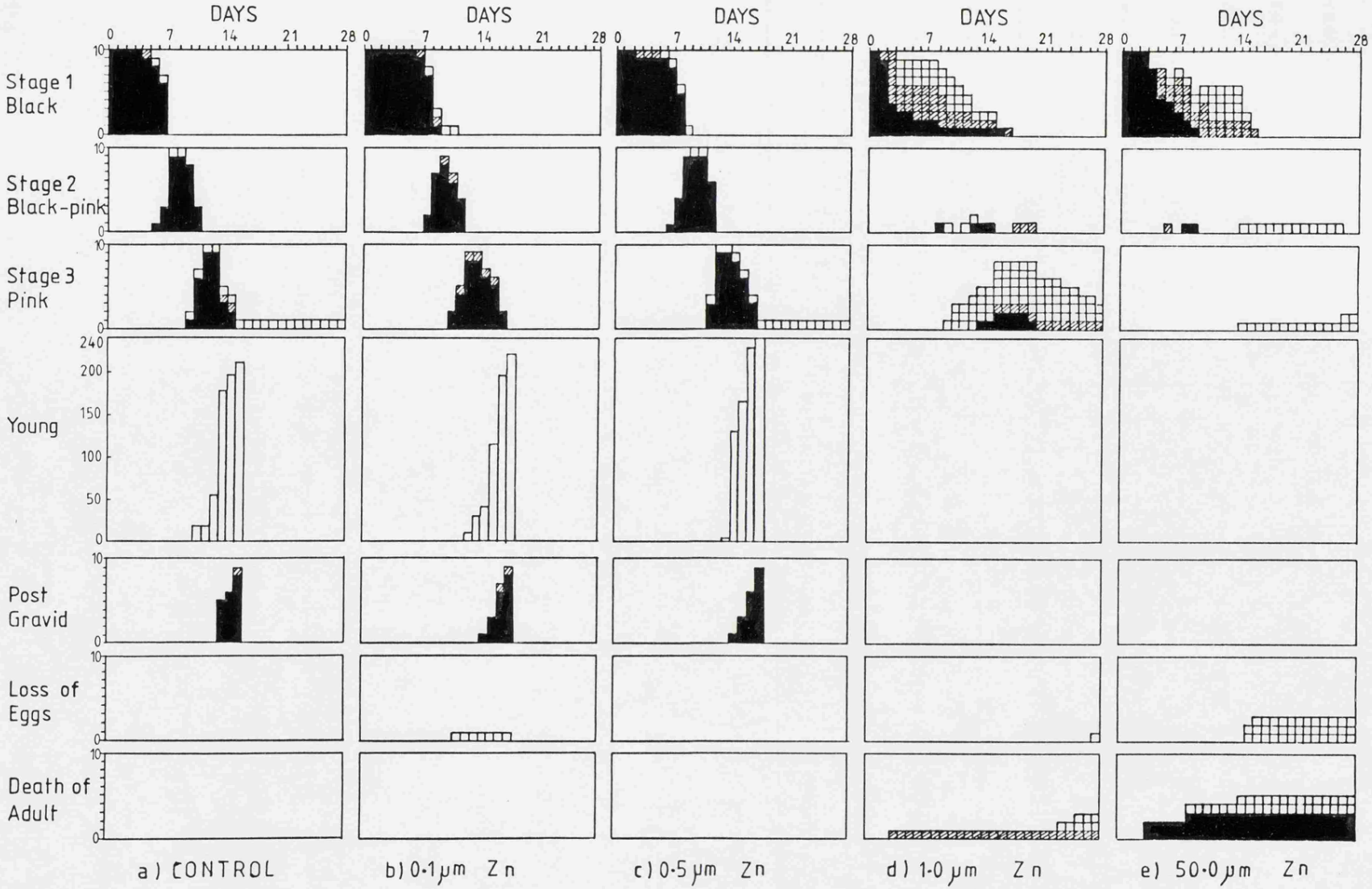


FIG. 6.8 C. pseudogracilis

A comparison of the control brood developmental records for both species reveals a basic similarity. Predominantly large broods were maintained throughout the three developmental stages culminating in the production of approximately 200 young. The slightly longer brood development time in *G. pulex* is shown by a greater time spent in each developmental stage, and a greater overlap between stages, compared to *C. pseudogracilis*.

In *C. pseudogracilis* eight of the nine females which successfully produced broods had maintained a full brood pouch throughout the incubation period, whilst the single female suffering partial egg loss produced only 3 young. The female producing no young had retained a brood pouch full of eggs which corresponded on visible inspection to the 'Pink' stage of development. Subsequent microscopic examination of these eggs revealed no coherent structure.

In *G. pulex* five of the seven females which successfully produced young had maintained full brood pouches throughout the incubation period. In contrast to *C. pseudogracilis*, no eggs were retained which did not develop and therefore the three females which did not produce young lost all their eggs before they were fully developed. In one female this loss was total on day 3 of the experiment but in the other two females the loss was gradual, with some eggs retained up to day 13.

In a 0.1 µM zinc solution the developmental trend of *C. pseudogracilis* was almost identical to the control with the exception that one female lost all eggs whilst they were still in the 'black' stage of development. In contrast, at this zinc concentration *G. pulex* showed considerable departure from the control 'pattern' of development. After 3-4 days several females had shed a proportion of their brood with the result that only four of the eight females producing young had maintained a full brood throughout the incubation period. This response in combination with the death of two females by day 7 explains the lower total number of young reared in comparison with that in the control (Table. 6.1a).

In a 0.5 µM zinc solution *C. pseudogracilis* again showed little deviation from the control pattern of development. The only female failing to produce young, had, as in the control, maintained a small brood of eggs for 28 days which showed no sign of internal organisation. At 0.5 µM zinc, however, *G. pulex* suffered serious egg loss and adult death. Egg loss began almost immediately and after 7 days only 3 females had retained full brood pouches. Two females died during the test and 3 suffered total brood loss. Of the five females with eggs entering the 'black-pink' stage of development only one retained a full brood. After further losses of the developing young only small or medium size broods were successfully released. Small brood size, total egg losses and death of two females therefore explains the small total of young produced.

In a 1.0 µM zinc solution all ten females of both species failed to produce any young (Table 6.1a). Their more detailed developmental records shown in Fig. 6.7 & 6.8 are, however, very dissimilar. *G. pulex* began to shed eggs almost immediately, and within 10 days all eight surviving females had completely lost their broods. A similar initial reduction in brood size occurred in *C. pseudogracilis*, but in contrast to the total egg loss seen in *G. pulex*, some eggs were retained by each female for the duration of the test (28 days).

Abnormal development was suspected from the prolonged black, and pink stages of development, together with the marked absence of an intermediate black-pink stage. After 20 days three females were removed and their remaining eggs examined under the microscope. Some were found to be granular with intact membranes (Fig. 6.9) whilst in others the membrane had begun to disintegrate and the eggs had taken on a globular appearance (Fig. 6.10). These observations combined with the absence of a normal black-pink stage of development indicated that no development had occurred.

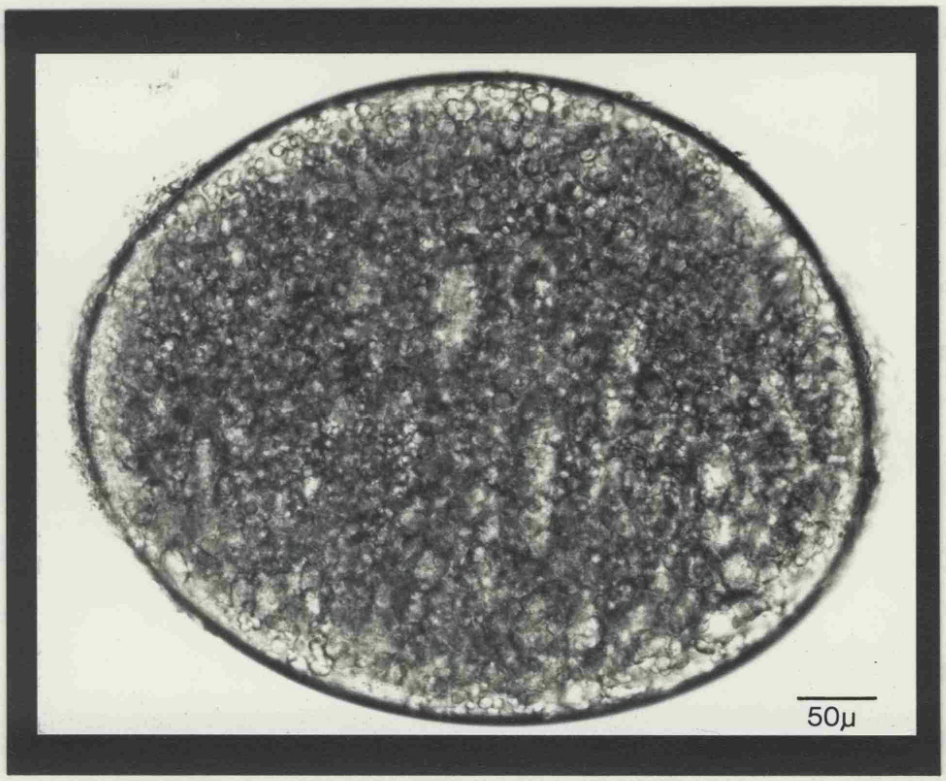


FIG. 6.9
Egg taken from the brood pouch of *C. pseudogracilis* after exposure to a zinc solution of 1 μM for 20 days; granular egg, membrane still intact.

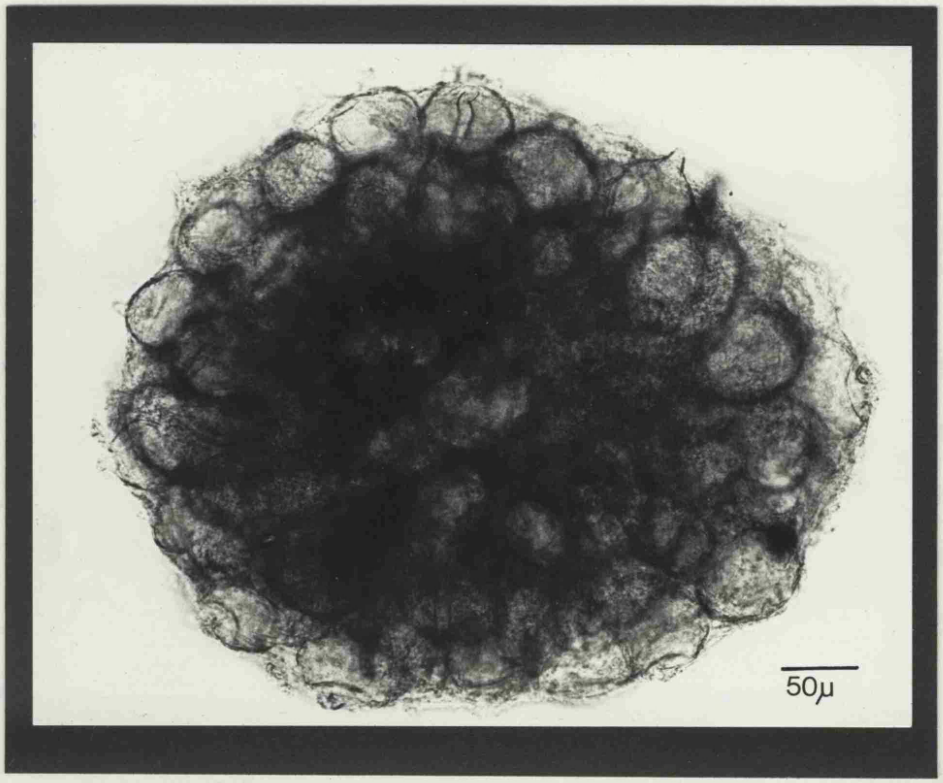


FIG. 6.10
Egg taken from the brood pouch of *C. pseudogracilis* after exposure to a zinc solution of 1 μM for 20 days; globular egg, membrane disintegrating.

Finally, within a 50.0 µM zinc solution five female *C. pseudogracilis* survived for the duration of the test. Three lost all their eggs after 14 days and two carried small inviable broods up to day 28. In *G. pulex*, only one female retained a brood for two days with the remaining females dying between days 2-4. Several attempts were made to replace these animals but the zinc concentration was too high for any female to live longer than 4 days.

Test 2 : Early survival of the young

C. pseudogracilis (Table 6.2a)

Under control conditions, 46% of the total young produced from ten females in TEST 1, died within 21 days of release, leaving only 113 individuals. An almost identical result was obtained with the young produced in a 0.1 µM zinc solution. After 21 days at this concentration only 119 of the 220 young produced remained, giving 45.9% mortality. In the 0.5 µM zinc solution, only 97 of the initial 238 animals survived for 21 days corresponding to a mortality of 59%.

G. pulex (Table. 6.2b)

The results obtained for *G. pulex* were less consistent than those for *C. pseudogracilis*. In the control medium only 73 of the 205 young produced remained alive after 21 days; this represents a mortality of 64%. In contrast, only 105 young were produced in a 0.1 µM zinc solution but 76 survived the following 21 days at this concentration, giving a mortality of only 28%. In a 0.5 mM zinc solution no 21 day total was obtained, but only 4 of the original 18 young were still alive after 17 days.

TIME	CONTROL			0.1 µM ZINC			0.5 µM ZINC		
(days)	+	cum+	actual	+	cum+	actual	+	cum+	actual
1	55	55		8	8		3	3	
2	18	73		21	29		128	131	
3		73		11	40		35	166	
4	116	189		73	113		63	229	
5	19	208		82	195		9	238	
6	3	211	210	25	220			238	
7		211			220			238	
8		211			220	204		238	216
9		211			220			238	
10		211			220			238	
11		211			220			238	
12		211	185		220			238	
13		211			220			238	
14		211			220			238	
15		211			220	182		238	167
16		211			220			238	
17		211			220			238	
18		211	138		220	156		238	137
19		211			220			238	
20		211			220			238	
21		211	113		220	119		238	97

a) *Crangonyx pseudogracilis*

TIME	CONTROL			0.1 µM ZINC			0.5 µM ZINC		
(days)	+	cum+	actual	+	cum+	actual	+	cum+	actual
1	23	23		19	19		1	1	
2	65	88		6	25			1	
3	19	107		26	51		15	16	
4	21	128		1	52			16	
5	13	141		5	57			16	
6	25	166			57			16	
7		166		33	90		2	18	
8	39	205	186	5	95			18	
9		205		10	105			18	14
10		205			105			18	
11		205	174		105			18	
12		205			105	97		18	
13		205			105			18	6
14		205			105			18	
15		205	157		105			18	
16		205			105	91		18	
17		205			105			18	4
18		205	108		105				
19		205			105				
20		205			105				
21		205	73		105	76			

b) *Gammarus pulex*

TABLE 6.2

Developmental record of young *C. pseudogracilis* and *G. pulex* over the first 21 days after release from the brood pouch.

Time = Real time in days from the first addition
\+ = Addition of animals from Test 1
cum+ = Cumulative total of animals added
actual = Actual number of animals remaining alive

DISCUSSION

The ability of both *G. pulex* and *C. pseudogracilis* to produce viable broods was greatly impaired at zinc concentrations well below those which were acutely toxic to the adult. Two components of impairment may be distinguished as an increase in brood development time between fertilization and release, and a decrease in the size of brood which was successfully reared.

Increase in brood development time

Models of normal brood development time have been constructed for both *G. pulex* and *C. pseudogracilis* from experimental data. Welton & Clarke (1980) found that the power law relationship;

$$\text{Log}_e D = a + b\ \text{Log}_e T \qquad (\text{or } D = 314.8T^{-0.962})$$

(D = Brood development time, T = Temperature, a = 5.752 and b = -0.962)

provided the best fit to their data on *G. pulex*. Substituting into this equation for a temperature of $18 \pm 1\ ^{o}C$, gives a brood development time of 18.53 - 20.62 days. The mean control development time of 17.56 days determined in this study is therefore 1 day below this range. Brood development time was extended in both 0.1 µM and 0.5 µM zinc solutions but by only about a day so that it just entered the predicted range shown above.

A similar model for incubation time in *C. pseudogracilis* has been constructed by Sutcliffe & Carrick (1981);

$$D = 601\ T^{-1.35}$$

(D = Incubation time, T = Temperature)

Substituting in this equation for a temperature of 18±1 ^{o}C provides a brood development time between 11.29 and 13.11 days. The control brood development time of 13.80 days determined in this study is just outside the top of this range. In contrast to *G. pulex*, retardation of brood development was the most obvious sign of impairment below 1.0 µM. In both 0.1 µM and 0.5 µM zinc solutions incubation time was over 2 days longer than that predicted from the above model.

The mechanism by which zinc retards development is unknown. One possible explanation is that the young animal hatches normally but remains for longer within the protection of the brood pouch. Embody (1911) observed that if the young are kept in the presence of the female they will re-enter the brood pouch. It is not inconceivable that this response may be further enhanced by a toxic external environment. In *C. pseudogracilis*, however, stages 1 and 2 of development were both markedly longer in zinc solution than in the control, indicating a retardation of the complete developmental process.

Reduction in brood size

Whilst complete reproductive failure occurred at the same zinc concentration of 1 µM for both *C. pseudogracilis* and *G. pulex*, in the former species only an increase in brood development time provided any indication of toxicity below this concentration. In contrast *G. pulex*, which showed less prolongation of development time, suffered a clear-cut reduction in brood size at a zinc concentration tenfold less than that which caused total reproductive failure.

Although *C. pseudogracilis* is therefore constrained by the same upper concentration limit, its ability to produce a full brood up to a zinc concentration of at least 0.5 µM must give it a reproductive advantage over *G.pulex* which did not produce a full brood at the lowest test concentration of 0.1 µM.

This difference between the impairment range of *G.pulex* and *C.pseudogracilis* is evidence for a difference in response to the toxicant between the two species. With increasing zinc concentration *G.pulex* retained fewer eggs for the necessary developmental period, and therefore the corresponding brood size was gradually reduced, until in a 1µM solution all eggs were lost within 10 days. At the lower zinc concentrations all eggs that were retained developed and were successfully released. It therefore follows that either the female can both distinguish and eject dead individuals from the brood pouch, or is non-selectively releasing potentially viable eggs in response to zinc stress. In view of the low acute tolerance level of adult *G. pulex* the latter hypothesis would appear more likely.

C.pseudogracilis showed little egg loss below a test concentration of 1µM. Since the tolerance level of the adult *C.pseudogracilis* is much higher than that of *G.pulex*, less stress on the female would be expected at these zinc concentrations and a full brood could therefore be supported. At a zinc concentration of 1 µM small broods of undeveloped, dead eggs were retained for at least 11 days longer than the normal development period. This suggests that the adult was not sufficiently stressed to eject eggs and therefore complete reproductive failure at this concentration must be ascribed to the direct toxic action of zinc on the eggs.

The experiments cited in the introduction to this section, concerning the toxicity of zinc to eggs of both fish and invertebrates, were all conducted on isolated eggs, and therefore the role of the adult in the toxic response was irrelevant. In this series of experiments with *G. pulex* it has been shown that the response of the gravid female to zinc may be as important as the tolerance of the egg in determining reproductive impairment.

Early Survival of the Young

In control conditions 54% of *C. pseudogracilis*, and only 36% of *G. pulex* survived for 21 days following release from the brood pouch. This is in agreement with the results of Welton & Clarke (1980), and Nilsson (1977), who discovered that the mortality of newly released young of *G. pulex* remains high for the first 28 days after hatching.

The absence of any evidence in either species for a higher mortality rate in a 0.1 µM zinc solution indicates that it is not immediately toxic to the young amphipods. There was some indication that a 0.5 µM zinc solution might reduce early survival, but without further testing this remains inconclusive. In addition, the low initial hatching rate of *G. pulex* provided insufficient data for a statistical analysis of mortality.

This experimental approach also differs from those cited in the introduction, since the young under test had been incubated within the test zinc concentration. It would therefore be of interest to measure the tolerance of young reared in control conditions to compare with these results.

In summary, it would appear that the most vulnerable period of the life cycle tested in this two part experiment was the development of young within the brood pouch from fertilization to release. If successfully released, the resultant young were sufficiently tolerant to survive at the same zinc concentration.

Limitations of the experimental technique

The experimental techniques used in this chronic toxicity test were largely dictated by practical considerations, and therefore several features can be identified which prevented optimal accuracy from being attained.

A continuous flow system for TEST 1 would have been desirable to guarantee that the test solution remained constant throughout the experiment. This was rendered impractical by the amount of equipment required to simultaneously service ten single animal chambers for all zinc concentrations and a control.

No observations were made during the nights, and hence loss of eggs, death, or the release of young were not recorded until the following day. In addition, no distinction was made between events which occurred at different times on the same day. These factors may partially account for the staggered hatching times recorded. Increasing the number of observations made during the daytime would only have enhanced the inaccuracy of omitting recordings throughout the night and added to possible disturbance effects.

Two methods were available for determining brood size and developmental stage of the young. In order to obtain an exact egg count and accurately define the developmental level attained, the eggs must be removed and examined under the microscope. If this approach is adopted the complete developmental record becomes a composite from several individuals, requires a large supply of animals and will be sensitive to individual variability. The approximate method for determining brood size and developmental stage used in this study was obviously prone to small inaccuracies, but allowed a single gravid female to be monitored throughout the test, using fewer individuals and eliminating inaccuracy from individual variability.

In TEST 2 a continuous flow system was used which relied on electricity to drive mechanical pumps. This type of system has been criticized as susceptible to power failure (Burke & Ferguson 1968; Stark 1973) but to service sufficient test chambers it was necessary to sacrifice absolute accuracy and reliability for a viable cost-effective system.

CHAPTER 7

BEHAVIOURAL TOXICITY TESTING

INTRODUCTION

According to Olla, Pearson & Studholme (1980), the rationale for the use of behavioural techniques to determine toxicity, is that an environmental disturbance will produce a measurable change in the normal behaviour pattern of the exposed species. An acute toxic disturbance may cause a behavioural reaction of sufficient intensity that will transcend the wide natural variation in behaviour, which is found in most species. More subtle changes, occurring at lower toxicant levels, may be harder to distinguish when time, equipment and experimental animals are limited. For this reason, behavioural methods have not commonly been exploited in assessing the effects of pollution (Cherry & Cairns 1982), with the result that no broad experimental base has been developed in an ecological context.

The most commonly used behavioural technique which has become sufficiently popular in the literature to allow tentative inter-specific comparisons to be formed, is the preference/avoidance study. The advantage of this type of behavioural test lies in its relatively uncomplicated methodology and apparently unambiguous results.

An avoidance response is successful if it removes or lessens the effects of a toxicant, thereby reducing the probability of death or energetic cost of compensatory responses. Three components comprise a successful avoidance response; the animal must be able to sense the change in its immediate environment, recognise that it is adverse, and respond accordingly. If any one of these components is missing the resultant exposure to the toxicant may be fatal (Olla 1980).

The majority of literature concerning avoidance studies pertains to fish Sprague (1973). Sprague (1968) found that *Salmo gairdneri* (rainbow trout) strongly avoided sub-lethal zinc sulphate concentrations. Jones (1947a) found that *Pygosteus pungitius* (10 spine stickleback) also detected and avoided zinc in concentrations at least as low as 150 µM, which was often exceeded in streams polluted by zinc mining.

The results of these two studies with zinc as the toxicant are not however representative of all the avoidance data obtained for fish. Sprague (1971) concluded that whilst the behaviour of fish in response to many kinds of pollutants had been widely studied no 'typical' avoidance response could be described. Jones (op. cit.) also found that *P. pungitius* showed no ability to either detect or avoid toxic concentrations of mercuric chloride.

There is a paucity of literature on similar avoidance experiments on macro-invertebrates. Costa (1966) was the first to study the avoidance behaviour of a mobile macro-invertebrate, *G. pulex*, to toxic solutions, using a choice chamber based on a design for testing fish (Jones 1947a; 1948). He reported a marked avoidance response to zinc sulphate solution down to the lowest concentration tested of 500 µM. Fifteen years later a further study on the same species clearly demonstrated a strong avoidance response to a zinc sulphate concentration of only 15 µM but no tests were conducted below this concentration (Abel & Green 1981). A similar investigation which was performed on the response of the estuarine amphipod *Gammarus daiberi* to a chlorinated effluent, showed that this gammarid can actively avoid chlorine concentrations well below the experimentally determined lethal level (Ginn & O'Connor 1978). In common with the fish studies however, no 'typical' avoidance response has been demonstrated. This point is further illustrated by a study in which it was demonstrated that *Gammarus lacustris* avoided copper concentrations between 1 and 2 times below

the 96 h LC50, but was actually attracted to concentrations 40 to 300 times higher (Maciorowski, Clarke & Scherer 1977).

The dearth of behavioural avoidance data for macro-invertebrates may reflect their lesser mobility in relation to fish, and consequently the impracticality of applying laboratory data to field situations. Weatherley, Lake & Rogers (1980) suggested that the study of pollutant induced behavioural modification in fish, notably the avoidance reaction, was probably more important than lethal or sub-lethal damaging effects. This was because disruption of spawning or territorial behaviour could profoundly affect the population dynamics of that species. Sprague, Elson & Saunders (1965) clearly demonstrated this in a study of the spawning behaviour of *Salmo salar* (atlantic salmon) within the partially polluted Northwest Miramichi River.

In his study on macro-invertebrate behaviour, Costa (1966) concluded that an avoidance of harmful substances was unquestionably adaptive, but failed to substantiate his assertion with field evidence of avoidance behaviour. Later Abel & Green (1981) hypothesised that whilst it was uncertain that invertebrates could show significant voluntary movement from a pollutant source as demonstrated for fish in lotic bodies, the avoidance response might result in an increased rate of downstream drift, reducing population densities in polluted stretches. An avoidance test is therefore an important component of this toxicty study since the high pH and hardness of the water in the brook to which the study relates, caused rapid downstream precipitation of metal. Hence any induced downstream movement of animals could be of survival value.

A further objective of fish avoidance studies has been to assess their suitability in the development of more sensitive indications of toxicity. This has resulted in the discovery that fish will avoid a wide range of pollutants, often at sub-lethal concentrations (Sprague 1971). As indicated above, however, there is wide variation of avoidance response in

relation to lethal concentration with each toxicant inducing its own particular pattern of strong, weak or variable response in each species studied. Since little is known of the limits within which macro-invertebrates can detect and respond to metal pollutants in relation to lethal concentration, the second aim of this experiment was to compare the avoidance response of the two macro-invertebrate species having very different acute tolerances, and to assess the sensitivity of this technique as a test for zinc toxicity.

Continued exposure to the pollutant will occur if the avoidance response is inadequate, or rendered incomplete by restricted mobility. In this case it becomes important to understand the effects of zinc on the exposed population but, as stated previously, studies of this nature are largely avoided because of difficulties of performance and interpretation. In a macro-invertebrate study where avoidance response is of indeterminate field significance, activity measurements in the presence of the pollutant may produce a more realistic indication of environmental stress.

The essential requirement of activity measurements is to establish behavioural base-lines derived from the animals' repertoire, so that any significant departure can be used as a measure of perturbation. This approach was used by Waller & Cairns (1972) who assessed the use of fish movement patterns, measured by light-beam interruption, as a technique for continuously monitoring the response of fish to zinc. Whilst this technique was found to give a reliable indication of the presence of a potentially lethal zinc concentration, the smaller size of invertebrate species places considerable technical restrictions on the use of automatic recording techniques of this nature.

It is obviously inappropriate to measure activity changes during avoidance experiments because the responses shown may differ markedly from those in a homogeneous zinc environment, and may also be dependent on the strength of the avoidance response. The second part of this investigation is

therefore concerned with measurable changes in activity which accompany exposure to a homogeneous lethal zinc solution. The suitability of this technique as a criterion of toxicity is assessed, for comparison with the avoidance reaction.

MATERIALS AND METHODS

The fundamental apparatus for both activity and avoidance experiments consisted of a glass experimental chamber within which either a homogeneous environment, or a choice between untreated water and zinc solution, was presented. This apparatus was basically a refinement of that used by Jones (1947a; 1948), Costa (1966; 1967a; 1967b) and Abel & Green (1981).

Apparatus design and construction

Two elevated 5 litre aspirators containing aerated control and zinc solutions respectively, each supplied bubble free medium by gravity to one or both ends of a cylindrical glass experimental chamber, depending on the relative setting of four 3-way stopcocks (Fig. 7.1). The chamber was 400 mm in length with internal diameter 20 mm and a total effective capacity of 120 cm^3.

A single short inlet at each end of the chamber had internal diameter 3 mm; each was recurved to aid dispersal of incoming medium, and prevent access to test animals (Fig. 7.2a). A short centrally placed outlet was fitted with a silicone rubber drain pipe of internal diameter 0.5 mm, to prevent entry of animals and enable waste solution to be conveniently drained into a measuring vessel (Fig. 7.2b). Animals were inserted through a second short central pipe at 90^{o} to the outlet. During operation this access was sealed with a quickfit stopper.

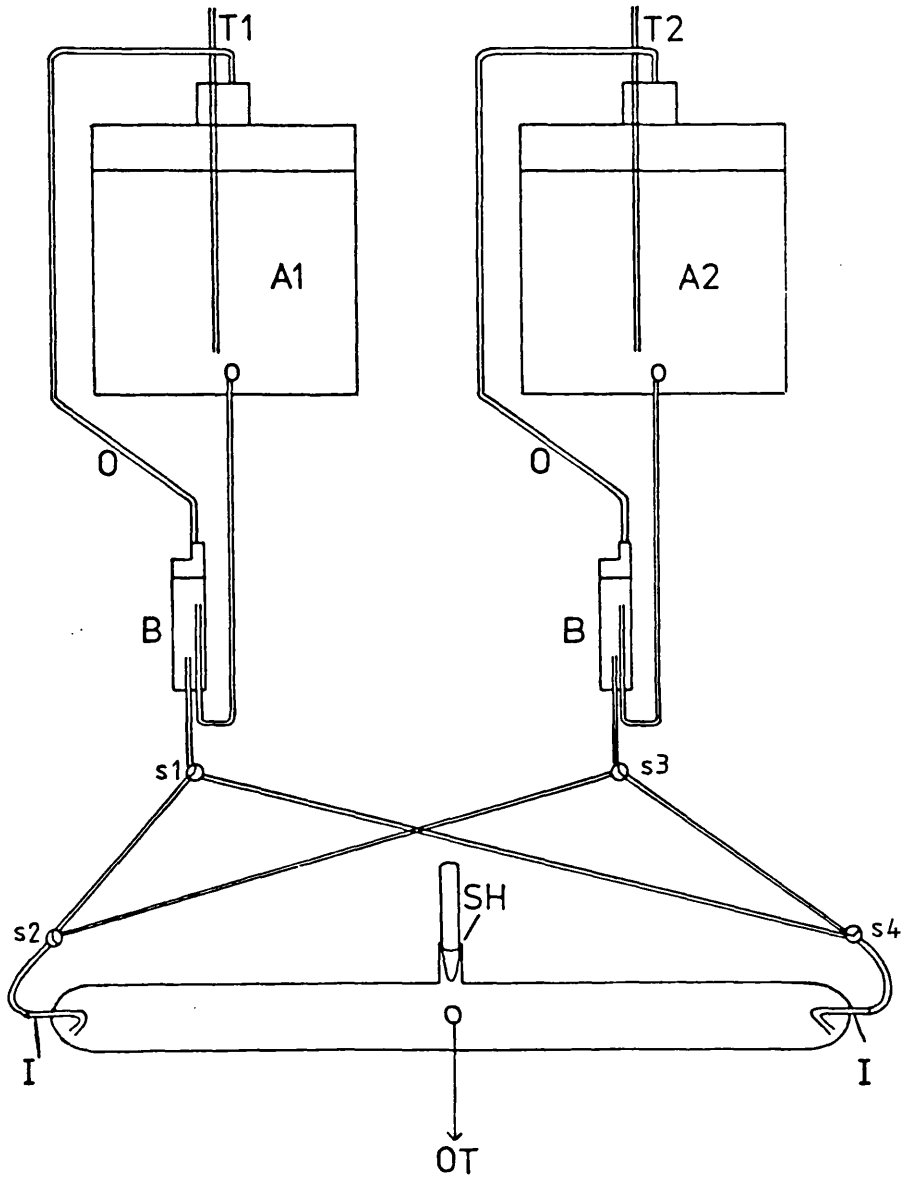


FIG. 7.1

Plan of basic apparatus (not to scale).
A1,A2 = 5 litre aspirators, T1,T2 = plastic aeration pipes, B = bubble traps constructed from inverted 50 cm^3 plastic syringe cases, O = overflow pipe, I = curved chamber inlets, OT = central outlet, SH = stoppered hole for insertion and removal of animals, S1-4 = stopcocks

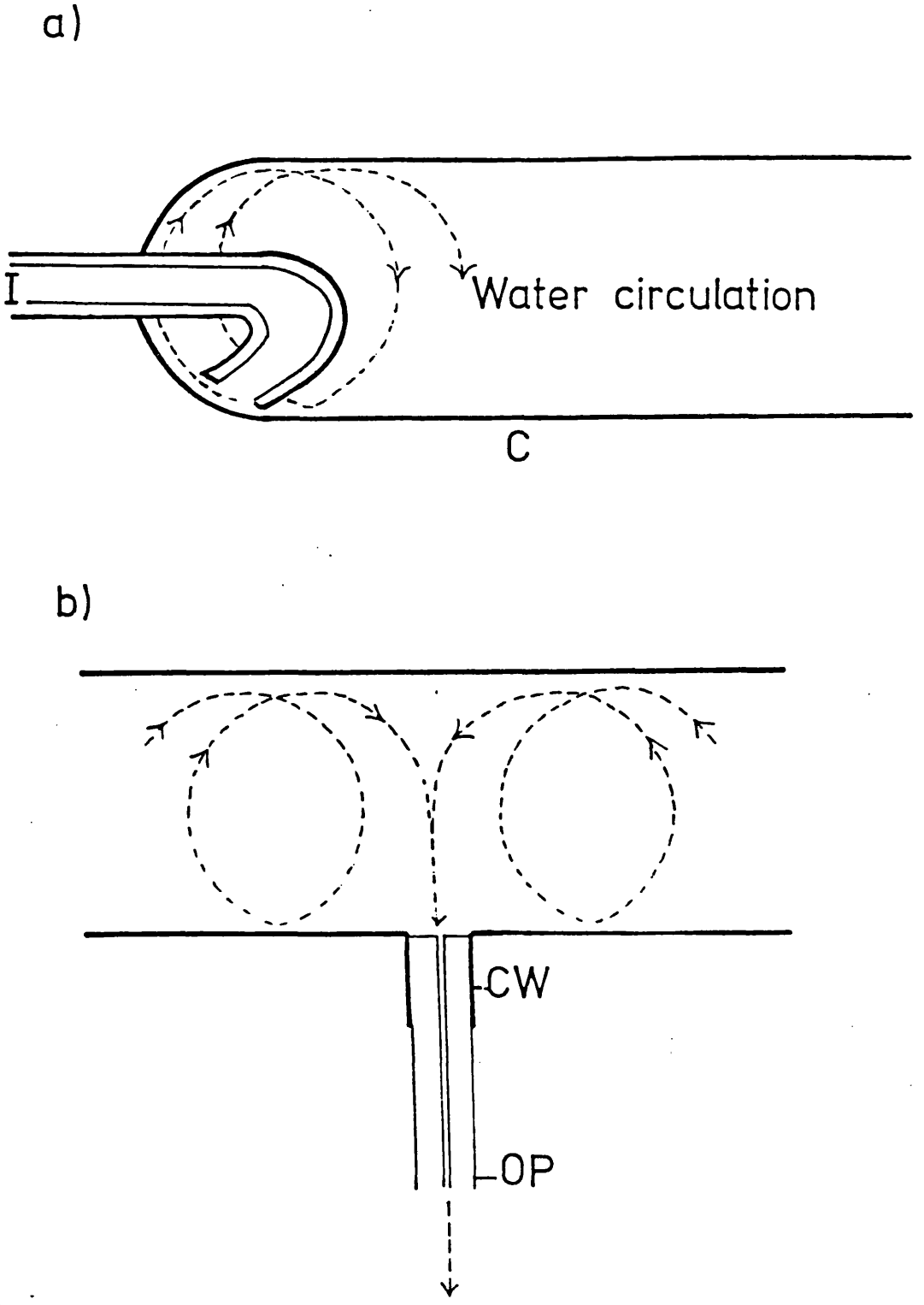


FIG. 7.2
Detail of experimental chamber.
a) Recurved inlet pipe, I = inlet, C = chamber
b) Insertion of outlet pipe, CW = chamber wall, OP = outlet pipe

The flow rates through both halves of the chamber were adjusted simultaneously by means of a single clamp on the outlet pipe. At a clearance rate of 50 cm^3 min^{-1} introduction of coloured water confirmed that total separation of the medium flowing through each half of the chamber was achieved, with a sharp central interface. (Fig. 7.2b & 7.4). After redirection of solutions a new separation was partially established in less than 2 min, and completed in 5 min with the formation of a distinct central interface.

Experiment 1: Avoidance behaviour.

Avoidance experiments were performed on replicated groups of 10 animals. Data were obtained from visual observation and no additional apparatus was required.

The experimental chamber was initially filled from both inlets with control reservoir water, and ten animals introduced through the central pipe. As soon as all air had been expelled from within the chamber, the stopper was replaced and the test animals allowed 5 min to acclimate before recording started. Only female *C. pseudogracilis* and 8-10 mm long *G. pulex* were tested after preliminary experiments had shown that smaller animals tended to become wedged in both inlet and outlet pipes.

The experimental chamber was externally subdivided into four equal quarters with thin tape strips. The number of animals in each quarter was recorded at 15 sec intervals for an initial 20 min period in control medium. Zinc solution of known concentration was then introduced into the left half of the chamber, and recording continued for a further 20 min period. In order to restore control conditions before the final test period, the chamber was flushed for 15 min during which time no recordings were taken. The same strength zinc solution was then introduced into the right half of the chamber for a final 20 min period.

Three tests were performed at each of 1000μM, 100μM, 50μM, 10μM, and 1 μM zinc sulphate solutions and in a 1000 μM sodium sulphate solution, on both *G. pulex* and *C. pseudogracilis*. Sodium sulphate was used as a control to determine the contribution of the sulphate ion to the avoidance response. Sodium was chosen as a suitably innocuous anion since sodium sulphate is one of the principle inorganic solutes in freshwaters (Sutcliffe 1978). The total numbers of animals in each quarter of the chamber were collated for the three replicate tests and averaged over successive 5 min intervals.

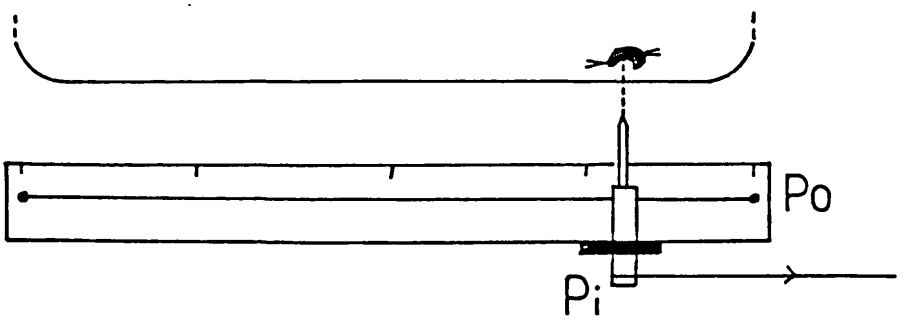
Experiment 2: Changes in activity.

Activity levels were obtained for single animals in homogeneous solutions of zinc sulphate and in control medium. In order to obtain a continuous record of activity, recording apparatus was required in addition to the basic chamber system.

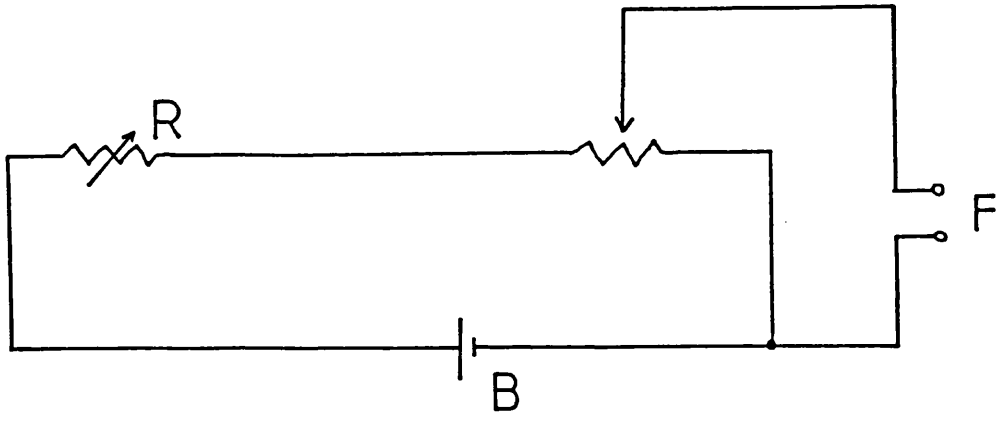
The experimental chamber was initially filled from both inlets with control medium. A single animal was inserted via the central pipe and after a 5 min acclimation period, recording was started. The horizontal position of the animal within the experimental chamber was continuously recorded for a 10 min period, by tracking it with a sliding contact on a potential divider placed immediately in front of the chamber. Preliminary experimentation had shown that if the chamber was viewed at eye level, the reflection within the chamber of the pointed contact could be aligned with the animal very accurately. Movements along the potentiometer wire were linearly echoed on a calibrated flatbed recorder, thereby producing a continuous record of horizontal movement within the chamber. The layout of this apparatus is shown in Fig. 7.3 & 7.4.

After 10 min, the control medium was replaced by a zinc solution of known concentration, and tracking continued for a further 20 min period. This test procedure was repeated for 10 animals of both species at zinc concentrations of 30 mM, 1 mM, and 0.1 mM.

30 mM zinc solution was chosen because of its acute lethality towards both species, 1 mM because of its acute lethality to *G. pulex* but not female *C. pseudogracilis*, and 0.1 mM as the lowest concentration at which a definite response could be elicited from either species.



a)



b)

FIG. 7.3
a) Arrangement for recording animal movements within the experimental chamber. (not to scale)
b) Circuit diagram for connection of potentiometer to flatbed recorder.

Pi = pointer, Po = Potentiometer, B = 9 v PP9 Battery, R = 2M Ohm resistance, F = Flatbed recorder

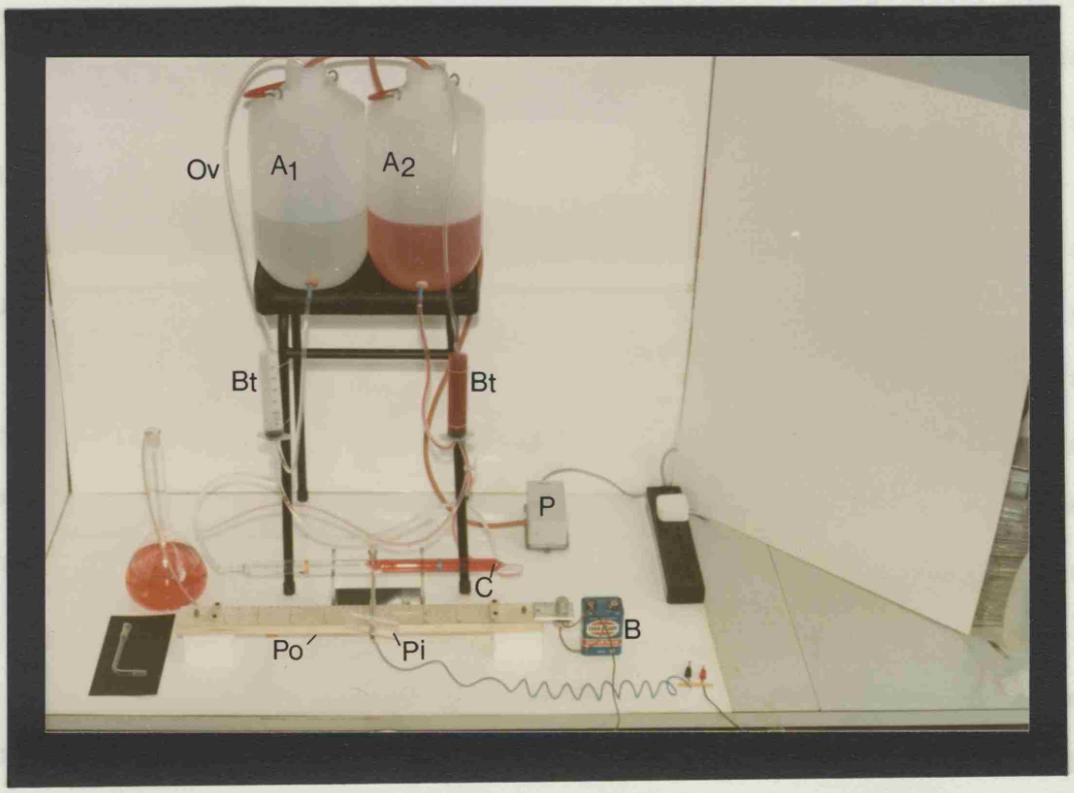


FIG. 7.4
Apparatus for recording the activity of a single animal within the experimental chamber.
A1,2 = 5 l aspirators, Ov = overflow, Bt = bubble traps, P = pump, C = chamber, Po = potentiometer, Pi = pointer, B = battery

Two different control experiments were performed. First, only 8 individuals of each species were used as identical controls for this test series because of a shortage of animals at the time of testing. The movements of these animals in a control medium for the total test time was recorded continuously as described above. Secondly, the activity of 10 animals of both species was recorded over a 15 min period in control medium, in order to characterize a 'normal' activity level within the chamber for both species.

Interpretation of the activity records could not be achieved manually with sufficient speed and accuracy, and therefore the data were analysed on a computer. The first stage in this process was conversion of the

graphical trace into a form capable of manipulation by computer. This was achieved by digitising the trace on a Ferranti Freescan Digitiser, accepting pictorial data up to 120 cm x 90 cm. A Newbury Laboratories VDU was used to run the menu driven digitiser program, and input the parameters necessary to define the resultant numerical output.

Each graphical trace was placed on the angled digitiser table and a co-ordinate system defined by allocating values to the axes of the trace. The activity line was then traced slowly with a cursor, generating co-ordinate values at 0.5 s time intervals. The end product of digitisation were data files of approximately 900 co-ordinates per 30 min of graphical trace, stored on a PDP11/44 computer. Data were transferred to a Cyber 73, for further analysis and interpretation.

In order to ensure that the numerical records accurately described the graphical trace, a random selection of files were reproduced in graphical form using an existing plotting routine, and compared to the original record. The original activity record was first traced on clean paper and superimposed with the computer generated plot. Whilst the difference between the two records was not quantified in is clear from the example in Fig. 7.5 that the digitised record correlated well with the original trace.

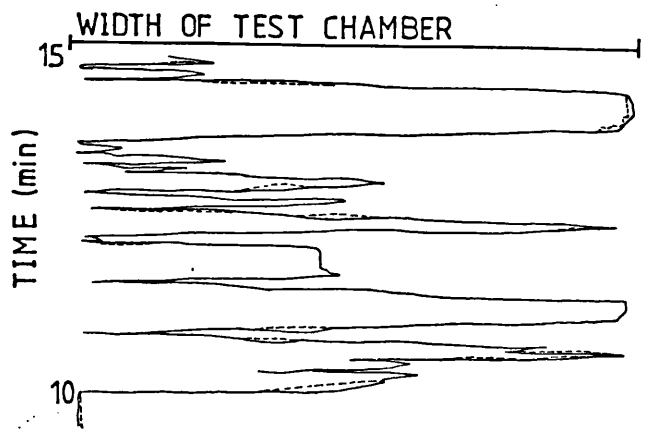


FIG. 7.5
A single animals movements within the test chamber.
———— Reproduced from digitised data
– – – – – – Traced from chart recorder graph

RESULTS

Experiment 1: Avoidance behaviour

Data from the three duplicate tests at each zinc concentration were combined to obtain a total number of animals in each quarter of the chamber, every 15 sec for 30 min. From this, mean values were calculated over successive 5 min intervals. These results are presented graphically in Fig. 7.6 & 7.7, and in the form of a significance table (Table 7.1).

Both species travelled freely within the test chamber during the initial 20 min control period and readily crossed the central interface from both directions without any visible behavioural response to the change in direction of flow. *C.pseudogracilis* showed a distinct tendency to crawl slowly along the bottom of the chamber, whilst *G.pulex* predominantly swam. Both species exhibited a preference for the end sections of the chamber during the control period of every test, but there was no significant preference for one half.

FIG. 7.6 & 7.7 (over page)

The avoidance behaviour of *G. pulex* (Fig. 7.6), and *C. pseudogracilis* (Fig. 7.7) in zinc sulphate and sodium sulphate solutions. The mean number of animals in each quarter of the test chamber has been plotted for each of the twelve 5 min test intervals.

0 - 20 min	Control period
20 - 40 min	Test solution in L.H.S. of chamber
40 - 60 min	Test solution in R.H.S. of chamber

————	Left end of chamber
— — — — —	Left middle of chamber
-·-·-·-·-·	Right middle of chamber
.....	Right end of chamber

A	1000 µM zinc sulphate
B	100 µM zinc sulphate
C	50 µM zinc sulphate
D	10 µM zinc sulphate
E	1 µM zinc sulphate
F	1000 µM sodium sulphate

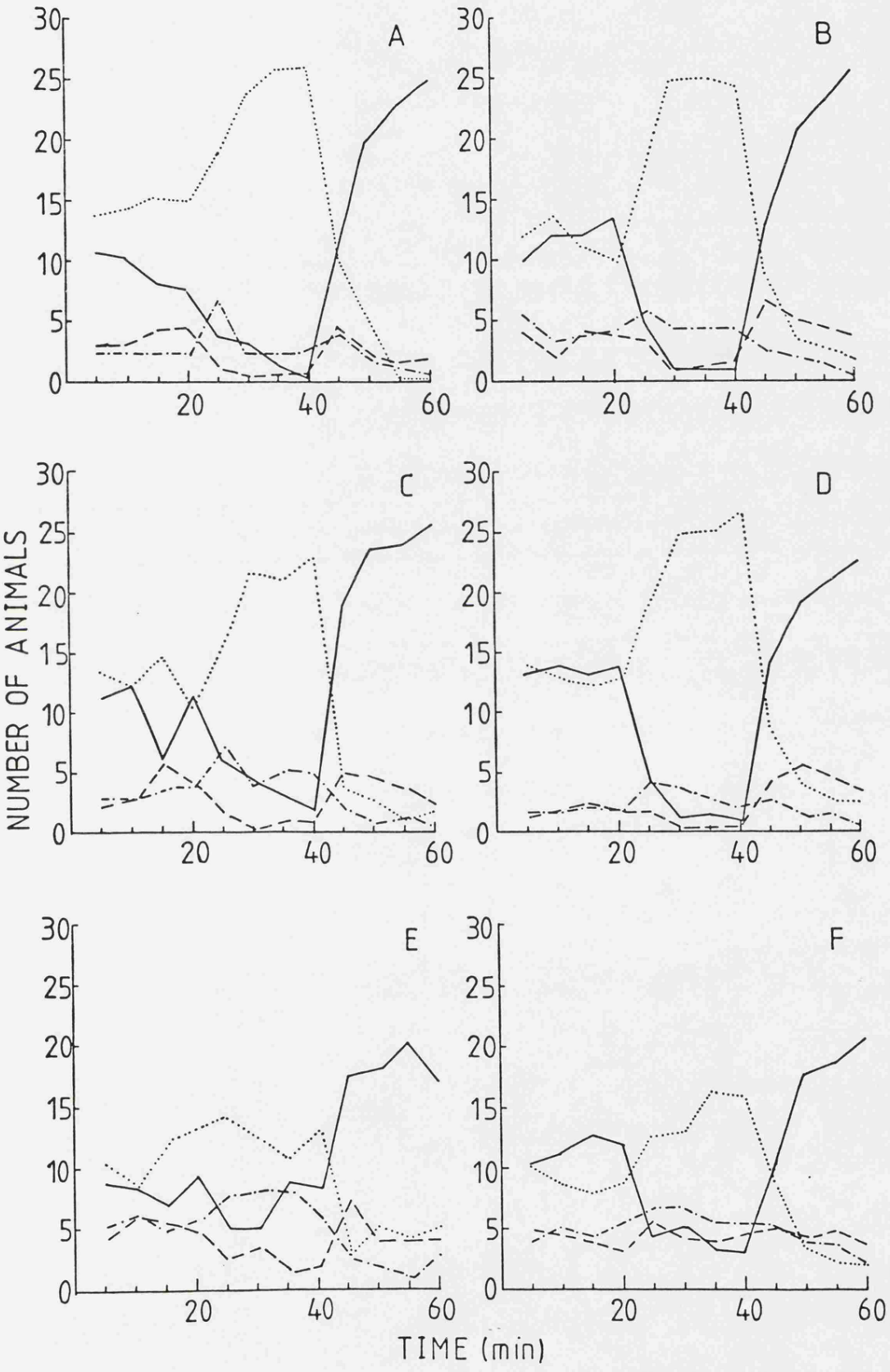


FIG. 7.6

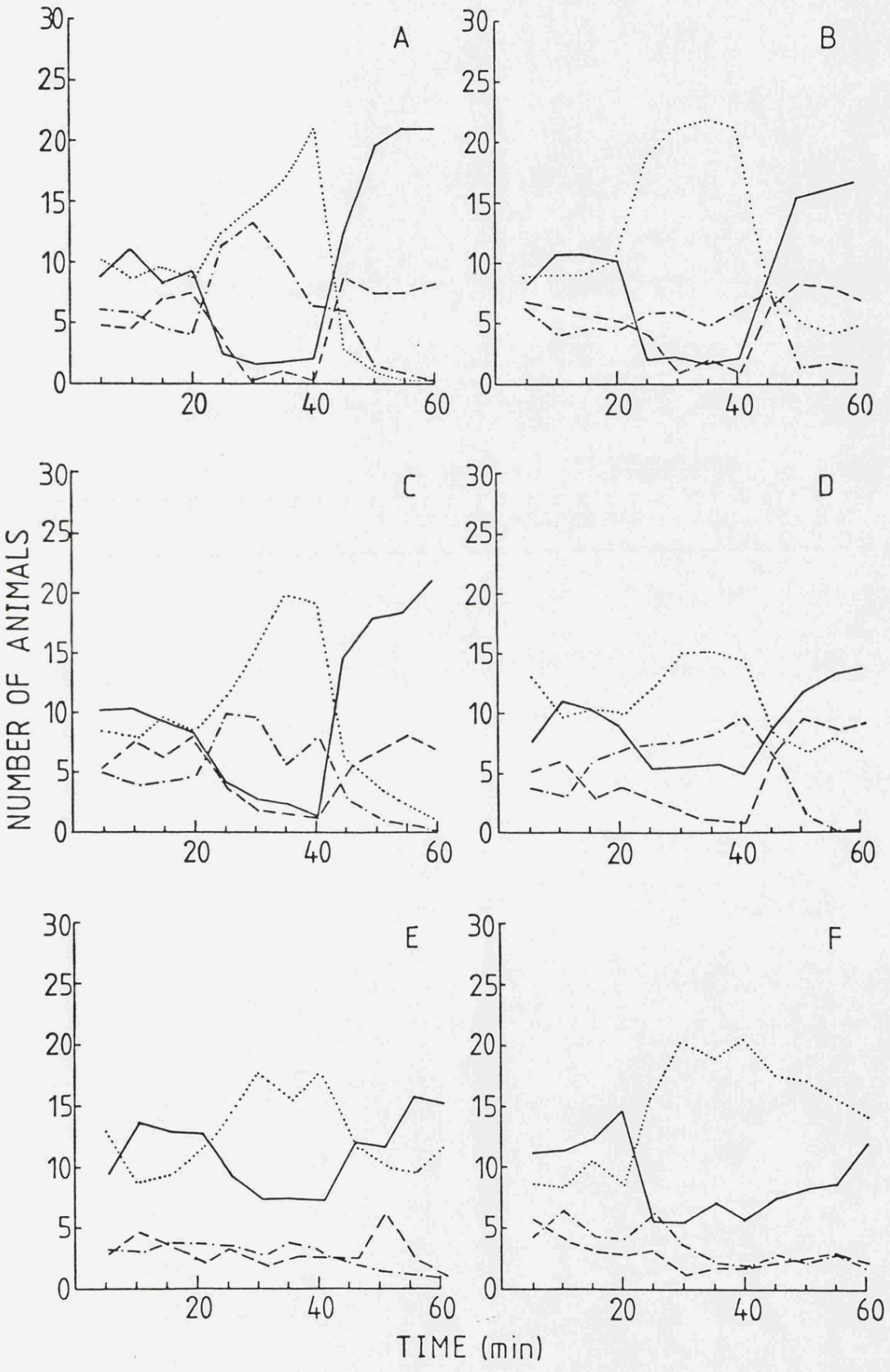


FIG. 7.7

Time (min)		$ZnSO_4.7H_2O$ (μM)										Na_2SO_4 (μM)	
		1000		100		50		10		1		1000	
		CP	GP	CP	GP	CP	GP	CP	GP	CP	GP	CP	GP
Control	5	N	N	N	N	N	N	N	N	N	N	N	N
	10	N	N	N	N	N	N	N	N	N	N	N	N
	15	N	N	N	N	N	N	N	N	N	N	N	N
	20	N	N	N	N	N	N	N	N	N	N	N	N
+Zn/Na	25	**	***	**	**	*	**	N	**	N	*	*	N
LHS	30	***	***	***	***	***	***	**	***	*	*	**	N
	35	***	***	***	***	***	***	**	***	N	N	*	**
	40	***	***	***	***	***	***	***	***	*	N	**	*
+Zn/Na	45	*	N	N	N	*	***	N	N	N	***	N	N
RHS	50	***	**	**	***	***	***	*	***	N	**	N	*
	55	***	***	**	***	***	***	*	***	N	***	N	**
	60	***	***	**	***	***	***	**	***	N	*	N	***

TABLE 7.1

The significant departures from equal numbers of animals within each half of the chamber for control and choice chamber conditions, at four zinc concentrations, using a chi-square test.

CP = *Crangonyx pseudogracilis*
GP = *Gammarus pulex*.

N Not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

On first encountering a 1000 µM zinc solution (Fig. 7.6A), the control behavioural pattern of *G. pulex* was quickly replaced by a period of increased swimming activity, characterized by short exploratory journeys across the interface, resulting in almost immediate retreat back into the control medium. After 5 min, formation of the central interface was completed, and a further behavioural change occurred. Short exploratory journeys were replaced by positive movement into the zinc free end of the chamber, accompanied by a rapid decrease in activity, and a strong tendency to become quiescent around the control medium inlet. Occasional violation of the interface between zinc and control solutions by more active individuals of this species invariably resulted in retreat with no further violations.

As a consequence of brief exposure to this zinc concentration, the control behaviour pattern of this species was therefore replaced by an initial increase in activity, a strong avoidance reaction and a subsequent marked reduction in 'normal' activity. After 10 min of choice chamber conditions most animals had settled around the control medium inlet, and very little positional change was recorded during the remaining 10 min. Those animals remaining within the zinc solution showed increased swimming activity, and appeared to have a faster pleopod beat rate whilst inactive.

After reversal of control and zinc inputs an almost identical avoidance response to the new zinc source occurred. Whilst the reaction of more than 50% of this species, was to instantly withdraw from the new zinc source, a significant avoidance was not recorded over the first 5 min period. This was due to the time taken for the zinc solution to totally permeate the right side of the chamber, since some animals positioned close to the central outlet did not experience the change to zinc solution until 3-4 min after reversal.

The avoidance responses of *G. pulex* to 100μM (Fig. 7.6B), and 50 μM (Fig. 7.6C), zinc solutions were very similar to those described for a 1000 μM zinc solution; a uniform distribution of individuals in each half of the chamber during the initial control period, followed by highly significant avoidance reactions to zinc solution first in the left half and then in the right half of the chamber.

In a 10 μM zinc solution (Fig. 7.6D), *G.pulex* maintained an avoidance response of equal strength to that obtained at higher concentrations, although a generally higher level of activity within the control medium during choice condition, indicated less behavioural modification than at the higher zinc concentrations.

When the test zinc concentration was reduced to 1 μM (Fig. 7.6E), little avoidance response was evinced by *G. pulex* during the first 20 min period of choice chamber conditions. After the choice chamber was reversed an immediate, highly significant avoidance reaction was obtained.

The avoidance response of *C. pseudogracilis* at zinc concentrations down to 50 μM were very similar to those of *G. pulex* (Fig. 7.7A-C). In a 1000 μM zinc solution, however, this species showed less tendency to cluster around the control inlet (Fig. 7.7A) In a 10 μM zinc solution *C. pseudogracilis* showed a much weaker avoidance reaction than *G. pulex* with less preference for the end section of the zinc free half of the chamber, and an almost equal distribution of animals throughout the control medium (Fig. 7.7D). Within a 1 μM zinc solution this species did not show any response that was significantly distinct from control behaviour (Fig. 7.7E).

Avoidance response to a 1000 μM sodium sulphate solution

Introduction of 1000 μM sodium sulphate solution into the test chamber produced an immediate movement of *C. pseudogracilis* into the control medium, although no initial clustering around the inlet pipe was observed, and the activity level generally remained similar to that in a homogeneous control

medium. On chamber reversal no immediate avoidance response was obtained as in the corresponding zinc concentration. After 20 min no further reaction had been obtained, with individuals of this species readily traversing the interface (Fig. 7.7F).

G. pulex responded less strongly than *C. pseudogracilis* on first encountering the 1000 µM sodium salt (Fig. 7.6F) but showed an easily detectable response when the choice chamber was reversed. However, reference to **Table 7.1** shows that the overall avoidance response was markedly less significant than that shown towards a 10 µM zinc sulphate solution (Fig. 7.6D).

Experiment 2: Changes in activity

Control Activity

A computer program was written in Fortran 77 (Appendix 2$), to calculate 7 parameters, chosen to comprehensively describe the control activity of each animal over a 15 min test period;

1) Total distance travelled
2) Mean speed
3) Resting time
4) Mean speed excluding resting time
5) Total number of turns
6) Mean length of journey
7) Time spent in each half of the chamber

The results of this analysis are shown in Fig. 7.8. *G. pulex* was generally more active than *C. pseudogracilis*. All 10 *G. pulex* travelled further than any *C. pseudogracilis*, at higher mean speeds, making longer individual journeys.

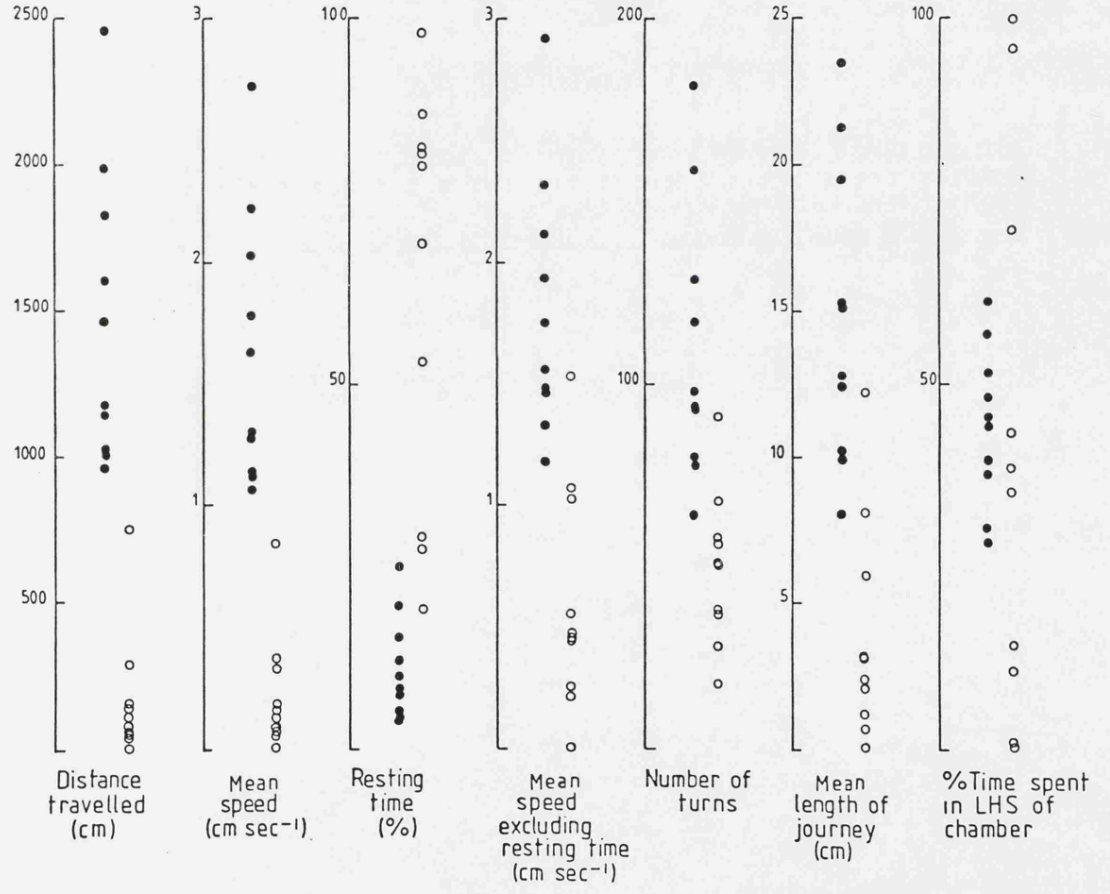


FIG. 7.8

Control activity patterns of ten animals of *G. pulex* ● and *C. pseudogracilis* o, within the experimental chamber.

No individual *G. pulex* was inactive for more than 25% of the test period and therefore the mean speed calculated by excluding resting time differed little from the overall mean speed. In contrast, *C. pseudogracilis* tended to crawl short distances with frequent periods of inactivity. Over half the individuals tested from this species were inactive for greater than 50% of the test time, and therefore the mean speed excluding resting time was correspondingly higher than the overall mean speed. Despite this fact, only one individual attained a mean travelling speed within the range shown by *G. pulex*.

The difference in activity level between the two species was similarly reflected in the time spent in each half of the experimental chamber. *G. pulex* travelled consistently and indiscriminately throughout the chamber. No less than 25% of the total test time was spent by any individual of this species in one half of the chamber. In contrast, the lower activity of *C. pseudogracilis* resulted in several individuals spending nearly all the experimental time in one half of the chamber. This distribution should not therefore be interpreted as indicative of a distinct end preference.

A distinct feature of this experiment is the wide variability of results obtained for different individuals of the same species under identical conditions, and illustrates the caution which must be shown when interpreting subsequent changes in activity in zinc solutions.

The effects of zinc on activity

Three basic parameters were selected as the most useful for quantifying changes in behaviour induced by the presence of zinc.

1) Distance travelled
2) Total time resting (Time spent inactive)
3) Number of turns

From these basic activity measurements it was possible to determine mean speed, mean speed excluding resting time, and length of journey. This restriction of parameters also rendered computer analysis of the results less complex (Appendix 2$).

The initial stage of data analysis was a simple cumulative plot of each of these three parameters against time, for every individual tested. Graphs showing all 10 test animals was produced for each species, in zinc solutions and in a zinc free control, for each parameter. This approach, producing 24 graphs, is illustrated by a single plot of the cumulative number of turns of ten individuals of *C. pseudogracilis* in a test zinc concentration of 1.0 mM (Fig. 7.9a). Every co-ordinate in the data file has been plotted.

Whilst it is possible from Fig. 7.9a to discern a general increase in the cumulative turning frequency approximately 1-2 min after addition of zinc, the magnitude of this increase is obscured by the background control level of activity. The next procedure in the analysis was therefore to average over the initial 10 min control period to obtain a mean control activity value for subtraction from each test data point. Any departure from a straight line along the time axis of the resultant graph thereby indicating a deviation from a mean control activity level.

This procedure has been carried out on Fig. 7.9a and the modified graph is shown in Fig. 7.9b. Between 0-10 min there was little departure from expected activity level, indicating an essentially linear control response. At approximately 11 min, 9 animals showed an increase in turning frequency above the expected control level. A single individual showed little change in control activity up to 15 min followed by a decrease in turning frequency below control level.

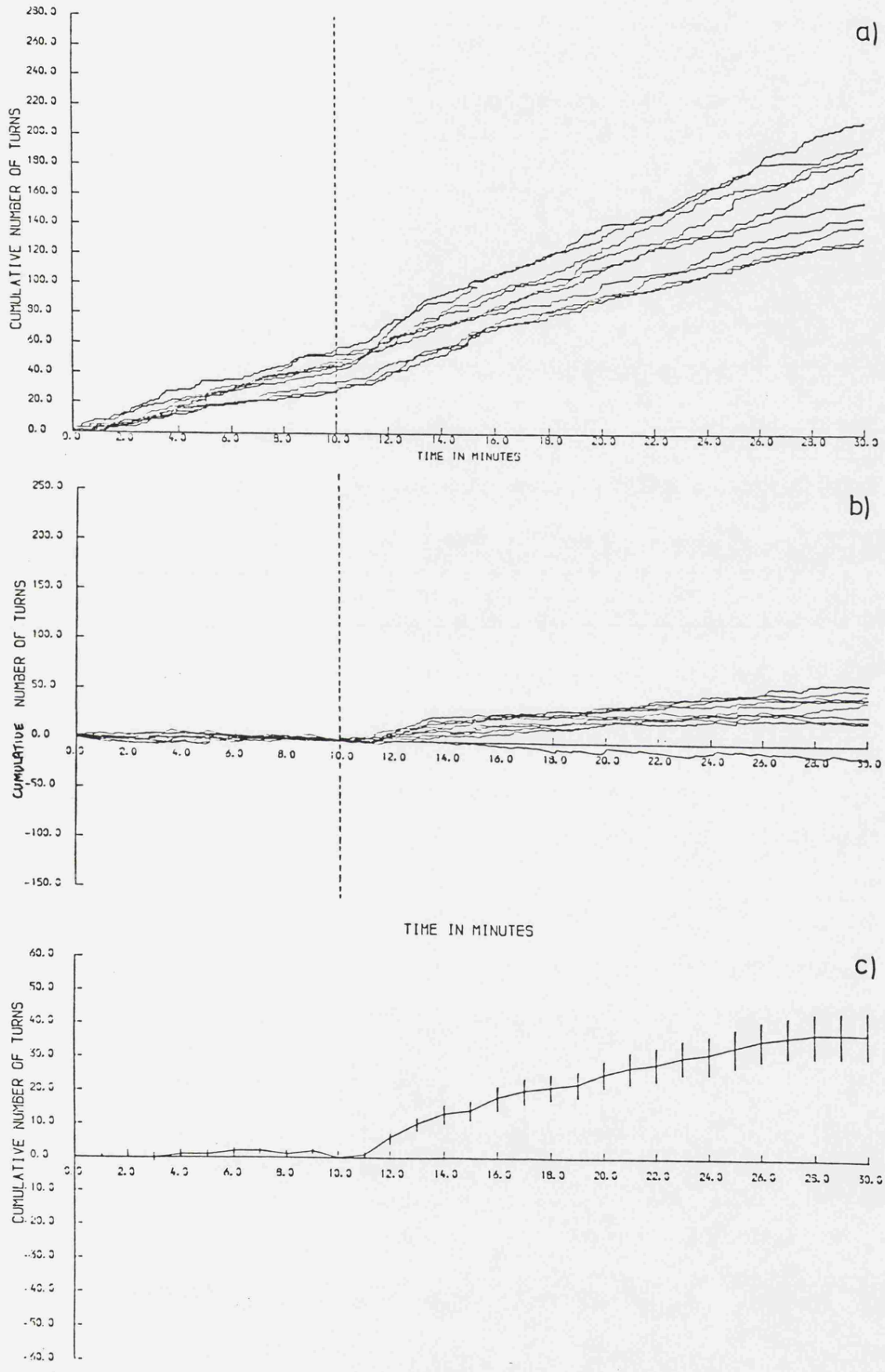


FIG. 7.9
Graphs to illustrate the steps in data manipulation

In order to condense these data into a more convenient form for comparative analysis a single line was plotted corresponding to the mean of all 10 animals. Fig. 7.9b has been redrawn in Fig. 7.9c using this technique against a reduced vertical axis for clarity, since the original data were all drawn to a larger identical scale for comparative purposes. Standard errors are shown as vertical lines at 1 min time intervals. The final stage of analysis was to plot the results for each test concentration on a single graph, with identical scales for both species in order to facilitate comparison. These are shown in Fig. 7.10 (Distance Travelled), Fig. 7.11 (Number of Turns) and Fig. 7.12 (Time spent resting).

In control experiments none of the 3 parameters were expected to change over the duration of the test. Small changes in activity were recorded however, for both species, further emphasising the inconsistent nature of the control response with time. *G. pulex* showed no significant deviation from the expected control distance travelled but turning frequency increased slightly after 15 min of testing and the time spent resting gradually decreased towards the end of the test. In *C. pseudogracilis* a similar increase in turning frequency and decrease in time spent resting was observed, resulting in a small increase in the final distance travelled above that predicted from the initial 10 min of the test.

In a 30 mM zinc solution both species showed marked increases in both distance travelled and turning frequency. The final amount by which both parameters exceeded the predicted control level was almost identical for the two species. Since *C. pseudogracilis* has been shown to have a substantially lower control activity level than *G. pulex* (Fig. 7.8), this similar real increase represents a greater percentage increase above the normal activity level.

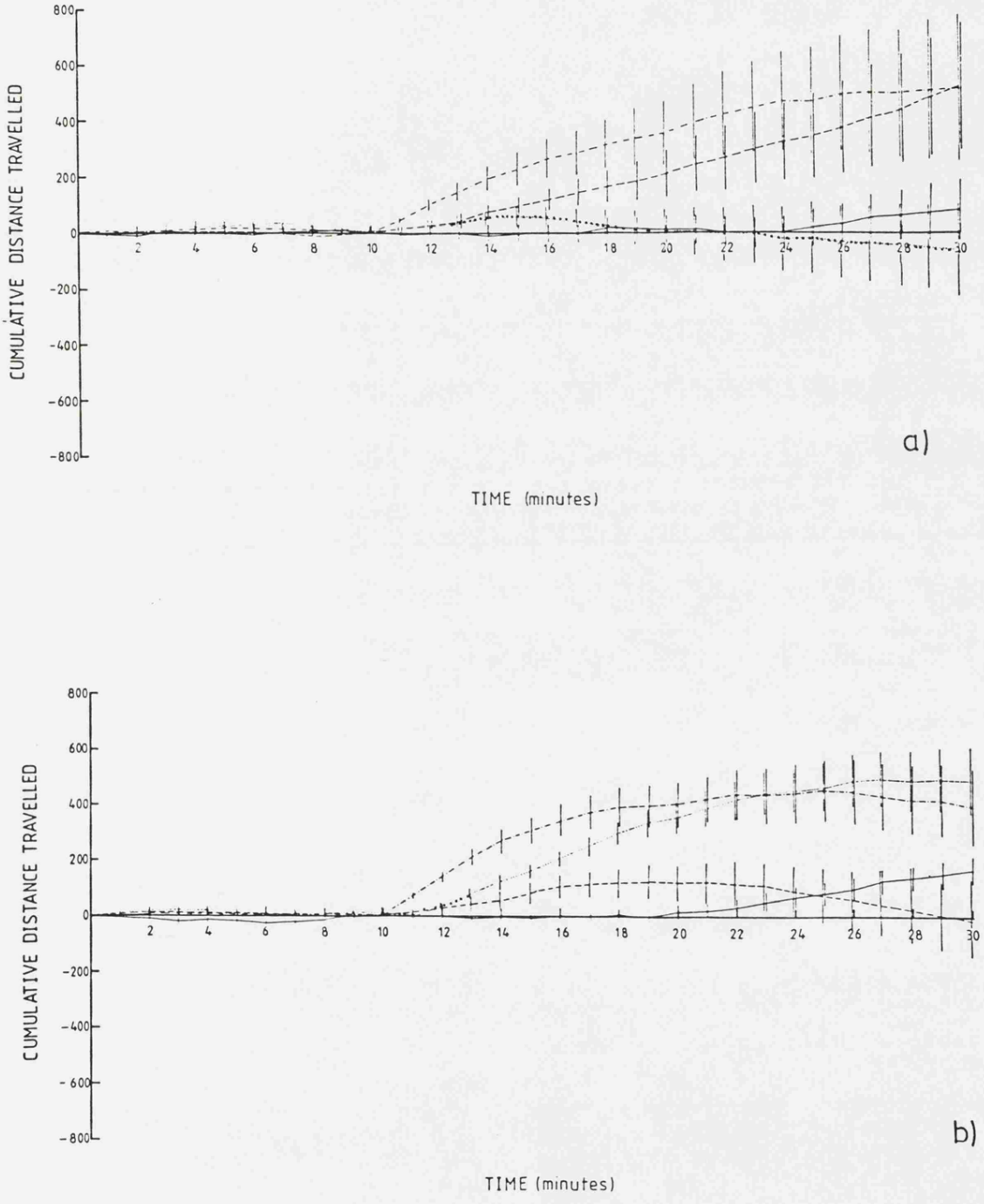


FIG. 7.10

Cumulative distance travelled by *G. pulex* a), and *C. pseudogracilis* b), in 3 zinc solutions and a zinc free control.

---------- 0.1 mM zinc sulphate

..... 1.0 mM zinc sulphate

—·—·—·— 30.0 mM zinc sulphate

———— Control

Error bars indicate standard error of the mean

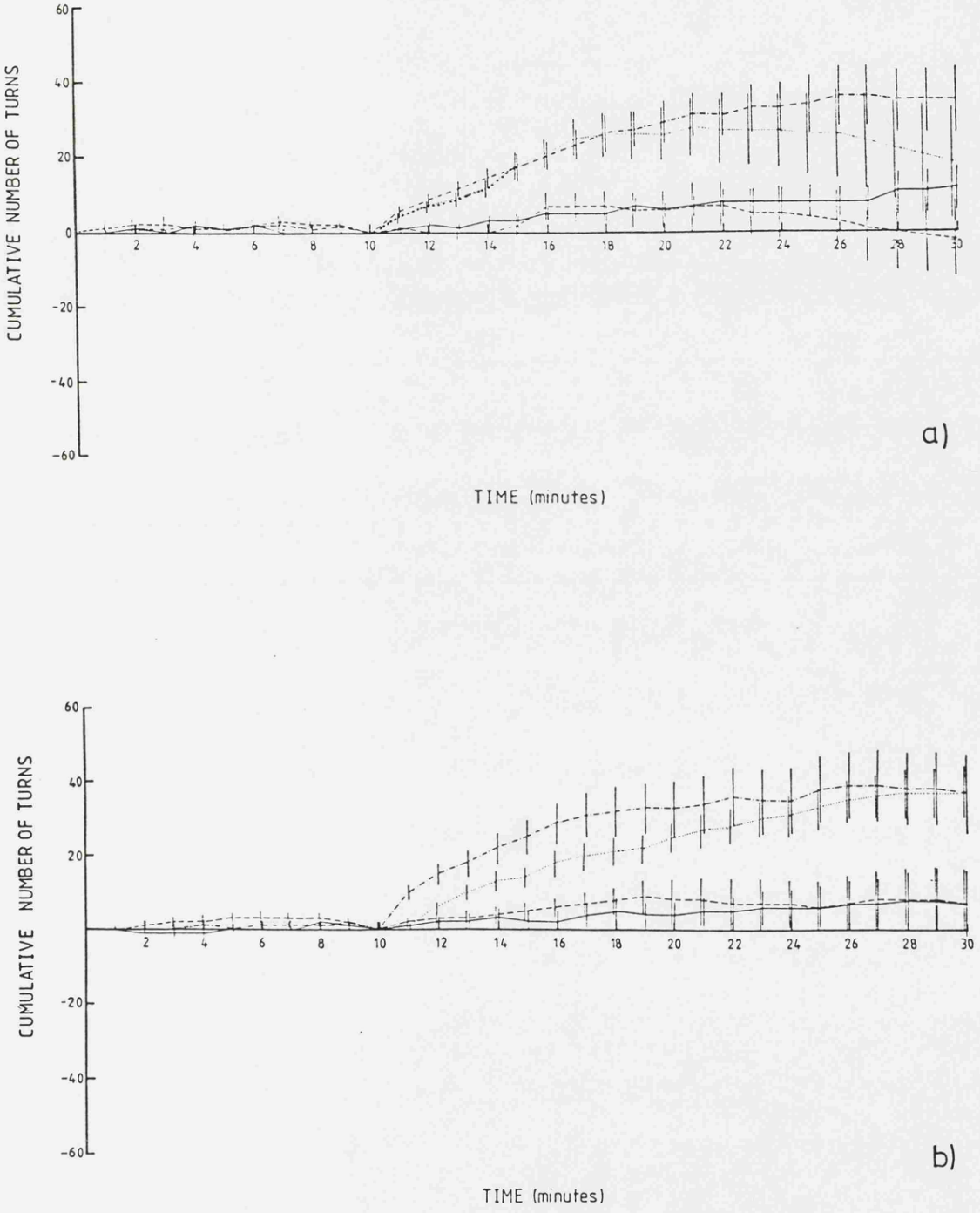


FIG. 7.11

Cumulative number of turns by *G. pulex* a), and *C. pseudogracilis* b), in 3 zinc solutions and a zinc free control.

---------- 0.1 mM zinc sulphate

..... 1.0 mM zinc sulphate

-·-·-·-·-·-·- 30.0 mM zinc sulphate

——————— Control

Error bars indicate standard error of the mean

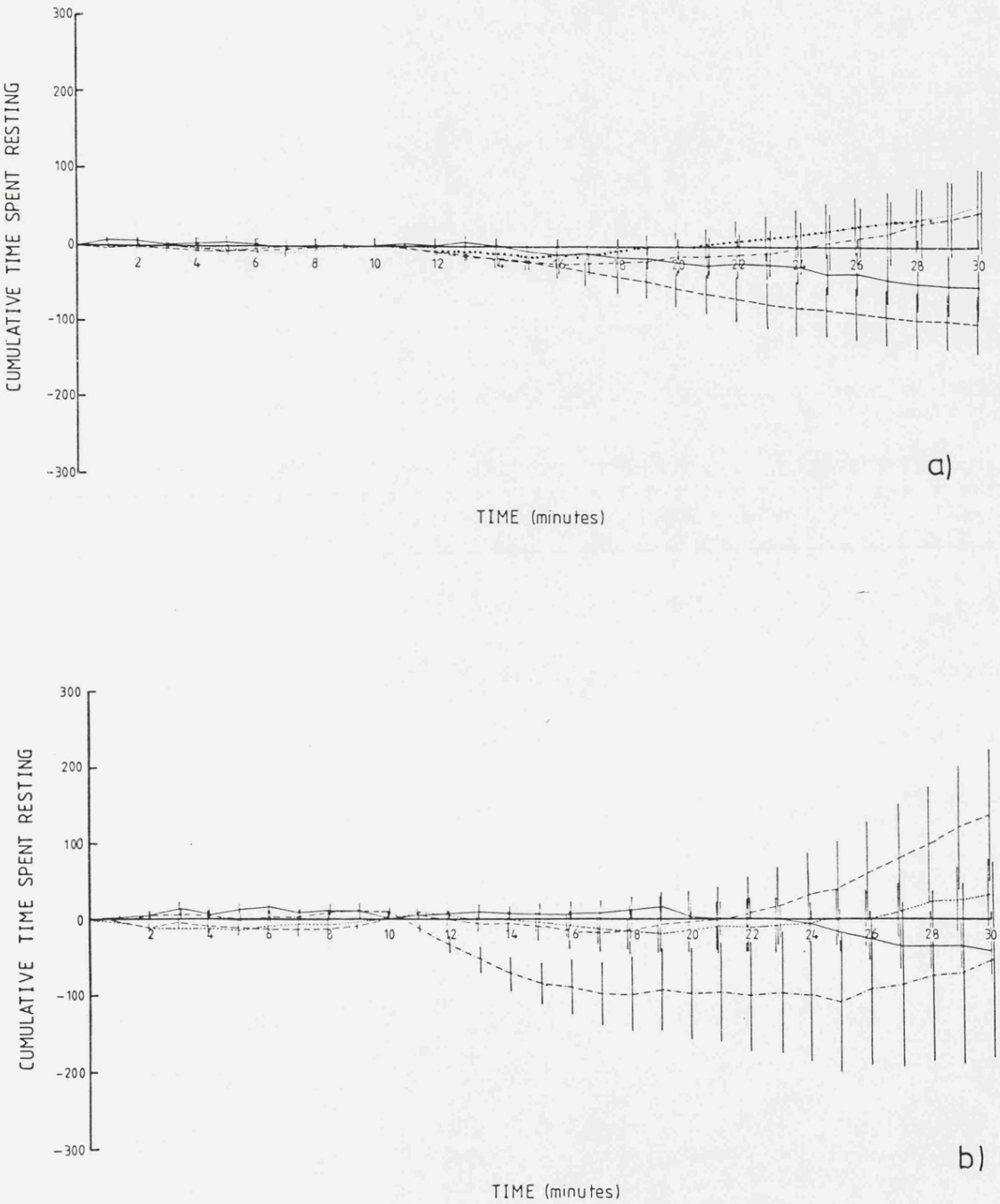


FIG. 7.12

Cumulative time spent resting by *G. pulex* a), and *C. pseudogracilis* b), in 3 zinc solutions and a zinc free control.

---------- 0.1 mM zinc sulphate

..... 1.0 mM zinc sulphate

-.-.-.-.-.-.- 30.0 mM zinc sulphate

———— Control

Error bars indicate standard error of the mean

An increase in distance travelled and turning frequency is indicative of a general increase in activity and therefore resting time was also expected to decrease for both species. Whilst this was true for *C. pseudogracilis* , (although there is a large associated standard error), a similar effect was not seen in *G. pulex* . This specific difference may be explained with reference to the control characteristics shown in Fig 7.8. *C. pseudogracilis* commonly remained inactive for greater than 50% of the control test time and therefore with increasing activity there was potential for a significant reduction in resting time. *G. pulex* characteristically rested for no longer than 25% of the control test time (median value 14%), and therefore less reduction in resting time was possible.

In a 1.0 mM zinc solution the only significant changes in the activity of *G. pulex* compared to the control was an increased turning frequency. In contrast *C. pseudogracilis* showed increases in distance travelled and turning frequency both of which had reached the same magnitude as in a 30 mM solution by the end of the test. Time spent resting was not significantly reduced and therefore the main influence of zinc on activity at this concentration was to increase mean travelling speed.

G. pulex responded to a 0.1 mM zinc solution with a totally different response to that obtained at 1.0 mM. Whilst turning frequency was only marginally higher than in the control the short journeys characterising the response at 1.0 mM were replaced by much longer journeys and a marked decrease in resting time.

In a 0.1 mM zinc solution *C. pseudogracilis* showed an increase in distance travelled and turning frequency in the first ten minutes after addition of zinc, but this was followed by a decrease in activity to give a final distance travelled equal to the control value. Cumulative time spent resting increased towards the end of the test but there was a large associated standard error in the mean measurement.

SUMMARY OF THE EFFECTS OF ZINC ON ACTIVITY

The effects of various zinc concentrations on the activity level of *G. pulex* and *C. pseudogracilis* were variable with large associated standard errors. The dominant trends can, however, be summarised as follows;

Both species became very active in a 30 mM zinc sulphate solution showing increases in both distance travelled and turning frequency. This effect was more pronounced in *C. pseudogracilis* which had a lower initial control activity level. Typical crawling locomotion of this species was almost completely replaced by continuous swimming.

The level of activity in a 1.0 mM zinc sulphate solution was generally lower in both species and distinctly different reactions were obtained. *G. pulex* responded with a slight decrease in average journey length, whilst in *C. pseudogracilis* crawling locomotion tended to be punctuated by periods of continuous swimming.

G. pulex swam almost continually in a 0.1 mM zinc solution with a longer average journey length than under control conditions. This is an apparently anomalous increase in activity level from that observed in a 1.0 mM zinc solution, illustrating that the intensity of the response does not decrease uniformly with decreasing concentration. *C. pseudogracilis* responded initially on introduction of the zinc solution with a slight increase in activity but had reverted to a normal control activity level by the end of the test.

DISCUSSION

Avoidance behaviour

Experimental method

Abel & Green (1981) reported that *G.pulex* taken from the River West Allen (Northumberland), were able to detect a 15μM zinc solution, the lowest concentration at which they were tested. In these experiments the same species has been shown to detect and avoid a 1 μM zinc solution. In comparison, *C.pseudogracilis* showed little avoidance below 10 μM.

This investigation differed from those of Costa (1966) and Abel & Green (op. cit.) in both the design of apparatus and the experimental technique, and therefore whilst the general characteristics of the avoidance response for *G.pulex* were relatively consistent with their results, several differences were detected. The apparatus used in both previous investigations was based on a design for testing fish, in which the flow rate through the experimental chamber was approximately 300 cm^3 min^{-1}. By the use of narrow curved inlets to enhance the even dispersal of incoming medium throughout each half of the chamber it was possible to reduce the flow rate to 50 cm^3 min^{-1} in this study and still achieve a complete solution reversal within 5 min. The currents within the chamber were insufficient to cause any involuntary movement of animals towards the outlet, with both species freely crossing the interface from both directions under control conditions. An additional advantage of this modification was that a 60 min test could be conducted without replenishment of the two 5 litre aspirators, an essential feature for an experiment of this type conducted by a single operator.

The distinct tendency for *G.pulex* to remain longer at the ends of the chamber during the control period of each test was also recorded by Abel & Green (op. cit.). This may be a positive response to the faster flow rate immediately adjacent to the narrow inlets, since more dissolved oxygen

would be supplied across the gill surface per unit time, than in the main body of the chamber. It is however interesting that this trend is also seen in *C.pseudogracilis* which is stated to prefer slow flowing or still water environments (Holland 1976). An alternative explanation for the apparent restriction of this species to lacustrine and slow water environments is that under favourable physio-chemical conditions *G. pulex* will predate *C. pseudogracilis* unless spatial separation is possible. This hypothesis has been explored in Appendix 2.

As the concentration of zinc offered during choice chamber experiments was increased, the avoidance reaction of both species became stronger, and a greater percentage of the time spent within the zinc free medium was occupied by clustering motionless around the inlet. It is therefore possible that above a certain concentration, exposure to zinc increases the oxygen requirement of the animal, possibly due to gill damage or an increase in general metabolic level, which causes it to show a strong affinity for the faster flow rate at the 'zinc free' medium inlet. This trend was not observed by Costa (1966) who reported that after an avoidance response 'normal' behaviour was resumed in the zinc-free half of the chamber.

Simultaneous reversal of zinc solution and 'zinc free' medium was rejected in favour of a two stage process in which zinc solution was totally flushed from one half of the chamber before its introduction into the opposite half. The transition period during simultaneous reversal, in which zinc solution must be present within both ends of the chamber was therefore eliminated. This was found to be especially important at the higher test concentrations where rapid avoidance of the new zinc input occurred before reversal of the choice chamber was achieved. Whilst both Costa (1966) and Abel & Green (op. cit.) used simultaneous reversal, the latter allowed a 3 min equilibration period for reversal to be completed before recording was recommenced. This assumes however, that animals which have been temporarily

prevented from entering the zinc free end of the chamber by a residual band of zinc solution, do not suffer any resultant behavioural modification. Preliminary experiments indicated that under these conditions individuals of both species tended to become confused and repeatedly swam into the zinc solution even after a new interface had formed.

At the lowest zinc concentration offered of 0.1 µM, *G.pulex* showed a clearer and more rapid avoidance response after solution reversal. Costa (1966) recorded the same trend at higher zinc concentrations and attributed it to learning. In contrast *C.pseudogracilis* responded to the first choice chamber period with a weak avoidance reaction but showed no response at all when the solutions were reversed, suggesting that in this species initial sensitivity at low concentrations is reduced with increasing time of exposure.

In addition to differences in the experimental apparatus Abel & Green (op. cit.) recorded avoidance data as the percentage time spent in control and toxic solutions. Whilst this allowed them to show that the time spent in zinc solution decreased with increasing zinc concentration it was necessary to test animals singly and therefore only five replicates were used at each concentration. In this experiment a less concise positional recording technique was used to allow ten animals to be tested simultaneously and a more statistically representative sample to be obtained.

A Summary of avoidance behaviour

The avoidance test is therefore a useful indication of sublethal sensitivity since both *G.pulex* and *C.pseudogracilis* show strong avoidance reactions to zinc concentrations below their previously determined 96 h LC50 values. *G.pulex* can detect and avoid zinc at a concentration at least ten times weaker than that which evokes a response from *C.pseudogracilis*. This difference may partially reflect behavioural as well as sensory differences between the species. Sprague (1968) performed toxicity tests on two

salmonoid species and found that whilst *Salmo salar* stayed predominantly on the bottom of the chamber, *Salmo gairdnerii* swam freely and therefore became more quickly aware of the choice between clean water and toxicant. The difference in toxicant detection between *G. pulex* and *C. pseudogracilis* may therefore be a partial consequence of a similar difference in their normal activity level within the test chamber. In view of the much higher tolerance of *C. pseudogracilis* in acute toxicity tests it has a greater margin for detection of zinc below its lethal limit than does *G. pulex*.

Activity change as an indication of zinc toxicity

Experimental method

Since the majority of behavioural toxicity studies reported in the literature are of the preference/avoidance type there is little activity data available which is directly applicable to this investigation, although some examples of pollutant induced behavioural change in marine macro-invertebrates are given by Olla, Pearson & Studholme (1980).

Several experimental difficulties were encountered in the performance of these tests. In order to obtain a continuous record of activity it was necessary to test animals singly. In consequence, fewer replicate tests could be performed in the time available than would have been desirable for greater statistical significance, with the resulting data showing considerable individual variability.

A further problem was encountered in the presentation of results. In control tests each measured behavioural characteristic was averaged over the duration of the experiment. After addition of zinc solution, however, the behavioural response could no longer be assumed to show a linear relationship with respect to time and therefore it was necessary to record the responses continuously throughout the experiment, producing a large volume of data which did not readily lend itself to statistical averaging procedures.

The results have therefore more value as predictive of possible detrimental toxicant levels rather than an accurate measure of either acute or chronic toxicity.

A SUMMARY OF BEHAVIOURAL TOXICITY TESTING

Two behavioural criteria of toxciity have been independently examined in this series of experiments. First, the ability of an animal to recognise and avoid a toxic zinc solution, and secondly, the behavioural modifications which occur if avoidance is prevented.

The avoidance response was found to be a good indicator of sub-lethal zinc toxicity for both species, having the additional advantage of being much quicker to apply than chronic tests on growth, development or reproductive success. Behavioural modification resulting from continuous exposure to the toxicant was only recorded at lethal zinc concentrations, and was therefore a less useful measure of toxicity than the avoidance response. This was partially attributable to the experimental difficulties associated with performance and interpretation of this type of test.

Under natural conditions a wide variety of environmental factors interact with the organism and toxicant to affect the outcome of a perturbation. For most laboratory studies the link between observed laboratory change and the impact on species dynamics in the ecological system is therefore difficult to ascertain (Olla *et al.* 1980). Such an outcome has been demonstrated by Laughlin (1978). Under natural conditions the juvenile *Callinectes sapidus* (Blue crab), was attracted to areas where storm run off had lowered the pH, but in laboratory tests they actively avoided similar pH levels. Whilst recognising the limitations of laboratory generated data, however, activity and avoidance studies provide a useful basis for further field observations.

CHAPTER 8

PHYSIOLOGICAL INDICES OF TOXICITY

I. OXYGEN CONSUMPTION

INTRODUCTION

According to Sprague (1971), an understanding of the physiological mode of action of a toxicant on an organism is the key to predicting its significant sub-lethal effects. In common with behavioural indices of toxicity, however, physiological studies on aquatic organisms have been primarily concerned with fish. Techniques drawn from biologically based disciplines including histopathology, histochemistry, haematology, biochemistry and physiology have all been employed in fish toxicology studies, to determine the mode and severity of toxicant action. Histology has been the most used of these investigative techniques but at least up to 1971 no detailed histological atlas was available for either fish in general, or for a given species (Sprague op. cit.). Physiology and biochemistry are also central disciplines for understanding toxicant action, with many of the component techniques adapted from the medical sciences (Sprague op. cit.).

A physiological examination of the effects of ionic zinc on the gammaridian respiratory system has been performed as part of this invertebrate toxicity study. Immediately prior to the start of this series of tests the population of *C. pseudogracilis* in Rutland reservoir underwent a rapid decline (see Chapter 3), and only sufficient individuals of *G. pulex* could be obtained for testing. The primary aim of the tests was therefore to assess their potential value as indicators of sub-acute zinc toxicity to *G. pulex*, with the secondary objective of gaining some understanding of the mode of zinc action on this species.

Two experiments to investigate the effects of ionic zinc on the oxygen consumption rate of *G. pulex* are described in this chapter. First, a comparison was made between the normal oxygen consumption rates of animals from Rutland Water, having no history of metal pollution, and animals from Willow Brook which had ceased to receive zinc effluent from Corby Steelworks in September 1980 (Chapter 2). The aim of this test, performed in a static system, was to identify any significant physiological difference between the two populations which might have resulted from the exposure of Willow Brook animals to dissolved zinc.

Secondly, the immediate effects of zinc sulphate solutions on the oxygen consumption rates of groups of animals from Rutland Water was investigated within a continuous flow system. This has been followed (Chapter 9) with a complementary analysis of the effects of identical zinc concentrations on gill ventilation rate of *G. pulex*.

Zinc toxicity and oxygen consumption in fish

As a consequence of the more extensive coverage given to physiological fish toxicity studies in the literature, and their comparative value with the limited number of invertebrate studies, the attempts which have been made to understand physiological and functional respiratory responses of fish to zinc are reviewed briefly.

Early investigations into fish toxicity indicated that in acutely lethal zinc concentrations coagulation and/or precipitation of mucus on the gills, secreted by cells or organs in the gills themselves, caused death by suffocation (Carpenter 1927, 1930). In later studies this effect was regarded as being of only minor importance with death actually resulting from direct damage to the gill membranes by ionic zinc. Separation of the epithelial layer from underlying central pillar cells of the gill lamellae was thought to increase the effective distance over which oxygen must diffuse to reach the blood. Death was therefore assumed to result from

collapse of weakened pillar cell systems and restricted blood flow through the capillaries. (Lloyd 1960; Skidmore 1964; Burton, Jones & Cairns 1972; Matthiessen & Brafield 1973; Hughes & Adeney 1977).

Damage to the respiratory system of fish has been identified under experimental conditions by an increase in respiratory activity, ie, breathing rates, volume and frequency of ventilation, and coughing. Skidmore (1970) in a study of *Salmo gairdneri*, found that in acutely toxic zinc solutions the rate of routine oxygen uptake for a quiet fish was unchanged, but oxygen utilisation decreased seven fold, gill ventilation increased six fold, and heart rate decreased two fold. He therefore suggested that gill permeability to oxygen was decreased, with death resulting from tissue hypoxia, when maximum gill ventilation was insufficient to supply the oxygen needs of the fish. Skidmore (1970) maintained that zinc was not a rapid internal poison to the trout and therefore whether there was histological damage to the gills, mucus accumulation on the gill surface, or a combination of both, respiratory incapacitation was the apparent major cause of acute zinc mortality.

Sparkes, Cairns & Heath (1972) found that *Lepomis macrochirus* (bluegills) responded to a 39 µM zinc solution with an increase in breathing rate and a change in breathing rate variance. Some authors have also described a corresponding decrease in oxygen uptake (Jones 1947b). This reduction was attributed to gill damage, leading to death from hypoxia accompanied by toxic accumulation of lactic acid (Burton, *et al.* 1972; Brafield & Matthiessen 1976).

Chronically toxic levels of zinc invoke a different set of symptoms from acutely toxic effects (Weatherley, Lake & Rogers 1980). Crandall & Goodnight (1963) demonstrated that sustained exposure of *Lebistes reticulatus* (common guppy) to zinc sulphate produced no gill deterioration, but caused other physical stresses such as vacuolated livers, expanded kidney tubules, and underdeveloped spleens. In addition, only 25% of the fish tested reached

sexual maturity. Chronically toxic levels of zinc have, however, been shown to affect fish gills. Matthiessen & Brafield (1973) found marked gill changes in *Gasterosteus aculeatus* in sublethal zinc concentrations. Vacuoles and myelin-like bodies appeared in the gill epithelium, and there was an increase in gill chloride cells. Sublethal studies by Hiltibran (1971) on the effects of zinc, manganese, cadmium, and calcium on the respiration of *Lepomis macrochirus*, and O'Hara (1971) on the effects of copper on oxygen consumption of the same species, both suggest that at sublethal levels, some metal toxicants can inhibit oxygen consumption at the mitochondrial level.

Zinc toxicity and oxygen consumption in invertebrates

Literature concerning the toxic effects of metals on invertebrate respiratory systems is not only less voluminous than that for fish, but also carries a noticeably different emphasis. Such studies have been mainly concerned with accurately recording changes in respiratory performance of the selected test species for bioassay purposes. Less attention has been paid towards clarification of the mechanisms which promote these changes, and consequently there is little reference in the literature to possible modes of toxicant action.

Jones (1941) measured the respiratory response of *G. pulex* to acutely lethal copper solutions and found an initial rise in the respiration rate, probably resulting from increased activity during the first part of the survival time. As the animals became incapacitated the respiration rate decreased accordingly. Hunter (1949) performed a similar study on *Marinogammarus marinus*, but at sub-lethal copper concentrations. and found a reduction in oxygen uptake. Increasing the copper concentration resulted in further depression of uptake rate. In contrast, a sub-lethal mercury solution had no effect on the same species. Hunter (1949) concluded that whilst mercury acted directly by poisoning protoplasm, copper affected the equilibrium of respiratory and/or osmoregulatory systems.

With the specific intention of designing a rapid and sensitive test for the detection of sublethal effects of toxicants, Brkovič - Popovič & Popovič (1977b) determined the rate of tubificid worm respiration over a range of zinc concentrations. An increase in respiration rate was found in a zinc concentration causing 100% mortality in 24 h, but no response was obtained at those concentrations below the 48 h LC50. This test was therefore insensitive at chronic zinc concentrations. Chinnayya (1971) was quoted by Weatherley *et al.* (1980) to have found that exposure of the freshwater shrimp, *Caridina rajadhari* to a 100 µM zinc solution for 30 min resulted in depression of respiration. Reference to the original paper, however, reveals a clear ambiguity between written text and results table, and any interpretation of this work must necessarily be treated with caution.

In a recent study, Costa (1980) described the effect of acutely lethal zinc sulphate solutions on the heart beat rate of *G. pulex*. In tests of 60 min duration Costa found that the heart beat rate rose sharply in 50 mM and 5 mM zinc sulphate solutions followed by a rapid decrease towards the end of the test. In a 500 µM solution the beat rate began to rise after 5-6 min and continued to do so until the end of the test. In a 50 µM solution no change in beat rate occurred for 50 min after which time there was a slight increase. According to the acute tests performed in this study (Chapter 5), a 50 µM zinc solution is approximately equal to the 24 h LC50 of this species. Costa concluded that the immediate aberrations of heart beat were caused by gill damage associated with absorption of toxicant via skin, gills and gasterointestinal tract into the circulatory system, although he offered no direct evidence to substantiate this inference. He also observed rapid and violent pleopod activity and suggested that zinc was in some way causing a reduction in the available oxygen.

Measuring oxygen consumption

Measurement of oxygen consumption for small samples has been performed in a variety of ways. There are, for example, some manometric techniques that give very precise results and these are comprehensively reviewed in Petrusewicz & Macfadyn (1970), and Umbreit, Burris & Stauffer (1972).

However, with most manometric methods the respirometer has to be shaken or the water agitated. Furthermore, the contained volume of medium is small and cannot be changed during measurement. These methods do not therefore fulfil the requirement that the experimental conditions be similar to natural conditions and are consequently not very suitable for ecological purposes. Chemical methods, especially 'Winkler' type methods have been popular in studies of invertebrate respiration (Lindemann 1935; Chinnayya 1971; Cairns, Calhoun, McGinnis & Straka 1976; Correa & Coler 1983), but the accuracy of this technique is readily impaired by the presence of ferrous iron, nitrite and other impurities in the test medium. It is therefore an unsuitable technique except when deionised water is used as the diluent.

In a respirometer with an open flow of medium the possibility of obtaining experimental conditions that are similar to those in nature are greatly enhanced. The medium around the animals is continuously changed, and there is no need to shake the respirometer. In addition the test medium can be changed during the experiment without dismantling the apparatus and stressing the animals.

A measuring technique which permits a continuous reading of the oxygen concentration of the medium flowing into and away from the experimental animals is more favourable than intermittent recordings where some portion of the response may pass unnoticed. Continuous recordings can be achieved with a polarographic technique in which the oxygen tension of the sample is measured electrically.

In view of the obvious advantages over manometric and chemical techniques, an open flow respirometry system incorporating dual polarographic electrodes was constructed for comparing the oxygen consumption rates of *G. pulex* in control and zinc solutions. Respirometric determinations using a polarographic electrode within a closed system were performed on animals from Willow Brook and Rutland Water using the apparatus and facilities of The Gatty Marine Laboratory, St Andrews University. In addition to the main object of this test outlined in the introduction, this enabled a comparison to be made between the respiration rates obtained from using closed and open respirometric systems, with principally similar recording techniques.

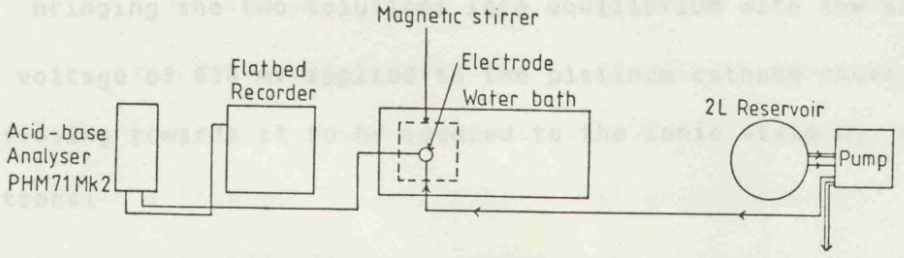
1) MEASURMENT OF OXYGEN CONSUMPTON USING A CLOSED SYSTEM RESPIROMETER

Principle of Operation

The apparatus consisted in principle of a small cylindrical plastic chamber of approximate volume 1.5 cm^3, fitted onto an E5046 polarographic oxygen electrode. Sterile reservoir water was supplied to the chamber throughout the acclimation period by a micro-pump. During operation the chamber was sealed. A continuous measurement of oxygen tension within the chamber was displayed on a flatbed recorder, connected to the electrode via an Acid-base Analyser PHM71 Mk 2, with an additional PHA934 unit (Fig. 8.1a & 8.1b).

Operation of the polarographic electrode

The E5046 polarographic electrode consists of a combined Pt - Ag/AgCl electrode mounted in an electrode jacket, and covered by a 20µm thick polypropylene membrane. A reservoir of phosphate buffer and potassium chloride is enclosed within the electrode.



a)

b)

FIG. 8.1

The Closed System Respirometer.

a) Basic plan of system (not to scale)

b) Photograph of apparatus in operation

A = acid base analyser, F = flatbed recorder,

M = magnetic stirrer, E = electrode, W = water bath

R = reservoir (partially obscured), P = pump

When a water sample is brought into contact with the electrode membrane a difference in partial pressure between oxygen in the sample, and at the cathode surface, causes oxygen to diffuse through the membrane into the electrolyte, bringing the two solutions into equilibrium with the same pO_2. A polarising voltage of 630 mV applied to the platinum cathode causes oxygen molecules diffusing towards it to be reduced to the ionic state by a supply of free electrons;

$$4e^- + O_2 + 2H_2O = 4OH^-$$

The hydroxyl ions formed, combine with KCl in the electrolyte to produce chloride ions;

$$OH^- + KCl = KOH + Cl^-$$

The chloride ions are attracted to the anode where they release an electron and deposit as AgCl;

$$Cl^- = Cl + e^-$$

$$Ag + Cl = AgCl$$

The overall result is a transfer of electrons from cathode to anode, representing a current flow that can be measured, and is proportional to the partial pressure of oxygen in the sample.[1]
This is a consumptive electrode and the water sample must be stirred to prevent local gradients of oxygen concentration from building up around the electrode tip when used within a closed system.

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[1] This is the oxygen tension of the sample, not its concentration.

Procedure

Calibration

The chamber was thoroughly cleansed with 'Milton 2' double concentrated sterilizing fluid (sodium hypochlorite 2% w/v, sodium chloride 16.5% w/v), and filled with oxygen saturated reservoir water. It was then pushed onto the base of the electrode where an airtight seal was formed between the electrode wall, and a thin cylindrical rubber seal around the inner top rim of the chamber. The assembled unit was immersed in an $18\pm1^{o}C$ water bath and positioned immediately above a magnetic stirrer to agitate a small magnetic flea in the bottom of the chamber. The micro-pump was switched off, and the inlet to the chamber clamped firmly. Oxygen tension within the chamber was monitored for 15 min after which time the apparatus was dismantled and the chamber refilled with a standard zero oxygen solution (pO_2 zero solution 54150: Radiometer Copenhagen). A further 15 min record of oxygen tension was taken following an identical procedure.

After calibration of the recording apparatus for 100% and 0% oxygen saturation the chamber was washed thoroughly in distilled water. A single *G. pulex* was placed within the chamber and it was re-fitted onto the base of the electrode, care being taken to ensure that no air bubbles remained attached to the cuticle of the animal. The assembled unit was replaced in the water bath and 5 min allowed for the animal to acclimate to test conditions. During acclimation fresh medium was supplied at a sufficient rate to maintain a fully oxygen saturated environment within the chamber. The pump was then switched off and the chamber isolated for a 15 min recording period.

On completion of the test the animal was removed and weighed damp, and the volume of water within the chamber measured with a micro-pipette.

Successive animals were tested using an identical procedure. Between each determination both chamber and electrode were thoroughly washed, first in sterilising fluid, and then in distilled water.

After the first and last animal of each experimental series had been respired a blank test was performed to ensure that no oxygen utilisation was occurring in addition to that of the animal and the electrode. Finally, the calibration procedure was repeated using saturated and zero solutions.

A total of 10 *G. pulex* from Willow Brook, and 10 *G. pulex* from Rutland reservoir were tested. Animals from each site were respired alternately to equalise any bias in experimental conditions over the test period. An individual respiration rate was calculated for each animal from the volume of water in the chamber, animal weight, and the rate of oxygen utilisation shown on the recorder trace, using a specific computer program supplied by the Gatty Marine Laboratory.

A COMPARISON BETWEEN THE RESPIRATION RATE OF *G. PULEX* FROM WILLOW BROOK AND RUTLAND WATER

The individual species specific respiration rates (S.S.R.R.) for *G. pulex* from Rutland Water and Willow Brook are given in Table 8.1. Only weak correlation was found between fresh weight (mg), and respiration rate ($\mu l\ O_2\ h^{-1}$) for both populations. (Willow Brook $r = 0.552$; Rutland Water $r = 0.721$). This may result in part from an intermittent fault in the electronic balance used to obtain the fresh weight of the animals, which was not detected until after the experiment was completed.

The mean respiration rate of $1.815 \pm 0.163\ \mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$ for animals from Willow Brook was significantly higher than that of $1.2060 \pm 0.110\ \mu l\ O_2$ mg dry $wt^{-1}\ h^{-1}$ for animals from Rutland Water (Mann Whitney $0.002<P<0.02$; 't' test $0.02<P<0.05$).

FRESH WT (mg)	RESPIRATION RATE (µl h^{-1})	S.S.R.R (µl O_2 mg fresh wt^{-1} h^{-1})	S.S.S.R (µl O_2 mg dry wt^{-1} h^{-1})
		WILLOW BROOK	
11.50	6.5451	0.5691	2.484
12.50	6.2929	0.5034	2.197
13.00	2.6426	0.2033	0.887
14.00	4.5009	0.3215	1.403
14.80	4.3642	0.2949	1.287
15.00	6.7926	0.4528	1.981
16.10	7.1882	0.4465	1.949
17.20	9.4117	0.5472	2.388
17.80	8.2387	0.4628	2.020
20.00	7.1048	0.3552	1.550
		RUTLAND WATER	
10.90	2.6515	0.2432	1.062
13.04	2.3800	0.1825	0.797
13.74	3.9356	0.2864	1.250
13.92	3.9477	0.2836	1.238
14.22	4.5335	0.3188	1.392
14.48	2.5244	0.1743	0.761
14.73	3.4056	0.2312	1.009
15.22	5.4470	0.3579	1.562
18.24	7.9660	0.4367	1.906
19.03	4.7243	0.2482	1.083

BAROMETRIC PRESSURE 775.0 mm Hg.

TABLE 8.1

Species specific respiration rates for G. pulex from Willow Brook and Rutland Water.

2) MEASUREMENT OF OXYGEN CONSUMPTION USING AN OPEN FLOW RESPIROMETER

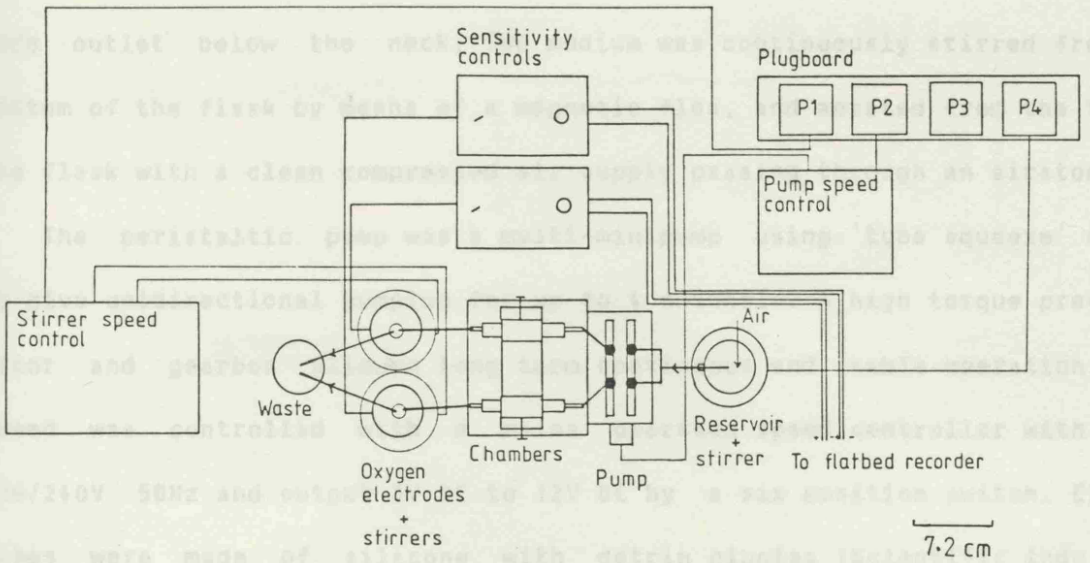
Principle of operation

Test medium was pumped unidirectionally through two identical test chambers, one of which contained test animals, and the other acted as a blank control. The oxygen content of medium leaving both chambers was measured with a polarographic electrode. While passing the animals, the oxygen content of the medium was reduced. This reduction corresponded to the difference between the electrode current in the medium measured leaving the test chamber, and that measured leaving the control chamber. The animals' oxygen consumption per unit weight was determined on the basis of percentage oxygen reduction, the quantity of water that had passed, and the total weight of animals within the respirometry chamber.

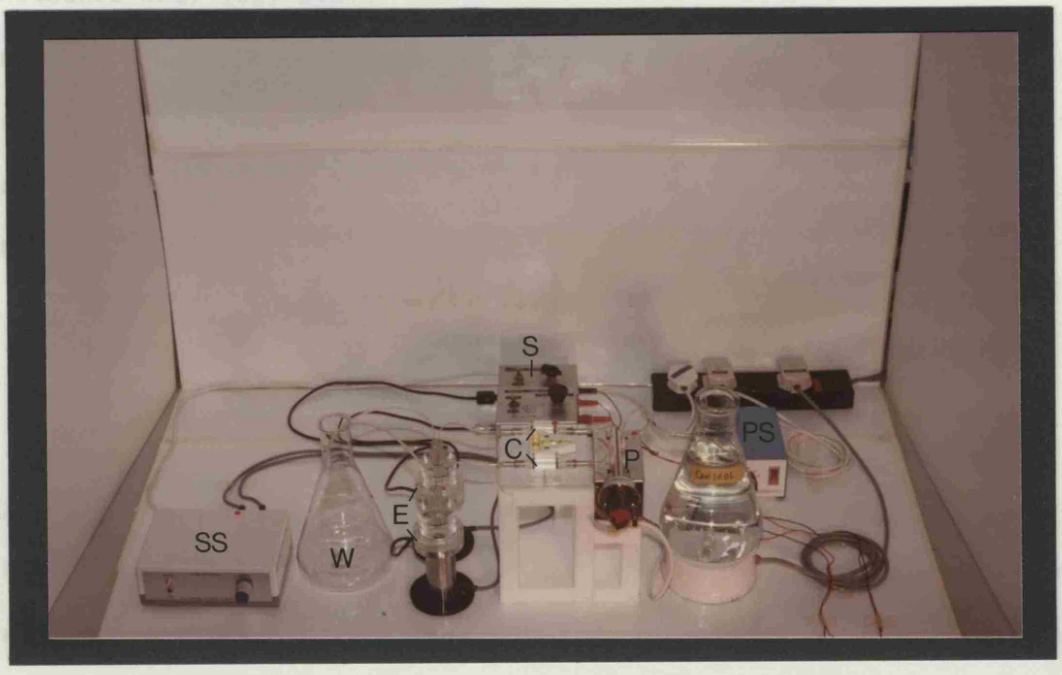
Construction of the respirometer

The principle features of the apparatus are shown in Fig. 8.2a, & 8.2b, and are based on a design by Nagell (1975) for testing the oxygen consumption of Plecoptera and Ephemeroptera larvae. There were four main components; a container of experimental solution, a peristaltic pump, dual respirometry chambers, and two corresponding Rank oxygen electrodes. These components were linked together by a system of short Esco rubber tubes (1 mm i.d.) and stainless steel 'T' junctions.

Test medium was withdrawn from the reservoir through a single tube which divided before entering the peristaltic pump to provide an individual supply for each respirometry chamber, and its corresponding electrode. On leaving the electrode outlet the medium was discarded as waste. An equal flow rate through both respirometry chambers was attained by the identical construction of each half of the system. The rate at which water passed through the two electrodes during measurements was theoretically always the same.



a)



b)

FIG. 8.2
The Open Flow Respirometer.
a) Basic plan of the system
b) Photograph of apparatus in operation
SS = stirrer speed control, S = sensitivity control
W = Waste, E = electrodes, C = chambers, P = pump
PS = pump speed control

The reservoir for test medium was a 1 litre vacuum flask with a narrow bore outlet below the neck. The medium was continuously stirred from the bottom of the flask by means of a magnetic flea, and aerated from the top of the flask with a clean compressed air supply passing through an airstone.

The peristaltic pump was a multi-minipump using 'tube squeeze' action to give unidirectional pumping for up to 4 channels. A high torque precision motor and gearbox allowed long term continuous and stable operation. Pump speed was controlled with a mains operated speed controller with input 220/240V 50Hz and output 3V DC to 12V DC by a six position switch. Element tubes were made of silicone with detrin nipples (Scientific Industries International inc. (UK) ltd).

The two respirometry chambers having identical dimensions were each connected to a modified Rank electrode (Rank Bros). Operation of this polarographic electrode was in principle similar to that described earlier in this section and details of the modifications are discussed below. The whole apparatus was enclosed within a polystyrene hood having a clear plastic front for inspection purposes.

Constructional details of experimental chambers and electrodes

The respirometry chambers were made from rectangular perspex blocks, internally machined to produce a cylindrical chamber (volume 1963.5 mm^3), designed to accommodate 10-15 *G. pulex* or *C. pseudogracilis*. Each end of the chamber had a wider cylindrical opening to accommodate a perspex plug with a short stainless steel outlet pipe (5 mm i.d.) for connection into the system. A thin cylindrical rubber gasket, 2 mm from the end of the plug made an airtight fit against the chamber wall. The stainless steel outlet pipe of each plug was fitted with a small silicone bung having a stainless steel pipe of equal diameter to the connective tubing. Fine mesh gauzes, clamped between the plug and main chamber, prevented escape of the test animals (Fig. 8.3).

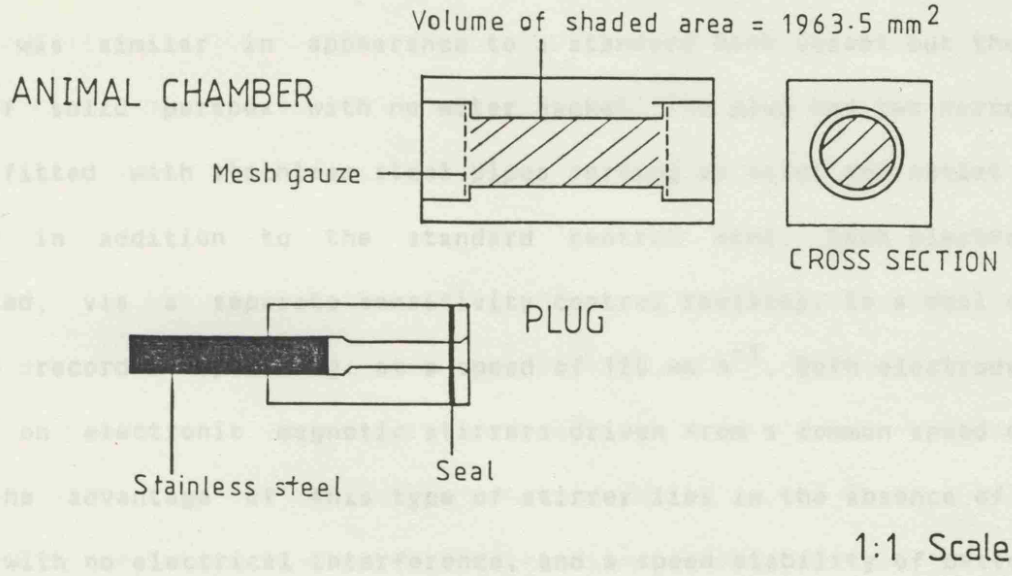


FIG. 8.3
Diagrammatic representation of chamber components.

Both chambers were mounted on a perspex block with a centrally placed spirit level to ensure that they remained horizontal during operation (Fig. 8.4).

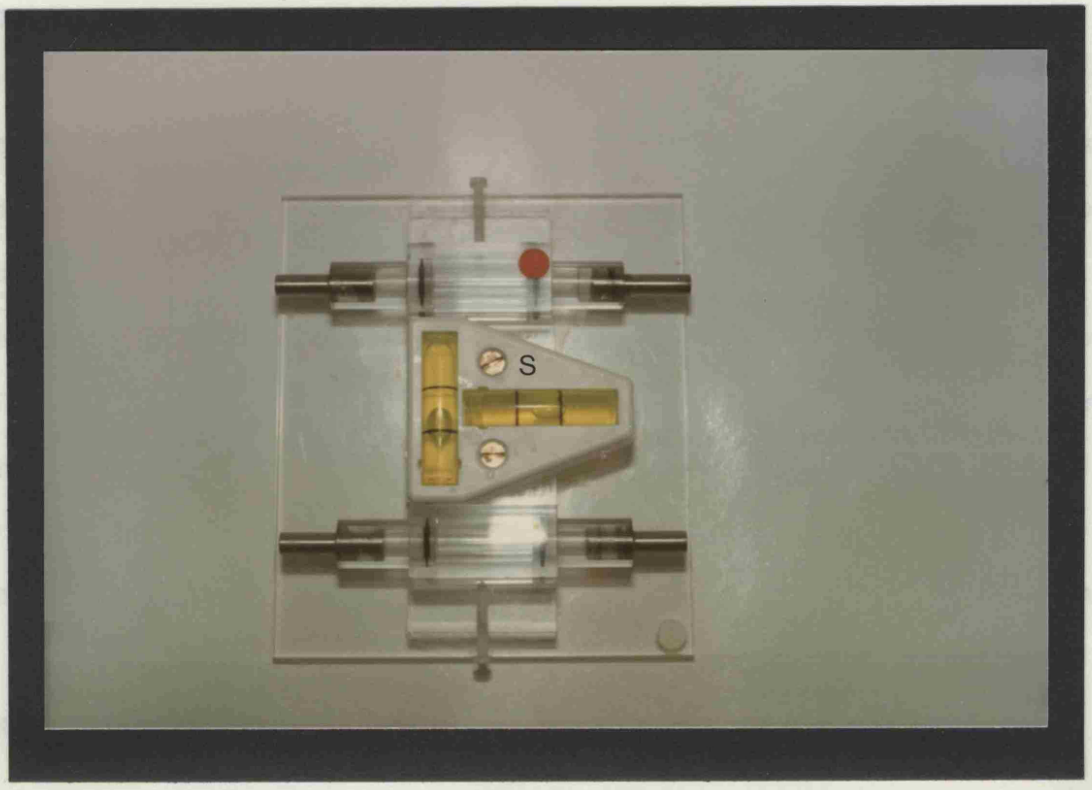


FIG. 8.4
Test chamber and control chamber mounted on a perspex base. S = spirit level

Each electrode comprised a standard Rank electrode base and a perspex locking nut, with a modified incubation vessel and plug. The incubation vessel was similar in appearance to a standard Rank vessel but the walls were of solid perspex with no water jacket. The plug had two narrow bore tubes fitted with stainless steel pipes serving as inlet and outlet to the chamber in addition to the standard central vent. Each electrode was connected, via a separate sensitivity control resistor, to a dual channel flatbed recorder operating at a speed of 120 mm h^{-1}. Both electrodes were seated on electronic magnetic stirrers driven from a common speed control box. The advantage of this type of stirrer lies in the absence of moving parts with no electrical interference, and a speed stability of better than 1%. The volumes of different sections of the respiratory system were used in combination with pump speed (determined experimentally), to calculate the time taken for water to travel throughout different sections of the system.

Procedure

The recording apparatus was calibrated for 100% and 0% saturation within the test chambers using saturated reservoir water and sodium dithionate solution respectively. The experiment was started when both electrodes recorded an identical response on the flatbed recorder.[2]

The vacuum flask was filled with 1 litre of fully saturated reservoir water and the pump switched on. Both chambers were held vertical during filling of the apparatus to prevent air from remained in the system. The inlet gauze between the plug and chamber was prodded gently with a glass rod to eliminate trapped air bubbles. As soon as each chamber was completely full, the outlet mesh was lowered into place with a pair of forceps and the outlet plug fitted into place.

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[2] Obtaining identical responses from the electrodes often required changing of membranes or adjusting the seating of the electrode on the stirrer.

The electrode plugs were pushed gently into place, ensuring that no air bubbles remained in the electrode chamber. The central vent was then plugged with plasticine and the electrode outlet pipe connected to an overflow dish. Pump speed was adjusted to produce an approximate flow rate of 0.2 cm^3 min^{-1} through both sides of the system. The apparatus was left to operate in this way for 1 h.

After 1 h the chamber to receive animals was quickly dismantled at the outlet plug and ten *G. pulex* each approximately 10 mm in length, introduced into the chamber with a wide bore glass pipette. Precursory experimentation had indicated that if the animals were too small eg. 20 *C. pseudogracilis* (♂), the largest reduction in oxygen saturation which could be achieved was too low to record accurately. If the test animals were too large eg. *G. pulex* > 15 mm long, the smallest reduction in oxygen saturation which could be achieved exceeded 70%, cannibalistic tendency was enhanced, and a stable oxygen saturation recording could not be obtained.

During introduction of the animals the outlet pipe to the electrode was plugged with a small piece of plasticine to prevent air from entering the electrode chamber. The chamber was then re-introduced into the system. Any air bubbles which had accidently entered the system during addition of the animals were eliminated from the electrode chamber via the central outlet vent in the plug. The test was continued for a further 3 h during which time the flow rate through both halves of the apparatus was measured by replacing the container at the outlet to the system with a measuring cylinder.

After 3 h, pumping was halted momentarily and the reservoir substituted for an identical vessel containing fully oxygen saturated solution of known zinc concentration. The test was then restarted for a further 4 h. On completion of the test the animals were removed for fresh weight and length determinations to be made. The apparatus was reassembled and allowed to run for a further 1 h. Six replicate tests on groups of 10 animals were performed at zinc concentrations of 500 µM, 100 µM, and 50 µM.

In order to reduce the level of experimental inaccuracy the following conditions were observed;

1) If either electrode failed to maintain a stable, reading of oxygen saturation during the initial control period of 1 h, the apparatus was dismantled and re-calibrated.

2) If the oxygen saturation level of the water leaving the animal chamber fell below 30% at any time during the test the experiment was discontinued.

3) If a desaturation exceeding 2% was recorded in the control chamber as a consequence of the change to a less well oxygenated zinc solution the experiment was discontinued.

4) If the oxygen saturation level within the electrode serving the animal chamber did not return to within $\pm$2% of that within the control chamber when the apparatus was reassembled for the final 1 h, the experiment was disregarded.

Calculation of fresh v dry weight ratio

Immediately after testing, all animals were blotted gently with absorbent tissue for 15 sec, and placed on a Sartorius balance (accuracy of $\pm$0.1 mg). The corresponding dry weight values were obtained by determining the mean water content of the specimens using the following procedure.

Fifty adult individuals of various sizes were weighed damp using the above procedure, and placed in foil pans of known weight. An initial drying period of 8 h at 60^{o}C in a normal oven was followed by 12 h at 60^{o}C in a vacuum oven operating at a pressure of 400 mm Hg. On removal from the oven after a cooling period, the animals were placed in a desiccator and transported to the balance for re-weighing.

A linear regression was performed of fresh weight against dry weight (Fig. 8.5), from which a percentage water content of 77.1 was obtained. This standard water content was then used for converting an experimentally determined fresh weight of *G. pulex* to a corresponding dry weight value. It is in good agreement with a figure of 79.8 ± 0.62 given for the same species by Sutcliffe (1971).

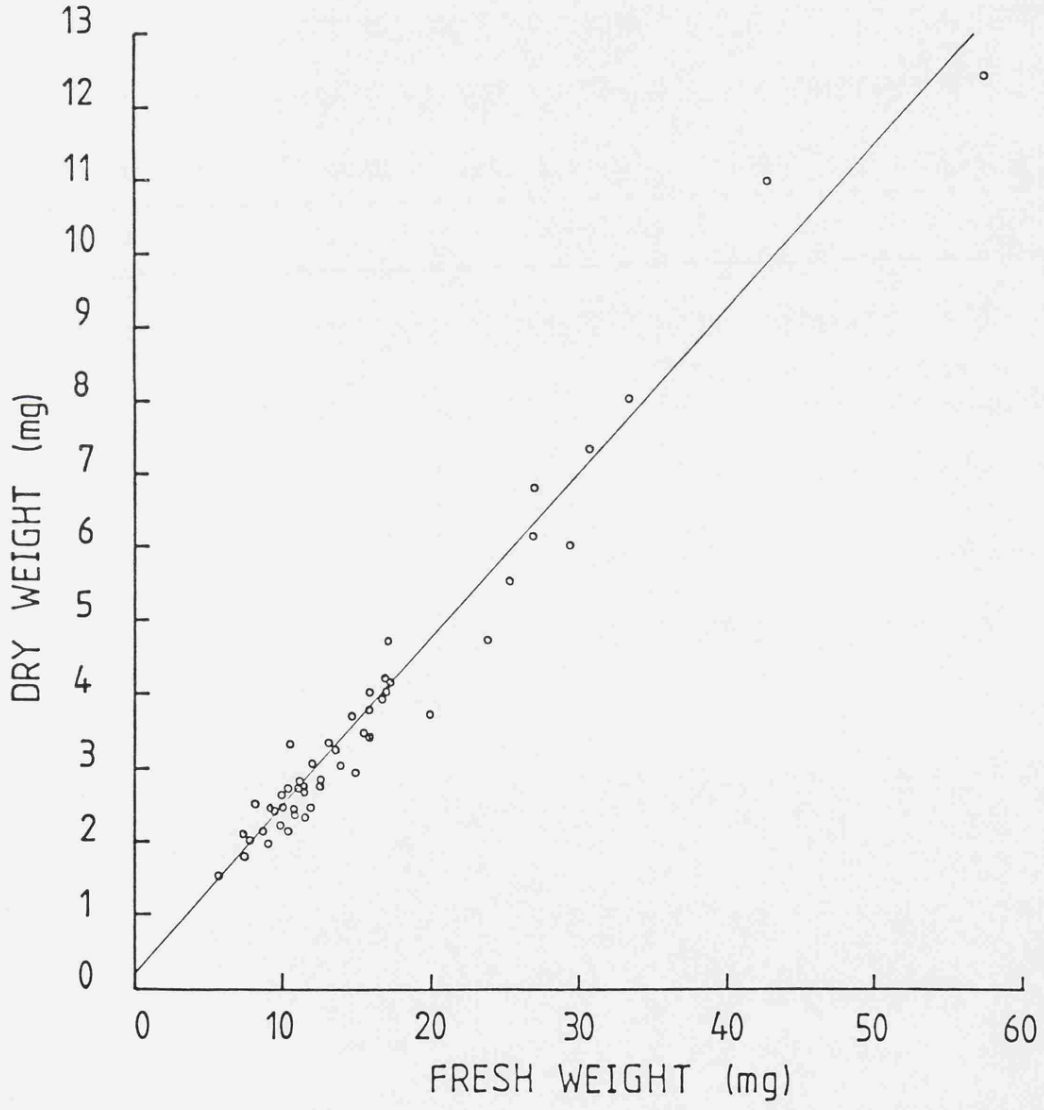


FIG. 8.5
Graph of fresh v dry weight for 50 *G. pulex*.
slope = 0.2272, intercept = 0.1801, r = 0.9842
Water content of *G. pulex* = 77.1%

ACTIVITY OF *G. PULEX* WITHIN THE RESPIROMETER

On introduction into the respirometry chamber at the start of each experiment, *G. pulex* swam continuously for the first 15 - 20 min of the control period, after which time they showed a distinct tendency to cluster on the mesh at the inlet and outlet to the chamber. In a 50µM solution no marked behavioural response was detected, either on introduction of the zinc solution, or throughout the remainder of the test. In a 100 µM zinc solution there was an initial increase in swimming activity lasting approximately 30 min. After this time the test animals resettled on the mesh pads at inlet and outlet to the chamber. In a 500 µM zinc solution there was a marked increase in initial activity which gradually decreased towards the end of the test when several individuals became incapacitated on the bottom of the chamber. The tendency to cluster on the mesh was less marked than during the control period.

THE EFFECT OF ZINC ON THE OXYGEN CONSUMPTION OF *G. PULEX*

The initial data produced from this series of experiments were six 7 h records for each of three experimental zinc concentrations, displaying the simultaneous percentage oxygen saturation within the control chamber and the chamber containing 10 *G. pulex*.

Despite the insulatory function of the polystyrene jacket, a temperature fluctuation of $\pm 1^{o}C$ (corresponding to the regular operation of the fan within the constant temperature room) was recorded in the air surrounding the test apparatus. This was detectable as a small regular oscillation ($\pm 1\%$) in the control oxygen saturation record. The corresponding record of oxygen saturation within the chamber containing the animals was corrected for a constant temperature of $18^{o}C$, and the oxygen saturation value for each 5 min time interval stored in a data file on the Cyber 73 computer.

A computer program was written in Fortran 77 utilising GHOST80 plotting functions, to reproduce the 18°C oxygen saturation curves for all six replicate tests at each of the three zinc concentrations (Fig. 8.6, 8.7 & 8.8). The flow rate through the apparatus during each test, and the mean length and fresh weight of the 10 animals is given in the accompanying legends.

The oxygen saturation curves for the 3 h control period of each test followed an essentially similar trend. In the first 30-50 min, the percentage oxygen saturation showed a sharp decrease (as compared with the control chamber). This effect was caused by gradual replacement of oxygen saturated water in the electrode chamber by water which had passed over the test animals.

The time taken to completely fill the electrode chamber was dependent on the flow rate and therefore the difference between individual tests shown in Fig. 8.6, 8.7 & 8.8 was the result of differences in the flow rate between individual experiments. With a lower flow rate the time taken for the saturation curve to reach a lower asymptote increased and the amount by which the medium was desaturated by the animals increased correspondingly.

In all tests the lower saturation was reached within 1 h, and the remainder of the control period measured only the oxygen consumption of the animals. In some tests the initial fall in oxygen saturation was followed by a steady rise to a higher level (Fig. 8.6-·-·-·-·-; Fig. 8.7------). In general, the percentage saturation varied during the control period by approximately ±2.5%, with some tests showing good stability (Fig. 8.7 ———), and others oscillations in percentage saturation of nearly 10% (Fig. 8.6 ———). The possible cause of these variations is discussed later.

FIG. 8.6, 8.7 & 8.8 (over page)

Graphs of the percentage oxgen saturation of water leaving the chamber containing 10 *G. pulex*, against test duration in hours.

8.6) 0-3 h = control period
3-7 h = 50 µM zinc solution

run	line	Flow rate ($l\ h^{-1}$)	mean animal length (mm)	mean animal weight (mg)
1	·—·—·—·	0.0152	7.9	13.4
2	— — — —	0.0138	9.2	18.2
3	- - - - -	0.0140	7.9	15.1
4	— — — —	0.0196	7.9	15.4
5	- - - - - - - -	0.0162	8.2	13.7
6	————	0.0094	8.9	17.1

8.7) 0-3 h = control period
3-7 h = 100 µM zinc solution

run	line	Flow rate ($l\ h^{-1}$)	mean animal length (mm)	mean animal weight (mg)
1	·—·—·—·	0.0133	7.3	9.6
2	— — — —	0.0158	8.8	15.2
3	- - - -	0.0148	8.1	13.5
4	— — — —	0.0080	8.8	21.1
5	- - - - - - -	0.0066	9.5	22.0
6	————	0.0071	8.5	17.5

8.8) 0-3 h = control period
3-7 h = 500 µM zinc solution

run	line	Flow rate ($l\ h^{-1}$)	mean animal length (mm)	mean animal weight (mg)
1	·—·—·—·	0.0163	9.5	15.0
2	— — — —	0.0171	9.1	15.4
3	- - - - -	0.0141	9.0	15.0
4	— — — —	0.0136*	8.7	14.0
5	- - - - - - - -	0.0136	8.1	13.9
6	————	0.0083	7.9	18.7

* no record for 30-60 min

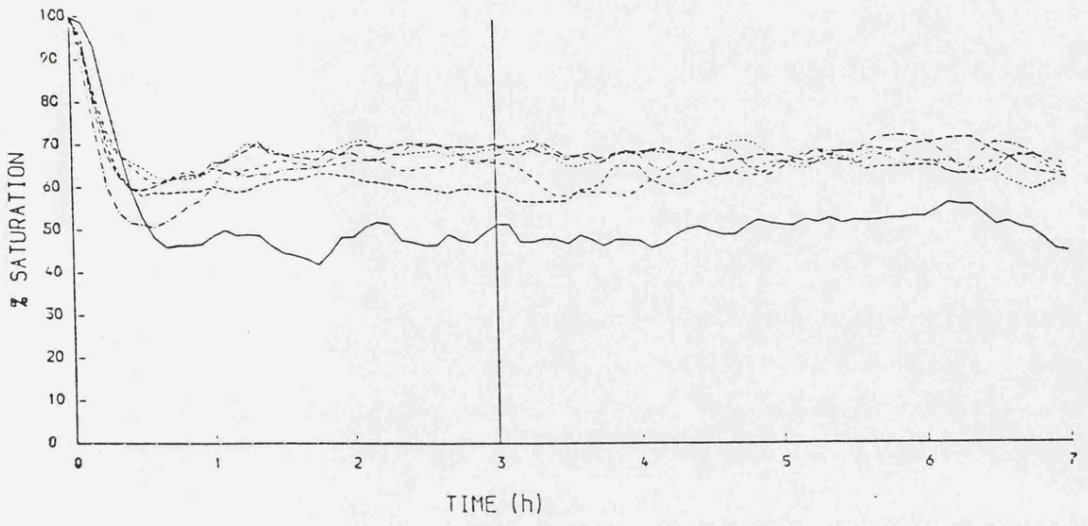


FIG. 8.6

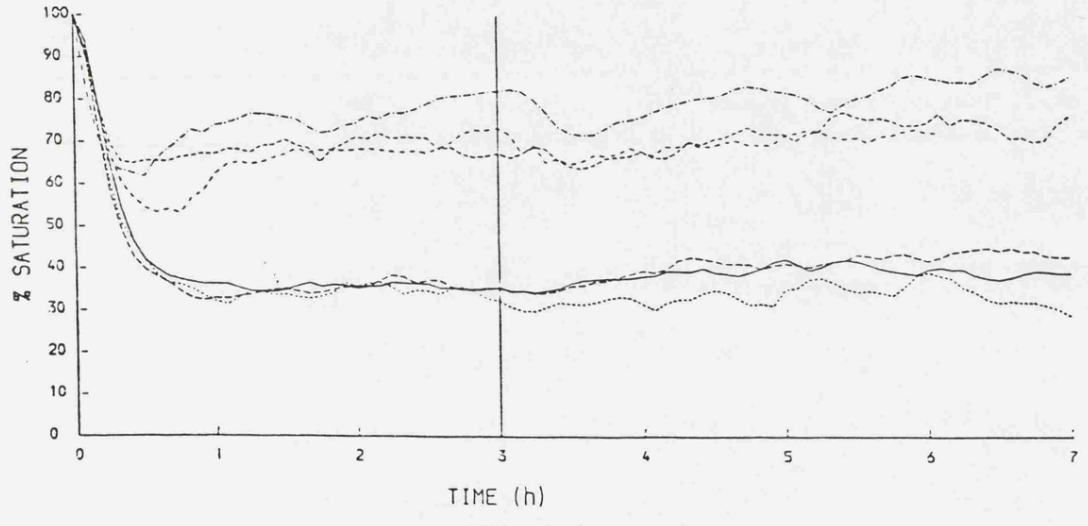


FIG. 8.7

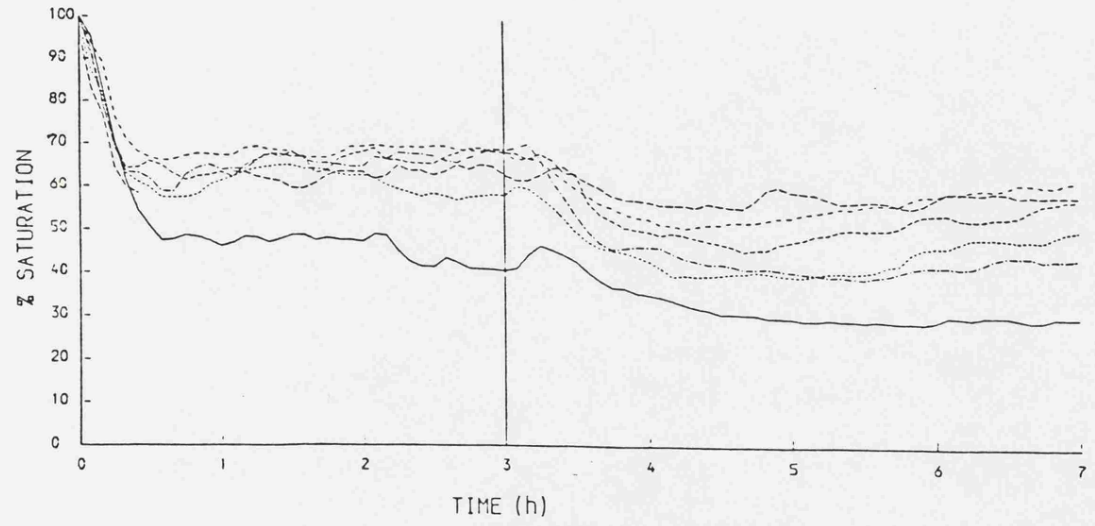


FIG. 8.8

A weight specific oxygen consumption rate was obtained for each group of test animals using the following procedure. The oxygen solubility in air-saturated freshwater at 18^{o}C was calculated from a nomogram (Hitchman 1978), and the resultant value converted from ppm into µl l^{-1}. Total available oxygen was calculated as the product of the flow rate and oxygen solubility. With reference to the oxygen saturation graphs (Fig. 8.6, 8.7 & 8.8), the amount of oxygen utilised by the animals was determined as the product of total available oxygen and percentage decrease in oxygen saturation. This value was divided by total fresh weight of animal to obtain the amount of oxygen consumed as µl O_2 mg fresh wt^{-1} hr^{-1}. To convert this figure to animal dry weight it was divided by the experimentally determined dry weight of the species (22.9%).

A Basic computer program was written for the RML 380Z to perform these series of calculations for every 5 min record of percentage oxygen saturation (Appendix 3). The resultant data were transferred to the Cyber 73 computer and a second series of graphs generated using GHOST80 plotting functions, with oxygen consumption (in µl mg dry wt^{-1} h^{-1}) plotted against time (in h) (Fig. 8.9, 8.10 & 8.11).

The first hour of the 3 h control period of each test corresponding to the maximum time required for the electrode chamber to become completely full of desaturated medium has been omitted in the presentation of results. A short period, dependent on the recorded flow rate was required for the electrode chamber to become completely filled with zinc solution. This time period was calculated for each test from measurements of the flow rate and internal dimensions of the apparatus, and has also been omitted.

The control oxygen consumption for each test was within the range 0.6-1.4 µl mg dry wt^{-1} h^{-1} (18 replicates). In addition, the marked differences in percentage oxygen saturation obtained for some replicate tests were effectively eliminated when the flow rate through the apparatus and the weight of test animals were taken into account.

On introduction of a 50 µM zinc solution no significant change in oxygen consumption was obtained from any of the six replicates although a more variable consumption rate was obtained in the later part of the test. In a 100 µM zinc solution there was a weak tendency for the rate of oxygen consumption to rise at the start of the test, followed by a steady fall to below the control value (Fig. 8.10 —·—·—, — — —, – – –). In a 500 µM zinc solution a definite increase in respiration rate was obtained in all replicate tests although the magnitude of this increase varied considerably. The highest oxygen consumption rate achieved was 2.1 µl mg dry wt^{-1} h^{-1} (Fig. 8.11—·—·—), almost doubling the control rate for this test. In general, the lower the initial control respiration rate, the lower the increase on addition of 500 µM zinc solution.

FIG. 8.9, 8.10 & 8.11 (over page)

Graphs of the mean oxygen consumption of groups of 10 *G. pulex* over a test of 7 h duration.

8.9) 0-3 h = control
3-7 h = 50 µM zinc solution

8.10) 0-3 h = control
3-7 h = 100 µM zinc solution

8.11) 0.3 h = control
3-7 h = 500 µM zinc solution

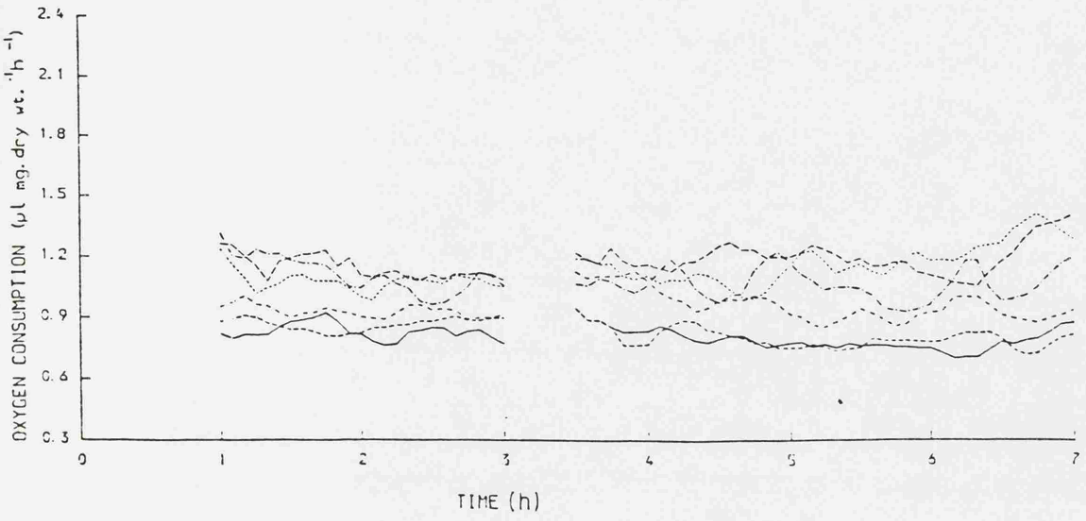


FIG. 8.9

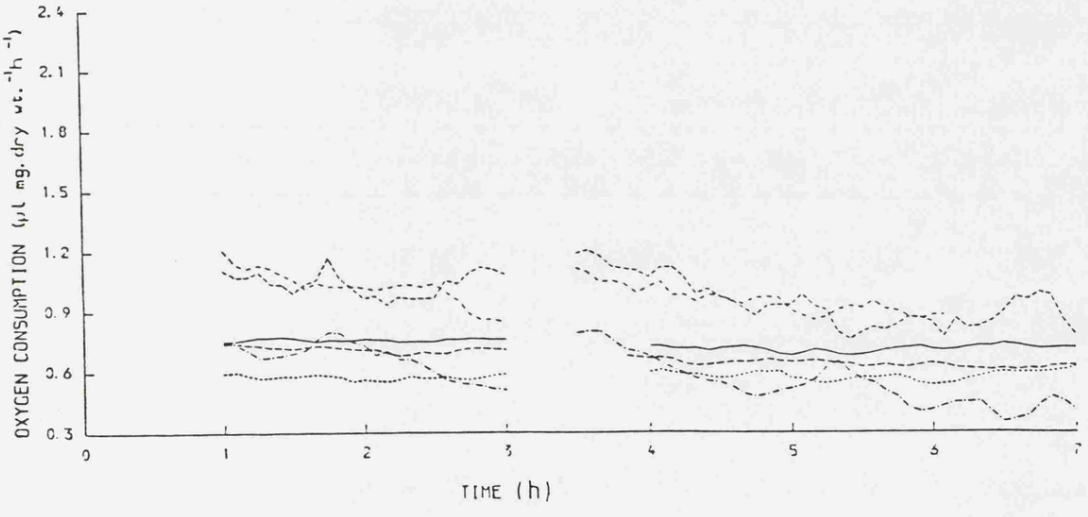


FIG. 8.10

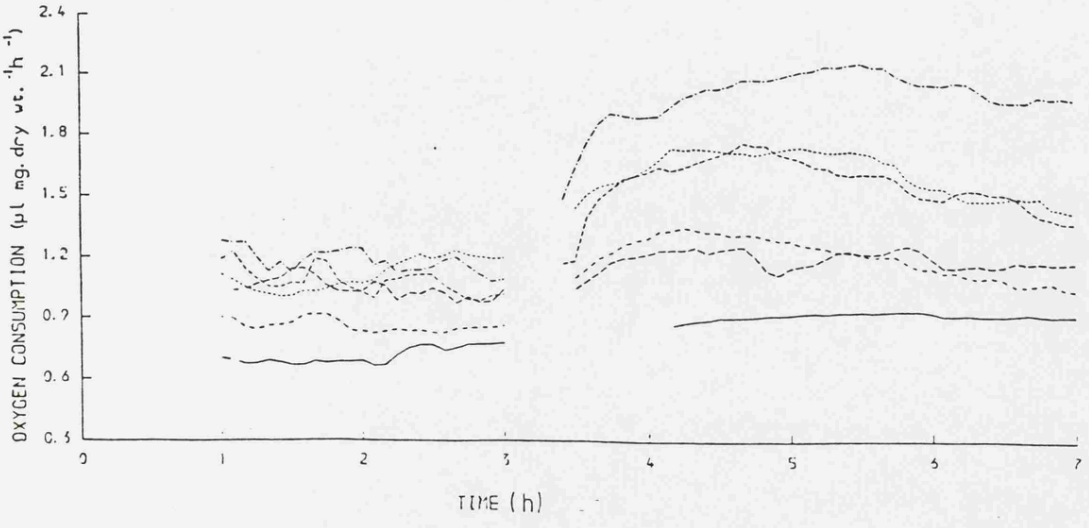


FIG. 8.11

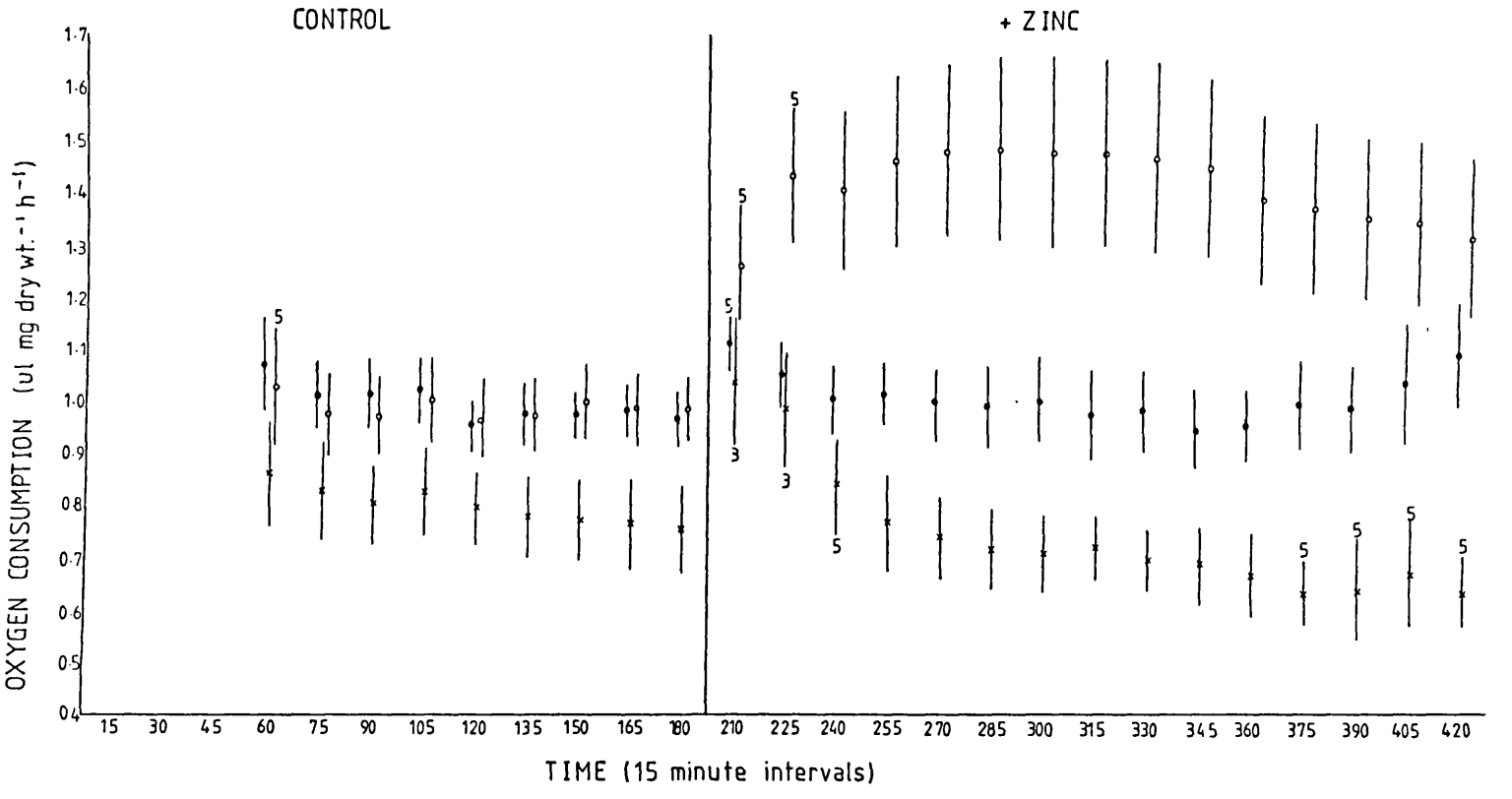


FIG. 8.12

Mean oxygen consumption of G. pulex against time for test zinc concentrations of 50 µM (●), 100 µM (×), and 500 µM (o)
0-180 min = control period. Each point is the mean of up to 6 replicate tests at that zinc concentration with the associated standard error. Means calculated from less than 6 replicate tests are shown with the number of replicates above or below the error bar.

The information from all three graphs has been collated in a single diagram by calculating the mean and standard error for each 5 min value from the six replicate tests at each zinc concentration (Fig. 8.12). Means calculated from less than 6 values are indicated with the number of replicates above or below the error bar. The variable response of replicate groups of animals is indicated by a large standard error in the determination of the mean.

General trends in the effect of zinc on the oxygen consumption of *G. pulex*

At a zinc concentration of 50 µM no apparent effect on oxygen consumption was produced by addition of zinc. At 100 µM there was an initial increase in oxygen consumption followed by a steady return to below the control level towards the middle period of the test. In a 500 µM zinc solution oxygen consumption increased immediately and remained approximately 0.4 µl O_2 mg dry wt^{-1} hr^{-1} higher than the mean control rate for the remainder of the experimental time.

DISCUSSION

If the oxygen consumptions of single *G. pulex* from Rutland Water (1.2060± 0.110 µl mg dry wt^{-1} h^{-1}) which were determined within a closed respirometry system, are compared with similar determinations made on groups of 10 animals within a continuous flow system (0.6-1.4 µl mg dry wt^{-1} h^{-1}), it is seen that the two techniques have given results which are in close agreement.

An increased oxygen consumption rate may be expected in a closed system respirometer for two reasons. First, the depletion of oxygen in the system will cause stress responses which will tend to increase oxygen consumption.

Secondly it is essential in such systems for continuous agitation to ensure uniformity of the medium. The agitator, usually a magnetic 'flea' will strike large animals if they are not isolated, resulting in increased activity and therefore a higher metabolic rate. Smaller individuals which settle on the side of the chamber out of contact with the 'flea' are much less active. This effect may partially explain the poor correlation between animal dry weight and respiration rate.

The closed system was therefore quick and easy to use with the advantage of being sensitive to the oxygen consumption of single animals. The apparatus was designed specifically for respirometric determination on juvenile sea slugs and minor adaptations including the provision of a platform for the animal to isolate the magnetic flea, and a slightly larger chamber size would have been desirable for testing amphipods.

The open system respirometer more accurately represented natural environmental conditions but had the disadvantage of being more difficult and time consuming to set up. A variability in oxygen consumption over the experimental period which was clearly identified in both saturation curves (Fig. 8.6, 8.7 & 8.8), and corresponding oxygen consumption graphs (Fig. 8.9, 8.10 & 8.11), may have been a consequence of changes in the activity level of the test animals. When initially introduced into the chamber they swam freely in both directions with little time spent resting. This initial excitability, similar to that shown by Jones (1941), probably explains the rapid decrease in percentage saturation followed by a steady rise to a more stable value observed in some tests. As the animals acclimated to test conditions there was a tendency to cluster on the gauze pads at inlet and outlet to the chamber. This activity partially blocked off the chamber causing the flow rate through that half of the system to decrease slightly. Tests in which this effect was most obvious had lower flow rates through the chamber and consequently the water passing out of the test chamber was more desaturated.

Value as a chronic test of toxicity

No change in the oxygen consumption rate of *G. pulex* was detected after 4 h exposure to a zinc concentration equal to the 24 h LC50 of the species. This test on *G. pulex* was even less sensitive than the similar test on *Tubifex tubifex* by Brokovič-Popovič & Popovič(1977b), and is consequently of little value as a measure of chronic toxicity in this species. A sustained increase in oxygen uptake was obtained in a highly toxic 500 µM zinc solution even though some individuals became immobilised at the bottom of the respirometry chamber towards the end of the experiment.

These results can now be assessed against the work of Costa (op. cit.), on the effects of zinc on heart beat rate in *G. pulex*, which is outlined in the introduction to this chapter. In zinc solutions greater than 5 mM there was an immediate increase in heart beat rate, followed by an equally rapid decline towards the end of the 1 h test (Costa op. cit.). Concentrations of this magnitude, which cause death in only a few hours, were not used in this series of experiments because of their extreme toxicity. The conclusions which Costa reached concerning the mode of zinc action at these concentrations may therefore accurately describe the observed effects, but must be considered of little value in extrapolating to lower, more realistic, toxicant levels.

In a 500 µM zinc solution *G. pulex* showed a steady rise in heart beat rate throughout a 1 h test (Costa op. cit.), and a sustained increase in oxygen consumption over 4 h (this study). Costa attributed the rise in heart beat rate to absorption of zinc into the circulatory system with associated gill damage. Whilst this hypothesis is not tested in the present study, the respiratory evidence to support gill damage is inconclusive. If gill damage had been extensive at this zinc concentration it would have been expected to impair functionality and therefore cause a decrease in oxygen consumption. Since oxygen consumption increased, an alternative hypotheses for zinc action must be sought.

In several toxicity studies the excitability of the animal has been seen to increase on introduction of zinc (Jones 1947b; Costa op. cit.; this study). It is therefore possible that zinc has no direct action on the respiratory system, but induces a general increase in metabolic rate which in turn demands a higher oxygen uptake. Alternatively, if the efficiency of oxygen utilisation is in some way impaired by the toxicant a higher oxygen consumption would be required to meet the respiratory needs of the animal. A series of experiments in which one of the aims was to distinguish between these two hypotheses are described in Chapter 9.

In a 50 µM zinc solution only a slight increase in heart beat rate occurred after 50 min (Costa op. cit.), and no change in oxygen consumption was observed in this study. This strongly indicates that at this realistic acute level (ie. 24 h LC50 value), zinc does not significantly affect the respiratory or circulatory systems directly. Thus, death may well result from direct poisoning of the protoplasm in a similar manner to that suggested by Hunter (1949). However, respirometric tests which cover the whole survival time are necessary to substantiate this observation.

In closed respirometry experiments in this study it was shown that animals from Willow Brook had a significantly higher respiration rate than animals from Rutland Reservoir. This may be attributed to the differences in flow rate between the two sites. Fox & Simmonds (1933) found that in the isopod *Asellus aquaticus* the oxygen consumption of individuals from a swift flowing stream was 1.5 times greater than that of individuals from a slow stream.

Changes in respiration rate as a consequence of adaption to previous zinc stress may therefore be obscured in this study, but it provides an interesting basis for further experimentation with an open respiratory system and animals from similar habitats.

The most obvious modification would be to run the test for a longer time period, either recording continuously, or with breaks in recording for recalibration of the electrodes. The later approach would be more acceptable since preliminary experimentation has indicated that the electrodes are prone to instability in tests of longer duration (in excess of 10 h). There are also several disadvantages to a longer test period. *G. pulex* is a cannibalistic species and larger individuals have been observed to attack and devour weakened animals during prolonged testing. The pump system described above is inadequate for longer tests since the element tubes compress with wear, causing changes in the pumping rates, and air diffuses through the rubber connective tubing causing bubbles to appear throughout the apparatus.

An alternative to addition of zinc during the test is to measure the oxygen consumption rate of the animals after a period of exposure to a sub-lethal zinc concentration, and in this way explore longer term chronic effects. This test is, however, less experimentally accurate than performing pre-exposure and exposed recordings during the same experiment, since the animals would need to be removed and replaced within the respirometer on different test occasions.

CHAPTER 9

PHYSIOLOGICAL INDICES OF TOXICITY

II. GILL VENTILATION FREQUENCY

INTRODUCTION

Rhythmical beating of the pleopods, for locomotory, feeding and respiratory functions, occur in many malacostracans, notably the Isopoda, Gammaridea and Stomatopoda (Lochhead 1961). The volume of water flowing over the gills is dependent on the frequency and amplitude of the pleopod beat. Under normal conditions this varies considerably depending on respiratory status, and locomotive and feeding activity (Wolvekamp & Waterman 1960).

Gill ventilation by the pleopods of gammarids such as *G. pulex* is an important supplement to diffusion for fulfilling the respiratory needs of the animal, but in order to study the effects of external conditions on ventilatory movements it is necessary to immobilise the animal and remove all potential food materials. In a study of this type on respiration and metabolic control in crustaceans, Walshe-Maetz (1956) measured the pleopod beat rate of immobilised *G. pulex* at $14^{o}C$ in water of different oxygen saturations, and found a smooth decrease in beat frequency between 140 min^{-1} at 20% to 12 min^{-1} above 65% saturation.

The aims of this investigation of gill ventilation frequency during exposure to zinc were first to investigate the use of this technique as a method for detecting acute or chronic levels of ionic zinc, and secondly to further assess the effects of zinc on the respiratory system of *G. pulex*.

The rationale behind these test objectives is that when locomotory and feeding movements of the pleopods are suppressed by immobilisation of the body, gill ventilation rate in a constant flow of fully oxygenated solution will be determined by the oxygen requirement of the inactive animal.

A change in beat frequency in the presence of zinc may therefore indicate a direct effect of zinc on the oxygen demand of the animal, independent from any response to changes in normal locomotory or feeding activity. Alternatively, changes in the rhythmic control of ventilation or the amplitude of beat may indicate impairment of ventilation and possible inability to supply the oxygen demand of the animal.

These series of tests were conducted at the same zinc concentrations as those used for respirometric determinations (Chapter 8), in order to relate the results.

MATERIALS AND METHODS

Test rationale

Single animals were immobilised in a clear perspex chamber with their pleopods clearly visible. Control and zinc solutions were pumped alternately through the chamber, whilst simultaneously monitoring the pleopods on video tape. Beat frequencies were subsequently calculated at regular intervals over the duration of the test, in order to determine the effects of zinc on the characteristics of pleopod movement.

Initial test procedure

Starved *G. pulex* measuring approximately 10 mm in length were dried rapidly on absorbent paper and the dorsal surface of the carapace pressed firmly for 3-5 seconds against a thin glass slide (12 mm x 4 mm), with a light covering of 'Superglue' adhesive. After a strong bond had formed between the animal and the glass, it was placed in a small glass holding dish containing 300 cm^3 of aerated filtered reservoir water for 24 h to adjust to the presence of the glass plate. Only specimens in which the appendages appeared undamaged by this preparation were tested.

A selection of individuals so treated were kept for up to 1 week to ensure that this process had no obvious harmful effect on their short term survival. Several individuals moulted during this time leaving the exuvium still attached to the glass surface. No visible effect of attachment could be seen on the new cuticle and all moulted animals appeared healthy.

In order to investigate whether the sensory role of the antennae had any influence on ventilatory rhythm in the presence of zinc, antennal tips were removed from a one test group, and the whole antennae from a second group. Antennaelectomised animals were maintained within the holding dish for a further 24 h in order for the wound to heal, before they were immobilised on glass slides.

Apparatus

The apparatus comprised two identical perspex chambers, each receiving an individually pumped supply of test medium from a common reservoir (Fig. 9.1). The chambers were essentially similar in design to those used for the respirometry experiments (Chapter 8), but were of smaller dimension (12 mm x 6 mm x 6 mm), and of rectangular internal cross section. The top of each chamber was highly polished to allow the contents to be viewed clearly. Both chambers were clamped horizontally on a perspex base to prevent movement during operation.

At a flow rate of 40.0 $cm^3\ h^{-1}$ the use of coloured dyes confirmed that medium from the reservoir reached both of the chambers in 65 sec, and completely filled them in a further 40 sec. Preliminary tests had indicated that this pump speed was the most convenient compromise between a rapid change over of medium within the chamber, and a sufficiently slow flow rate to stimulate a rhythmic control ventilatory pattern in most test animals.

The perspex base was placed on the stage of a binocular dissecting microscope with one chamber immediately beneath the oculars. The microscope was then focussed on the animal through the polished top panel. Illumination was provided from two fibre optic lamps. A black and white video camera was attached to the microscope for the purpose of recording pleopod movement on video cassette tape, and relaying the image of the animal to a television screen (Fig. 9.2).

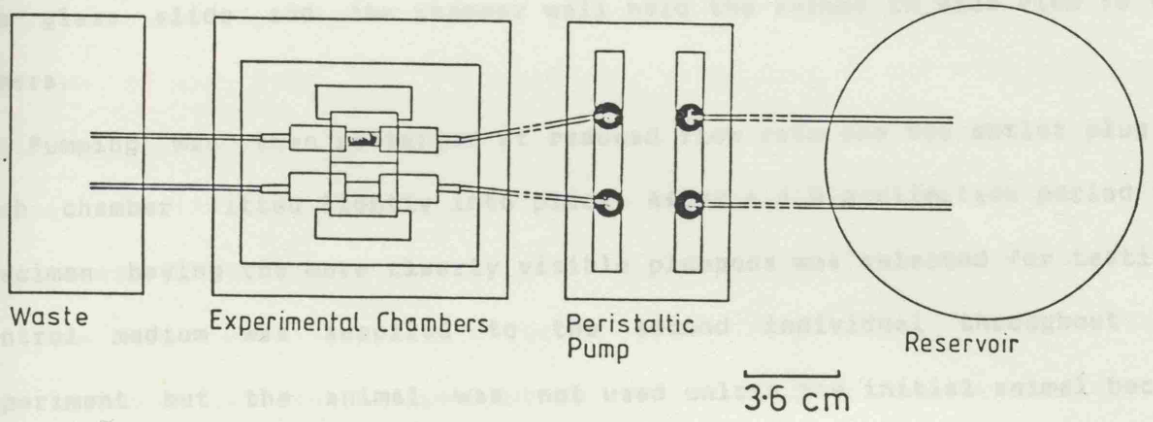


FIG. 9.1
Diagram of ventilatory system illustrating the flow of medium through the experimental chambers.

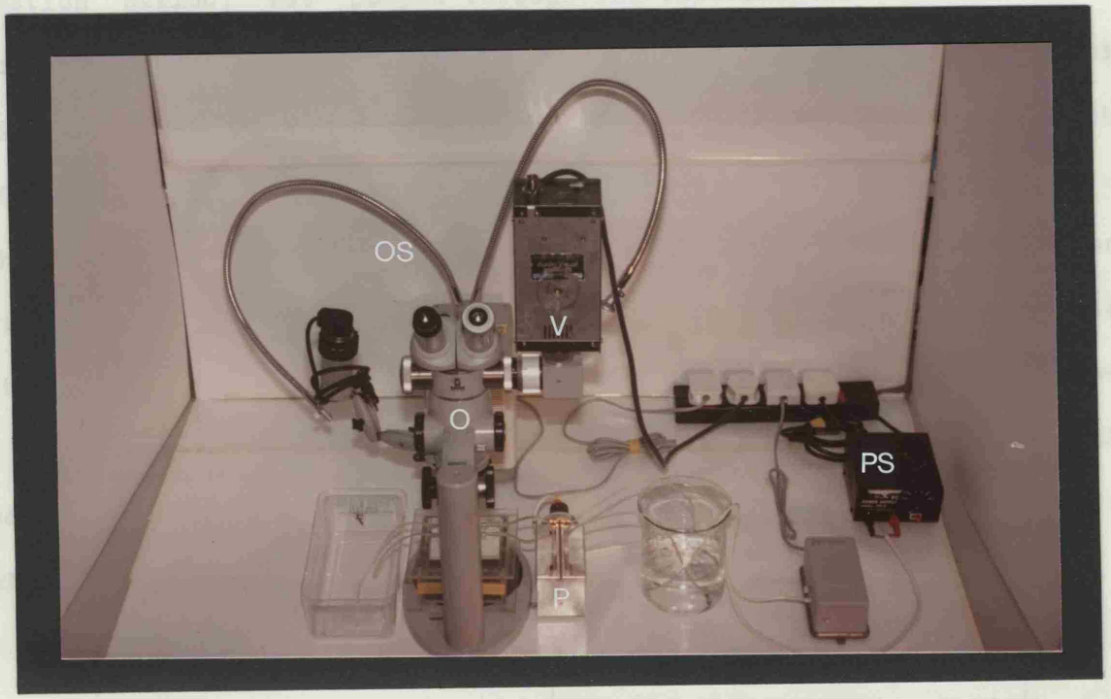


FIG. 9.2
Photograph of complete system for recording pleopod movements. O = optical binoculars, V = video camera PS = pump speed control, P = peristaltic pump, OS = fibre optics

Procedure

The experimental chambers were filled rapidly with filtered reservoir water by switching the pump to maximum speed. During filling the outlet plugs were removed and the chambers held vertically to prevent air from remaining within the system. The pump was then switched off whilst an animal mounted on a glass slide, was put into each chamber. The animal was positioned with the head end adjacent to the inlet. Suctional force between the glass slide and the chamber wall held the animal in side view to the camera.

Pumping was then restarted at reduced flow rate and the outlet plug of each chamber fitted tightly into place. After a 1 h acclimation period the specimen having the more clearly visible pleopods was selected for testing. Control medium was supplied to the second individual throughout the experiment but the animal was not used unless the initial animal became unsuitable. Filtered reservoir water identical to that used during the acclimation period, was pumped through the experimental chamber for a 1 h control period. The pump was then momentarily turned off and control medium substituted by zinc sulphate solution of known concentration for a further 1 h. Control medium was then reintroduced for a final 1 h period.

Throughout the experiment the image of the test animal was viewed on a television screen. If the animal moulted or became detached from the plate the test was restarted with the second animal. 5 min video recordings were taken every 15 min commencing at the start of the test. This procedure thus produced four 5 min records for each of the three 1 h periods and was repeated for 16 animals according to the schedule shown in Table 9.1.

N^{o}. of replicates	Zinc concentration (μM)
3 : no antennae	500
2 : no antennal tips	500
3 : antennae	500
4 : antennae	100
4 : antennae	50

TABLE 9.1

Data preparation

The first stage in data analysis was to convert each visual 60 min record of pleopod beat into an interpretable numerical form. Ideally, direct evaluation of beat frequency could have been obtained by counting the number of pleopod beats over constant time periods. Without an audible timing device, however, it proved impossible to count pleopod beats accurately whilst simultaneously monitoring time. It was thus necessary to select a standard number of beats over which to record the cumulative time intervals.

Each 60 min tape was replayed on an editing consul with a built in timimg device. Cumulative time was recorded (in min, sec & frames) over each separate 5 min time section, for consecutive groups of 10 beats. A variable speed control allowed the tape to be replayed at normal speed during periods when the animal was inactive, and to be slowed down for accurate analysis of faster movements. During periods of rapid pleopod beating, the record was analysed frame by frame ($25\ sec^{-1}$).

At the start of the first control period the amplitude of the 'normal' pleopod beat was marked on the video screen. A central point was calculated corresponding to a beat of half the normal amplitude. During subsequent counting any perceptible beat which did not pass this central point was recorded as a 'half beat'.

Each 5 min time record was converted into seconds and entered together with the corresponding record of half beats as a data file on the Cyber 73 computer. This procedure was repeated for all sixteen, 60 min video records.

Analysis and presentation of results

A computer program was written in Fortran utilizing GHOST80 plotting routines to produce graphs of cumulative recording time against cumulative number of pleopod beats. In order to simplify the presentation of this unmodified data the 10 min interval between each 5 min record has been omitted.

'Half beats' are indicated on a shortened vertical scale down the right hand margin of the diagram. Control and zinc sections of the test are demarcated by dotted lines, parallel to the ordinate. Breaks in some records occur when the last group of < 10 beats in any one 5 min period has been disregarded. The horizontal scale for each animal was obtained by counting the total number of beats and rounding up to the nearest 1000. The data files and computer program, together with a brief description of its operation are given in Appendix 3$.

RESULTS

1) 500uM No antennae (Fig. 9.3 a-c)

It can be seen from Fig. 9.3a & 9.3b that two of the three *G. pulex* tested showed little change in pleopod beat frequency or amplitude, during either exposure to this highly toxic zinc solution, or on subsequent removal to control medium. A third individual with a low control beat rate, showed a marked increase in pleopod beat rate almost immediately on changing from control to zinc solution, followed by a return to control beat frequency towards the end of exposure to zinc. After changing back to clean solution a second increase in beat frequency was obtained (Fig. 9.3c). No change in the amplitude of the beat was observed throughout any test.

2) 500uM No antennal tips (Fig. 9.4 a-b)

Both *G. pulex* responded to the introduction of zinc solution with a slight increase in pleopod beat frequency. In one animal (Fig. 9.4a), beat frequency remained high for the duration of exposure to zinc, but returned to control level when the toxicant was removed. In a second individual (Fig. 9.4b), an initial increase in beat frequency on addition of zinc, was followed after 30 min exposure by a drop to below the control value.

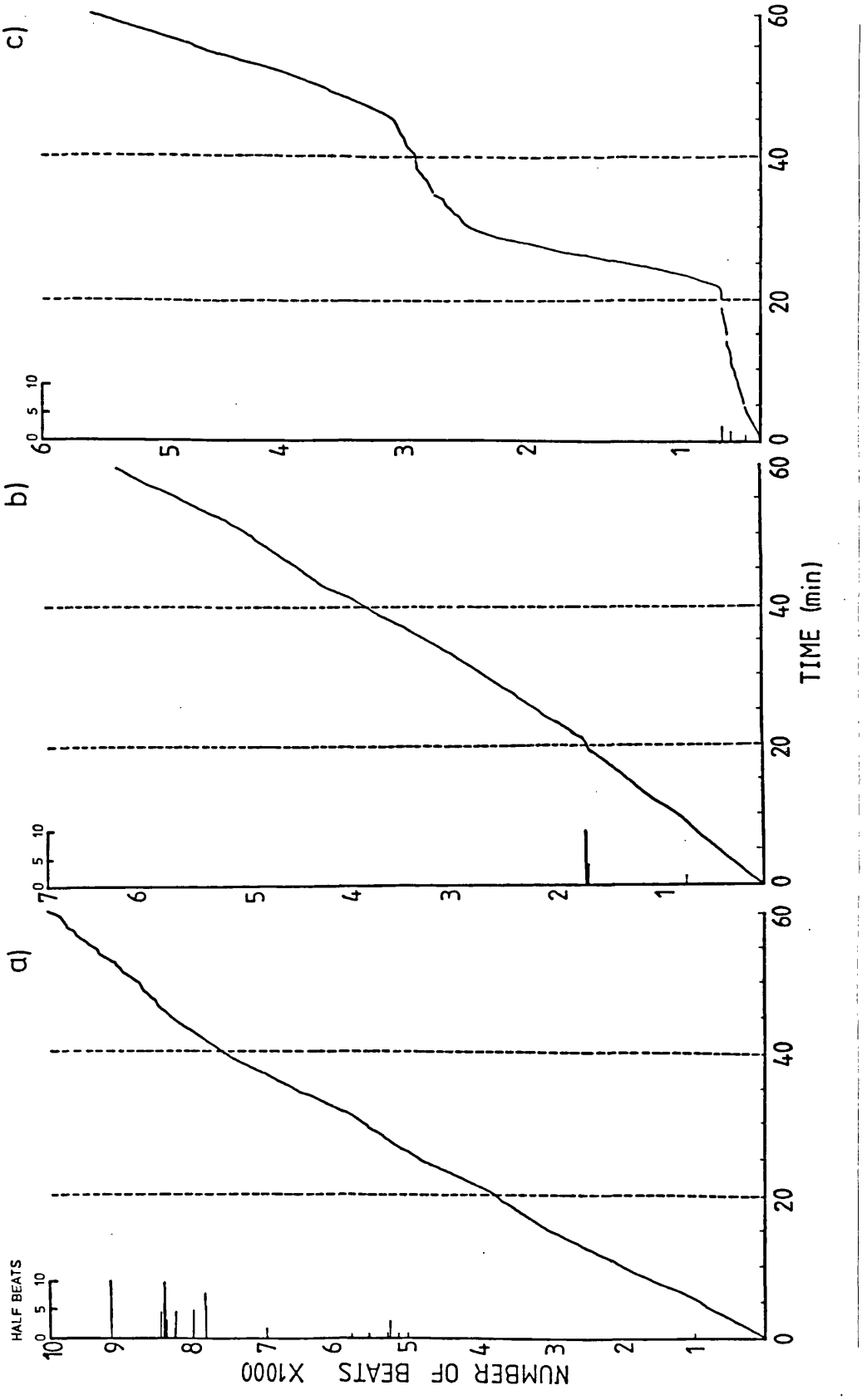


FIG. 9.3 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with no antennae, in 500 µM zinc sulphate solution.

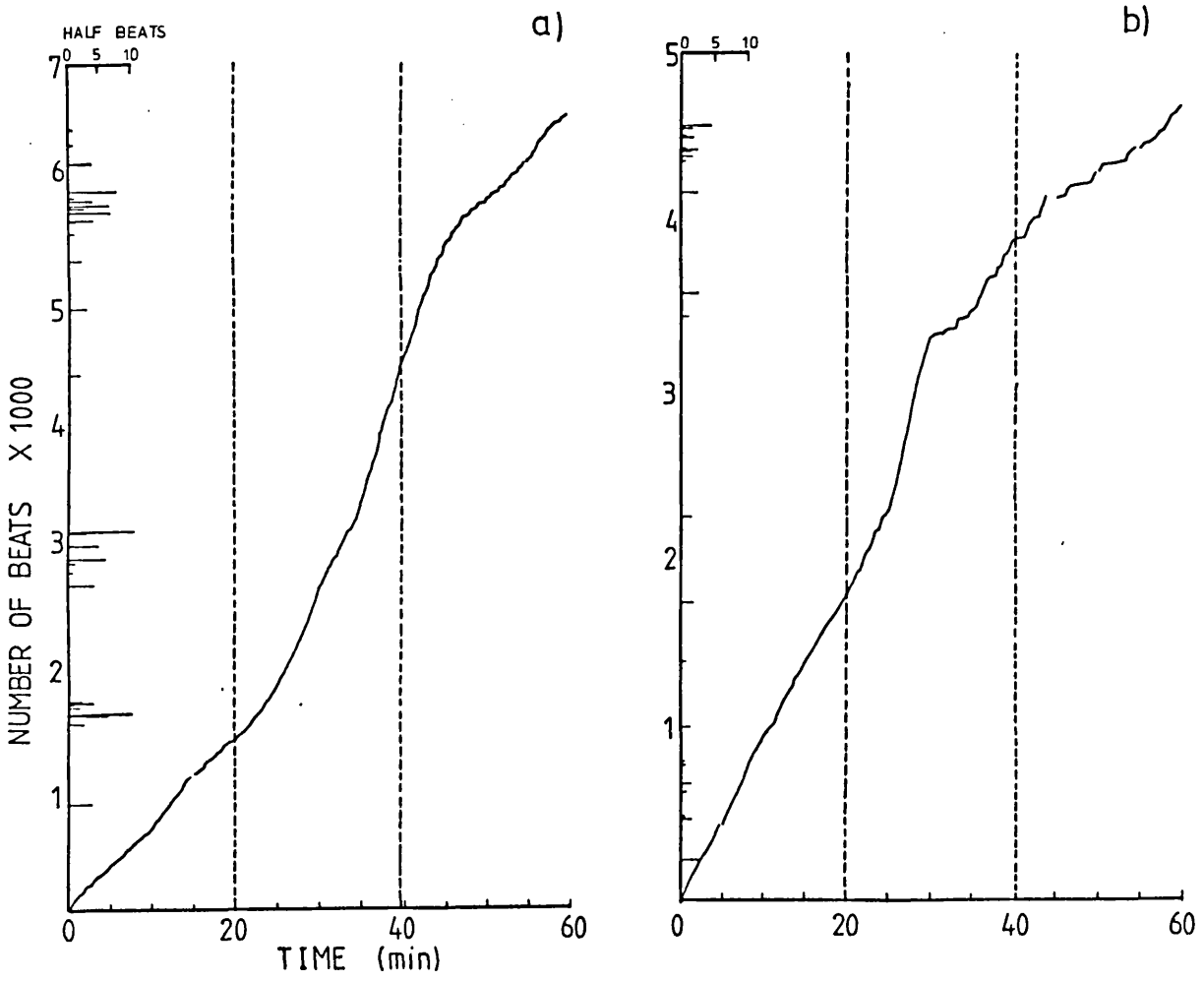


FIG. 9.4 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with no antennal tips, in 500 μM zinc sulphate solution.

A marked difference in the pleopod beat pattern of this animal was also observed. Rhythmical beating was replaced by irregular periods of violent stretching movements and wiping of antennae and pleopods, interspersed with periods of total inactivity in which the pleopods were held rigid and vertical to the body. This behaviour pattern continued after restoring control solution.

3) 500µM Antennae Present (Fig. 9.5 a-c)

Whilst the three animals tested at this zinc concentration showed no common response, large changes in pleopod beat frequency were not obtained. In two animals (Fig. 9.a & 9.5b) the addition of zinc resulted in irregular beating which continued throughout the second control period. In the first of these animals (Fig. 9.5a), this was accompanied by a decrease in beat frequency and amplitude which lasted for the duration of the test. In the animal shown in Fig. 9.5b the pleopod beat amplitude also varied during this period of exposure to zinc, but returned to normal on restoring control conditions. Additional symptoms of zinc exposure (as described above for antennaelectomised animals) were short bursts of intense stretching movements followed by periods of total inactivity. This behaviour was often correlated with wiping of antennae and pleopods in all 3 animals.

4) 100µM Antennae Present (Fig. 9.6 a-d)

A similar response was obtained from all four animals tested at this concentration, and can be described with reference to Fig. 9.6b. During the first control period, beat frequency remained relatively smooth with only slight fluctuations, but on addition of 100 µM zinc solution it decreased and became much less regular. On returning to control solution little further change in beat frequency was observed, but the irregular beat pattern persisted for the duration of the test. This is clearly illustrated by the step wise appearance of the graph.

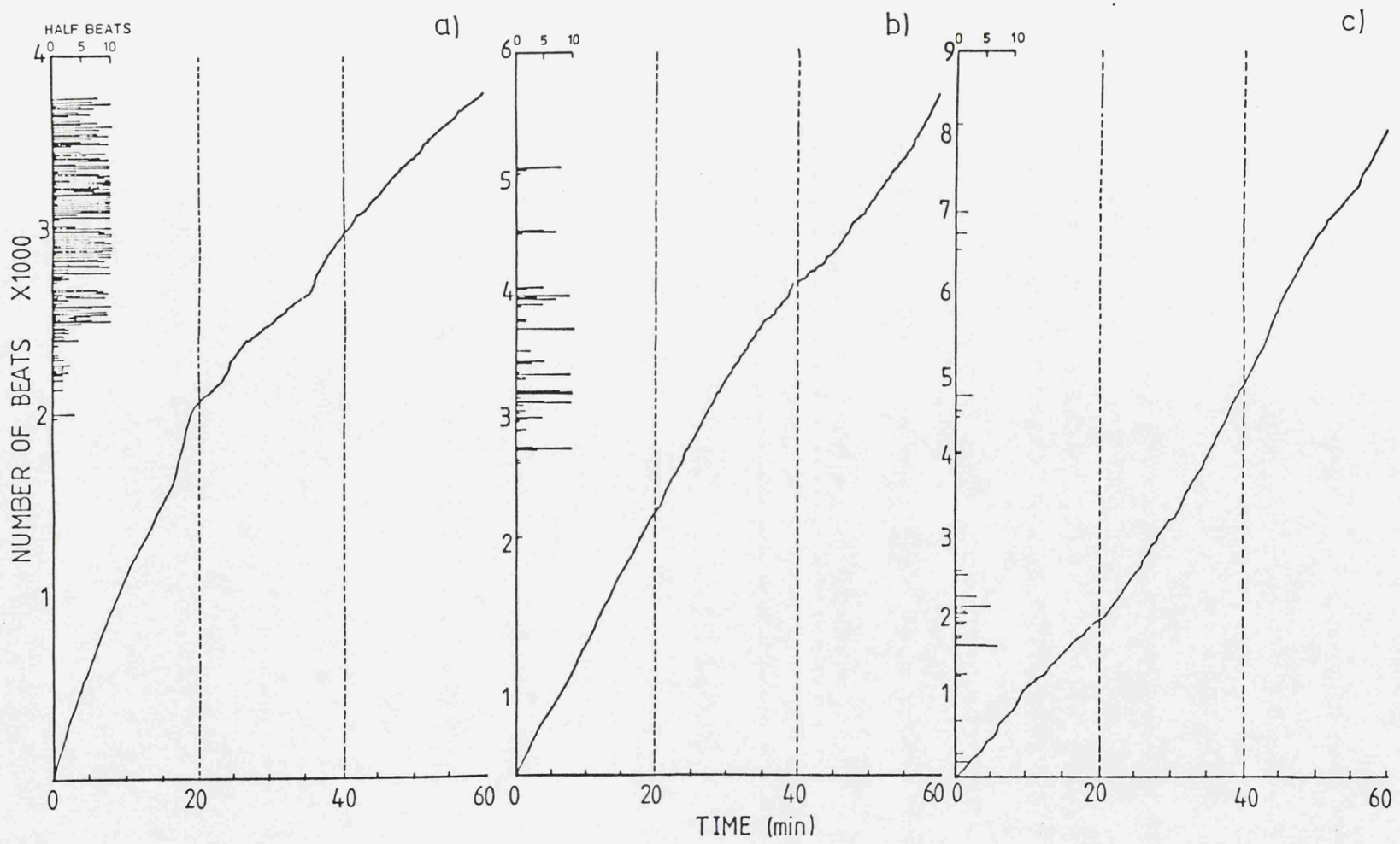


FIG. 9.5 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with antennae, in 500 µM zinc sulphate solution.

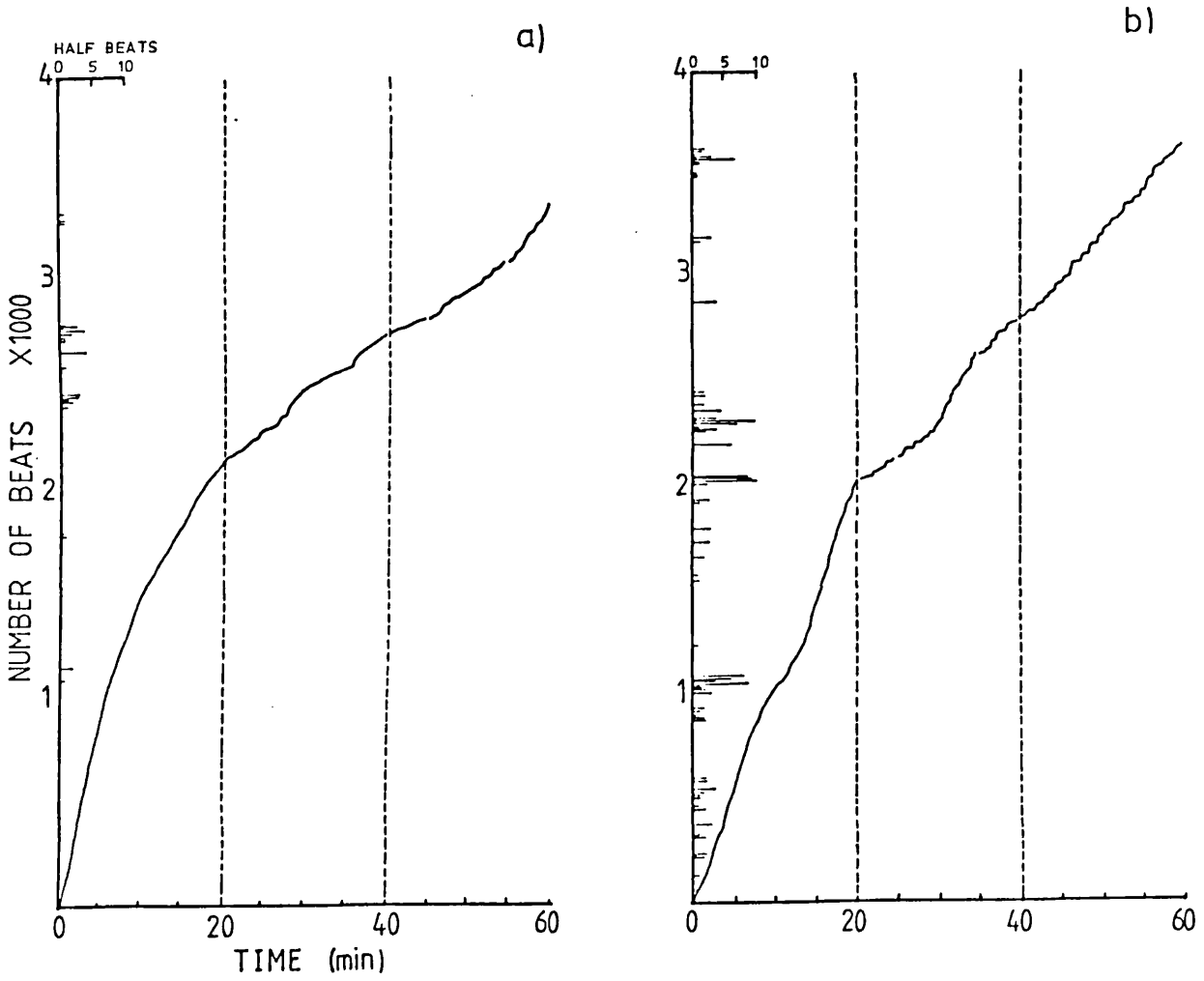


FIG. 9.6 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with antennae, in 100 μM zinc sulphate solution.

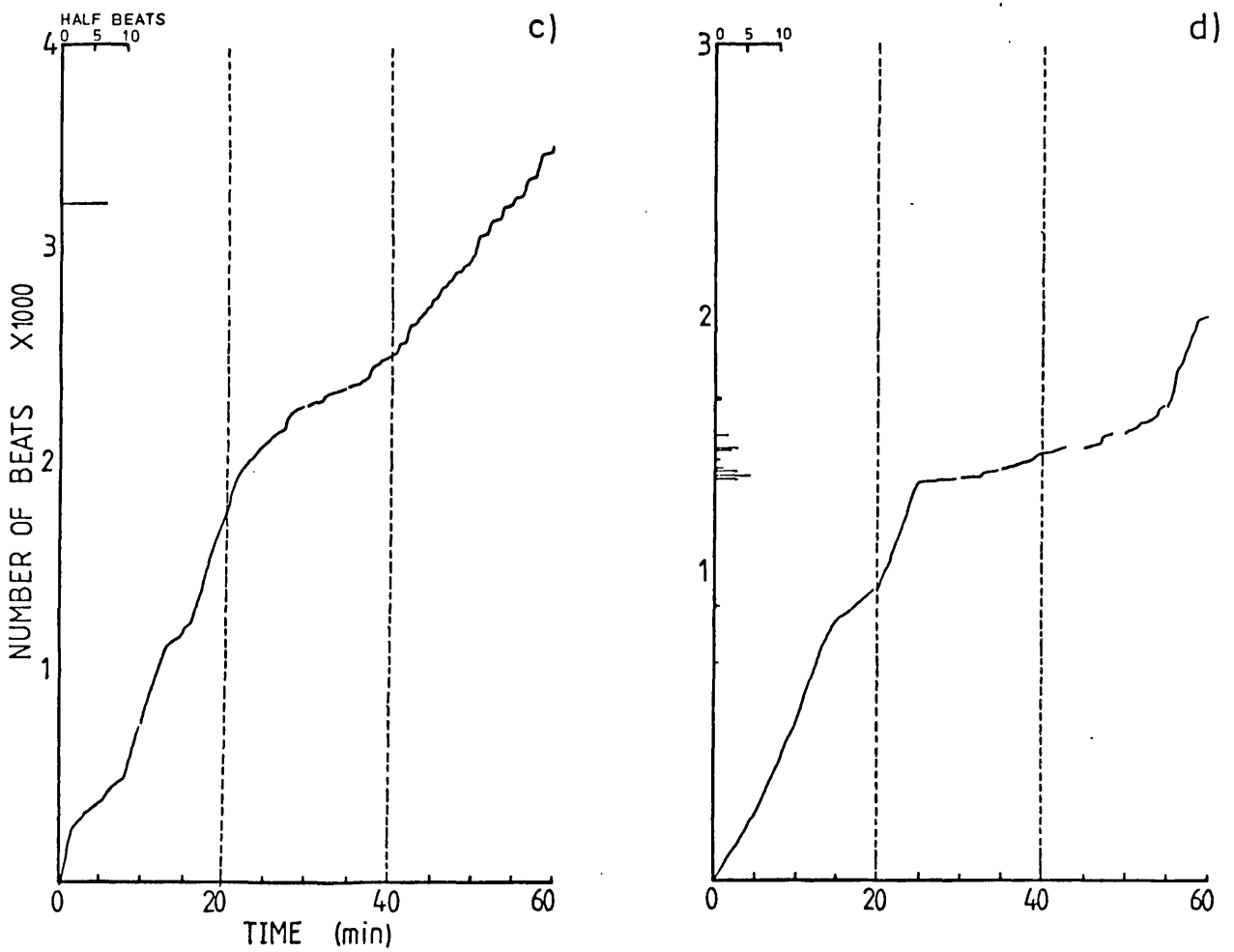


FIG. 9.6 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with antennae, in 100 μM zinc sulphate solution.

In one animal (Fig. 9.6d), the control beat frequency was maintained during the first 5 min in zinc solution, but was followed by a sharp decrease to a level at which it remained until the last 15 min of the control period when a sudden increase occurred. This was the only animal tested at this zinc concentration to reattain a beat frequency as high as that during the initial control period.

5) 50 µM Antennae Present (Fig. 9.7 a-d)

This zinc concentration had the least effect on pleopod beat frequency, although there was some tendency for the beat rhythm to become variable after the addition of zinc (Fig. 9.7a & 9.7d). In one animal (Fig. 9.7c) the beat frequency was low and irregular during the initial control period but this behaviour pattern was maintained throughout the test.

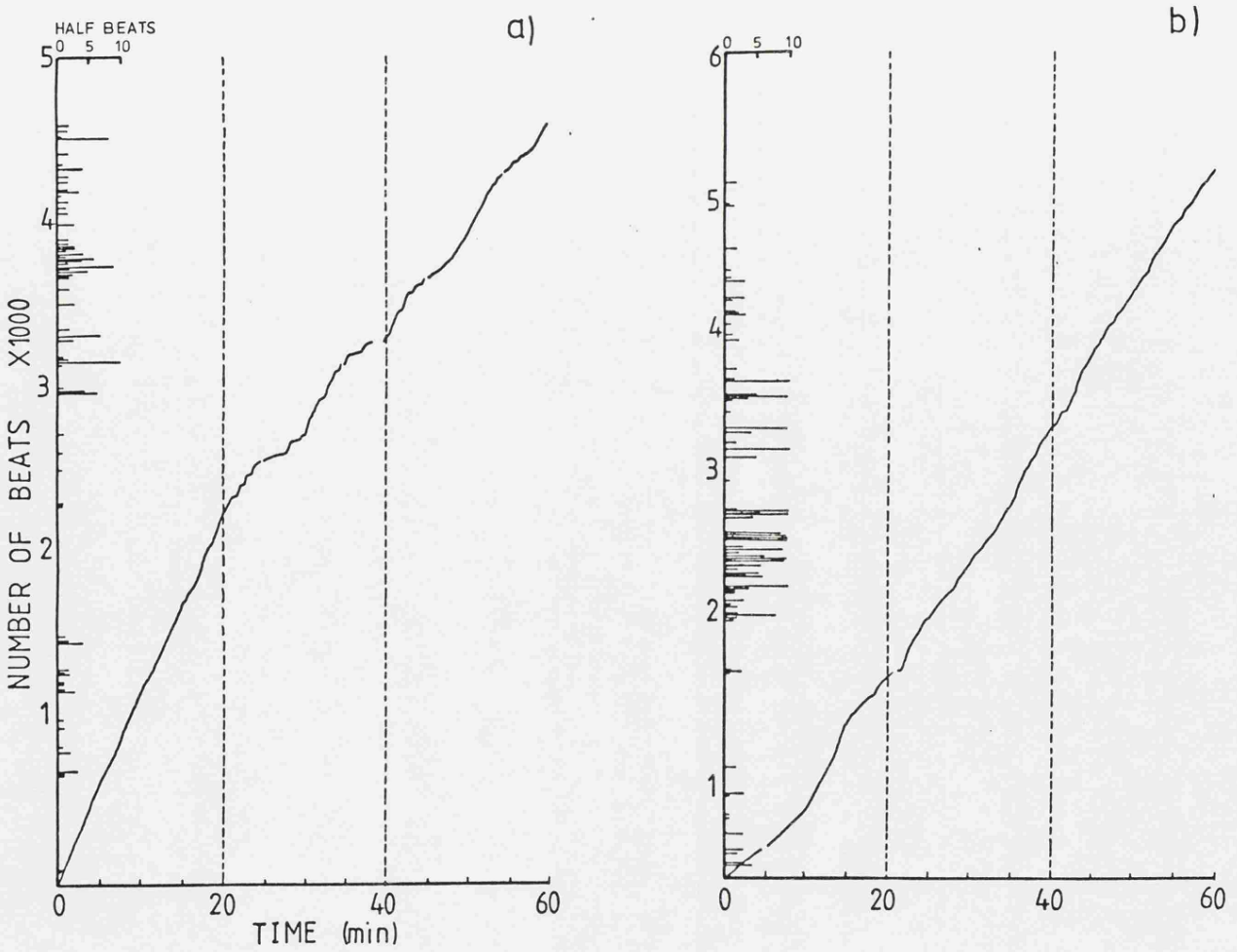


FIG. 9.7 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with no antennae, in 50 µM zinc sulphate solution.

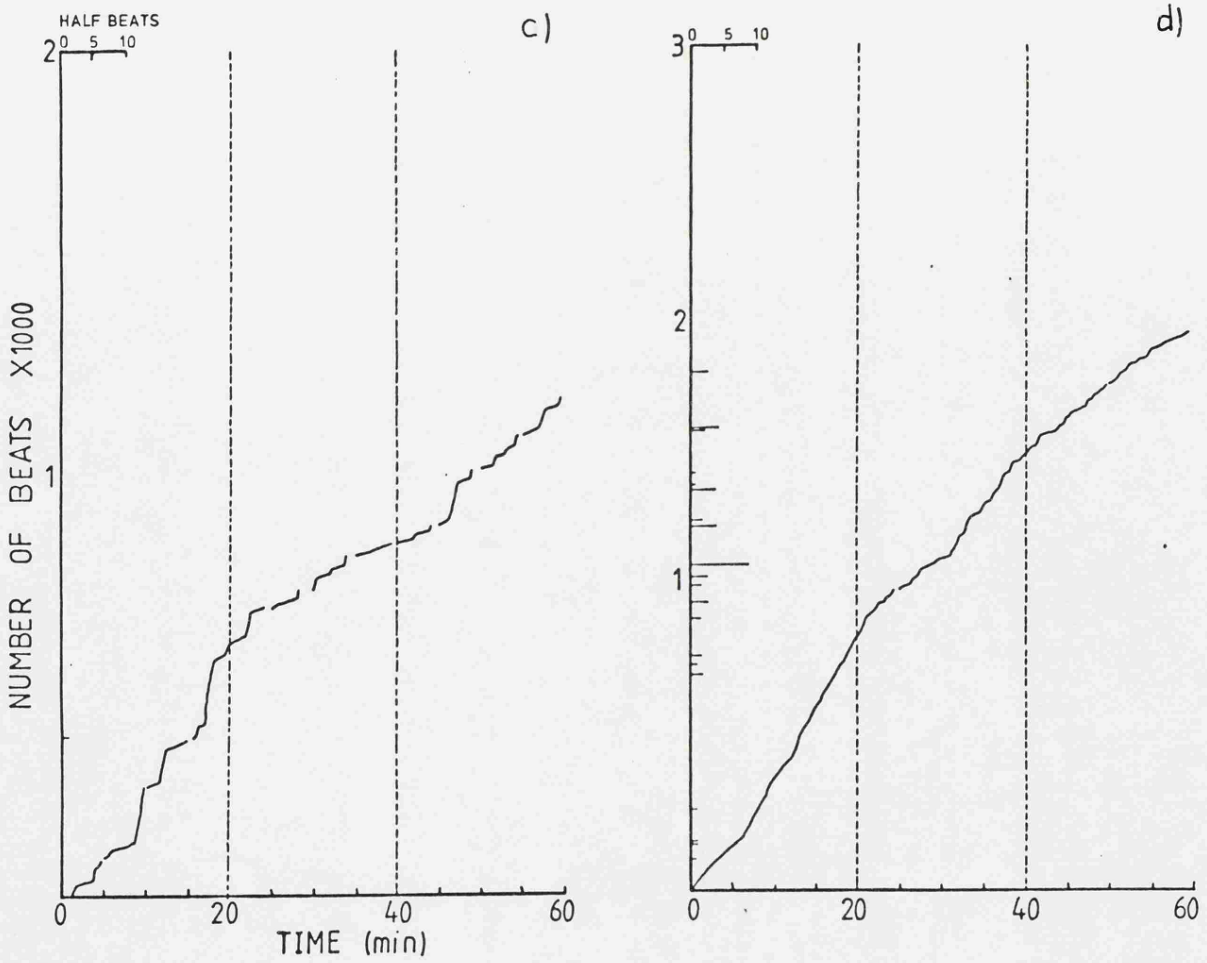


FIG. 9.7 Plot of cumulative number of pleopod beats against recording time for *G. pulex* with antennae, in 50 µM zinc sulphate solution.

General Trends

These results are summarised in Fig. 9.8 . Mean beat frequency has been calculated over each 5 min time section and the results, expressed in the form of pleopod beats per minute, plotted against total test time. Each animal has been individually identified. Control and zinc sections of the test have been delineated by solid vertical lines at 1 h and 2 h time intervals. Whilst the generally high level of individual variability in both control and zinc solution make it impossible to distinguish definite evidence of zinc toxicity from this test, weak trends can be identified between the concentration of the zinc solution and changes in pleopod beat frequency and amplitude.

Antennaelectomised animals tended to show an increase in pleopod beat frequency in response to zinc. This was most marked in an animal with a low resting beat frequency (Fig. 9.8[1] animal c). Little change was seen in an animal with a high resting beat rate (Fig. 9.8[1] animal a). In non-antennaelectomised animals there was no consistent response but any changes in beat rate which did occur were not as large as for antennelectomised animals. Animals exposed to a 100 μM zinc solution showed a general decrease in beat frequency which appeared to be independent of the initial control beat frequency, and which persisted on return to control solution. A 50 μM zinc solution had no obvious effects on mean beat frequency or amplitude. At all three zinc concentrations the most obvious effect on the ventilatory system of *G. pulex* was the loss of the rhythmic beat pattern, and its replacement by irregular periods of inactivity with pleopod and antenna wiping.

FIG. 9.8 (over page)
Summary of pleopod beat rates in control and zinc solutions (see text for explanation of diagram construction).

(1) Animals with no antennae
1-2 h = 500 µM zinc sulphate solution

animal	corresponding graph
a	Fig. 9.3a
b	Fig. 9.3b
c	Fig. 9.3c

(2) Animals without antennae tips
1-2 h = 500 µM zinc sulphate solution

animal	corresponding graph
a	Fig. 9.4a
b	Fig. 9.4b

(3) Animals with antennae
1-2 h = 500 µM zinc sulphate solution

animal	corresponding graph
a	Fig. 9.5a
b	Fig. 9.5b
c	Fig. 9.5c

(4) Animals with antennae
1-2 h = 100 µM zinc sulphate solution

animal	corresponding graph
a	Fig. 9.6a
b	Fig. 9.6b
c	Fig. 9.6c
d	Fig. 9.6d

(5) Animals with antennae
1-2 h = 50 µM zinc solution

animal	corresponding graph
a	Fig. 9.7a
b	Fig. 9.7b
c	Fig. 9.7c
d	Fig. 9.7d

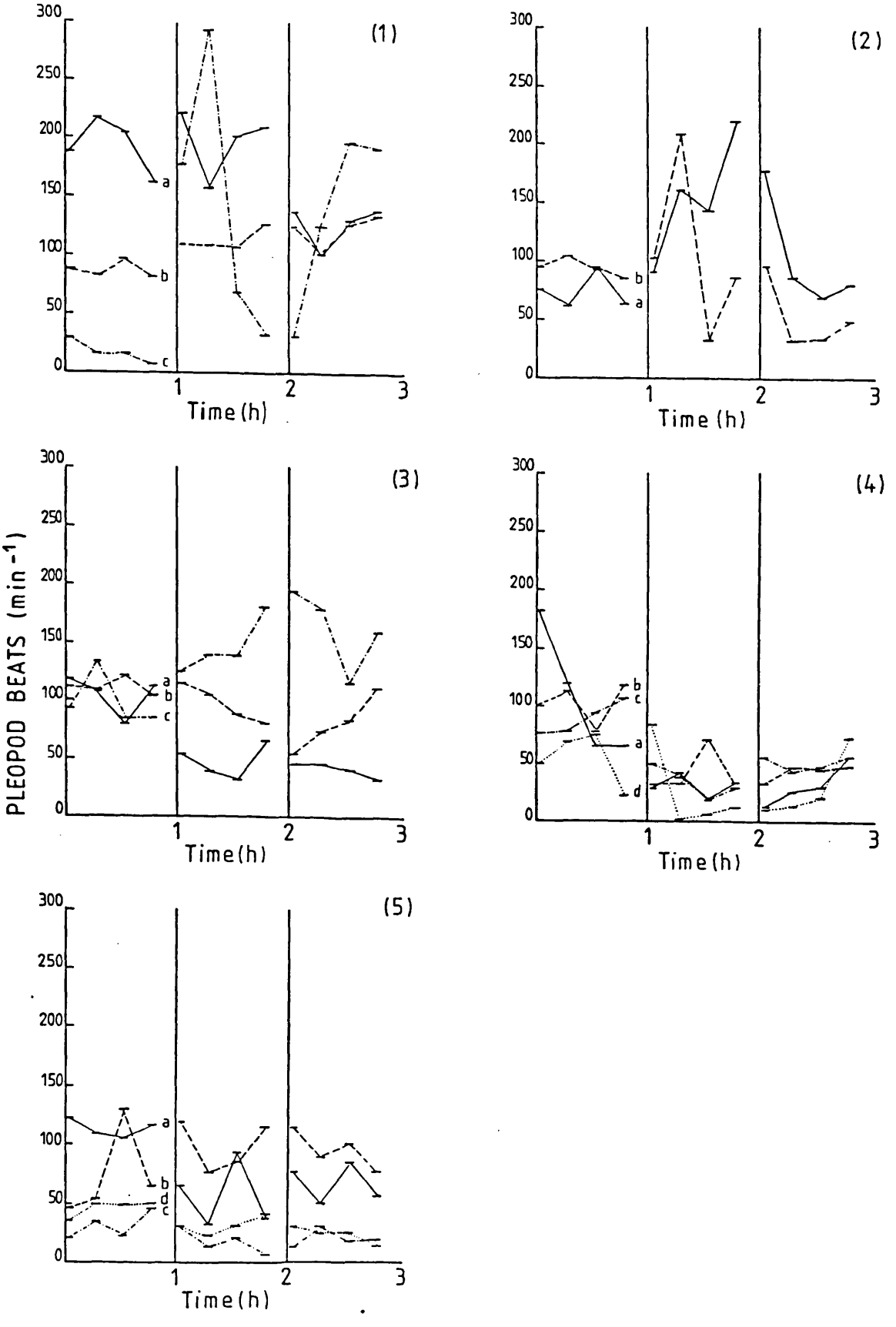

(1)
(2)
(3)
(4)
(5)
PLEOPOD BEATS (min⁻¹)
Time (h)
300
250
200
150
100
50
0
1
2
3
a
b
c
d

FIG. 9.8

DISCUSSION

Gill Ventilation as a criterion of toxicity

The large control variability in beat amplitude and frequency shown both by single individuals over the duration of a recording period, and between different animals exposed to identical test conditions may be identified as originating from two main sources;

1) Variability produced by the test procedure.
2) Natural variability within and between individuals of the same species.

Several stages of the test procedure can be diagnosed as potentially stressful for the animal and therefore likely to upset the natural beat frequency. The procedure used to immobilise the animals was confirmed to have no directly harmful effects on the animals and whilst some individuals showed a high pleopod beat frequency immediately after attachment, no signs of stress were observed after a 24 h acclimation period. It is possible, however, that by preventing pedal contact and therefore forcing the animals to keep 'swimming' by means of pleopod movements, the normally rhythmical pleopod beat was affected.

Violent struggling activity associated with pleopod and antenna wiping was observed in zinc solutions, and was nearly always accompanied by a temporary increase in beat rate. This suggests that the animal was expending energy in trying to release itself from the glass plate.

Several changes in ventilation rate were observed which could not be readily explained as experimentally induced. Immobilised animals within both the holding dish and the respirometry chamber showed marked changes in both frequency and amplitude of pleopod beat with no accompanying changes in activity level.

In addition, animals having almost identical beat frequencies when in the holding dish (assessed subjectively), often showed marked differences when they were placed within the twin animal chambers, and treated identically. In individuals with low beat frequencies the time between successive groups of beats was often irregular.

From the difficulties encountered in this study two aspects of the experimental procedure used by Walshe-Maetz (1956) make the stated precision of her determinations doubtful. First, Walshe-Maetz determined beat frequencies up to 140 min^{-1} from direct counts. In these tests beat frequencies of 200 min^{-1} were recorded in the control period, and as great as 300 min^{-1} in zinc solutions. These could only be measured by video recording and it must be doubtful whether even rates of 100 min^{-1} could be accurately determined without such a facility. Secondly, only five replicate groups of 50 pleopod beats were recorded for each animal by Walshe-Maetz (op.cit.). At a beat frequency of greater than 125 min^{-1} this represents a total recording time of less than 2 min. In this series of experiments the pleopod beat frequency was recorded for three 20 min periods irrespective of initial beat frequency. In several cases changes in beat frequency were detected throughout the control periods which might have been undetected in shorter tests. A longer test period was considered to be especially necessary when the initial beat frequency was low and irregular.

The large variability in the pleopod beat rate in the control medium can therefore be considered as a combination of experimental technique and natural variation both within the normal ventilatory beat pattern of a single individual and between different animals. These factors must considerably restrict the accuracy in measuring changes in pleopod beat frequency and amplitude in response to changes in the respiratory environment such as pO_2 or the presence of toxicants.

EFFECTS OF ACUTE ZINC CONCENTRATIONS ON THE RESPIRATORY SYSTEM OF *G. PULEX*

The results of respirometry and ventilatory experiments have been combined to assess the effects of zinc on the respiratory system of *G. pulex*, It must, however, be stressed that the ventilatory experiments were subject to considerable theoretical and technical difficulties which have resulted in marked variations in the results obtained. The following hypothesis, whilst of value in establishing likely effects, must therefore be considered tentative until improved methods have been developed for testing amphipods which overcome at least some of these problems.

In a 500 µM zinc solution the oxygen consumption of *G. pulex* was found to increase significantly above the control level. Two hypotheses were presented to explain this result. First, it was suggested that zinc was not acting as a poison on the respiratory system but causing an increase in metabolic rate, thus indirectly increasing the oxygen demand of the animal. The second hypothesis was that zinc in some way impaired the efficiency with which oxygen was utilised, which was counteracted by an increase in oxygen consumption.

It was not possible to distinguish between these hypotheses using the respirometry apparatus and techniques described in Chapter 8, since it was impractical to immobilise all 10 test animals to be simultaneously respired. Experiments on the ventilatory system, however, provided an alternative method of assessing the effect of locomotory activity on the oxygen consumption within zinc solutions between 500 µM and 50 µM.

The interpretation of ventilatory experiments in a 500 µM zinc solution centred on the assumption that the increase in oxygen consumption seen at this concentration, had been met by an increase in the rate at which oxygen was supplied to the gills (ventilation rate), rather than an increase in the efficiency with which oxygen was extracted from the water.

Animals with antennae showed no distinct increase in gill ventilation frequency within a 500 µM zinc solution, and cannot therefore have reacted to zinc in the same way as the mobile animals used in respirometry experiments. This strongly suggests that it was an increase in locomotory activity which was responsible for a faster ventilation rate and therefore a higher oxygen consumption of mobile animals at this zinc concentration.

In antennaelectomised animals the gill ventilation frequency showed a tendency to increase in a 500 µM zinc concentration. This was contrary to the expectation that removal of the antennal setae which are thought to have auditory and chemo-sensory functions (Cussans 1904), may impair the detection of zinc and therefore reduce the excitability of the animal.

In a 100 µM zinc solution there was a drop in gill ventilation frequency although no corresponding decrease in oxygen consumption was detected for mobile animals. This again suggests that locomotory activity was responsible for stimulating a higher level of gill ventilation, therefore maintaining a constant level of oxygen consumption. In a 50 µM zinc solution no marked change in either oxygen consumption or gill ventilation rate was observed, which supports the conclusion reached in Chapter 8, that the respiratory system will function normally for at least part of the survival time, in a zinc concentration equal to the 24 h LC50 of the species.

At all zinc concentrations tested there was some indication that zinc may affect the regularity and coordination of the pleopod beat. This effect was most marked at the highest zinc concentration with changes in beat amplitude and frequency. In addition several animals were observed to constantly brush the pleopods and antennae, suggesting that zinc may be acting as an irritant to these appendages. Any loss in ventilatory rhythm within a zinc concentration of 50 µM had no obvious effects on the overall frequency of ventilation.

The effects of zinc on the respiratory system of *G. pulex* can therefore be summarised as follows. In those concentrations which are lethal to the animal within several hours, the oxygen consumption rises, mainly as a result of an increase in locomotory activity. Whilst the ventilatory system may successfully respond to the increased demand for oxygen with an overall increase in the pleopod beat frequency, rhythm and co-ordination are at least partially impaired. Tests of longer duration are necessary to determine the progressive severity of this impairment.

At a zinc concentration equal to the 24 h LC50 (ie lethal to 50% of the animals in 24 h), zinc would appear to have little direct effect on the respiratory system other than by causing a slight loss of pleopod beat co-ordination.

CHAPTER 10

CONCLUSION

The ultimate objective of this laboratory based toxicity study was to obtain consistent acute and chronic toxicity data for the freshwater amphipods, *G. pulex* and *C. pseudograciis*, in order to determine the likely effects of zinc pollution on each species in the more complex field environment. The study was conceived after a relatively short-term, but highly deleterious, incident of zinc pollution in Willow Brook, a small Northamptonshire river, from which both amphipod species had been recorded.

The first part of this concluding chapter examines the extent to which the collated results of laboratory toxicity tests performed on *G. pulex* and *C. pseudogracilis* from the unpolluted, but otherwise chemically similar, Rutland Reservoir can explain recorded changes in the distribution and abundance of the two species in Willow Brook, whilst heavily polluted by zinc, and during the subsequent recovery phase. The second part of the chapter is devoted to a discussion of ways in which the methodology of this relatively simplistic approach towards toxicity testing may be developed to have greater application to specific field situations.

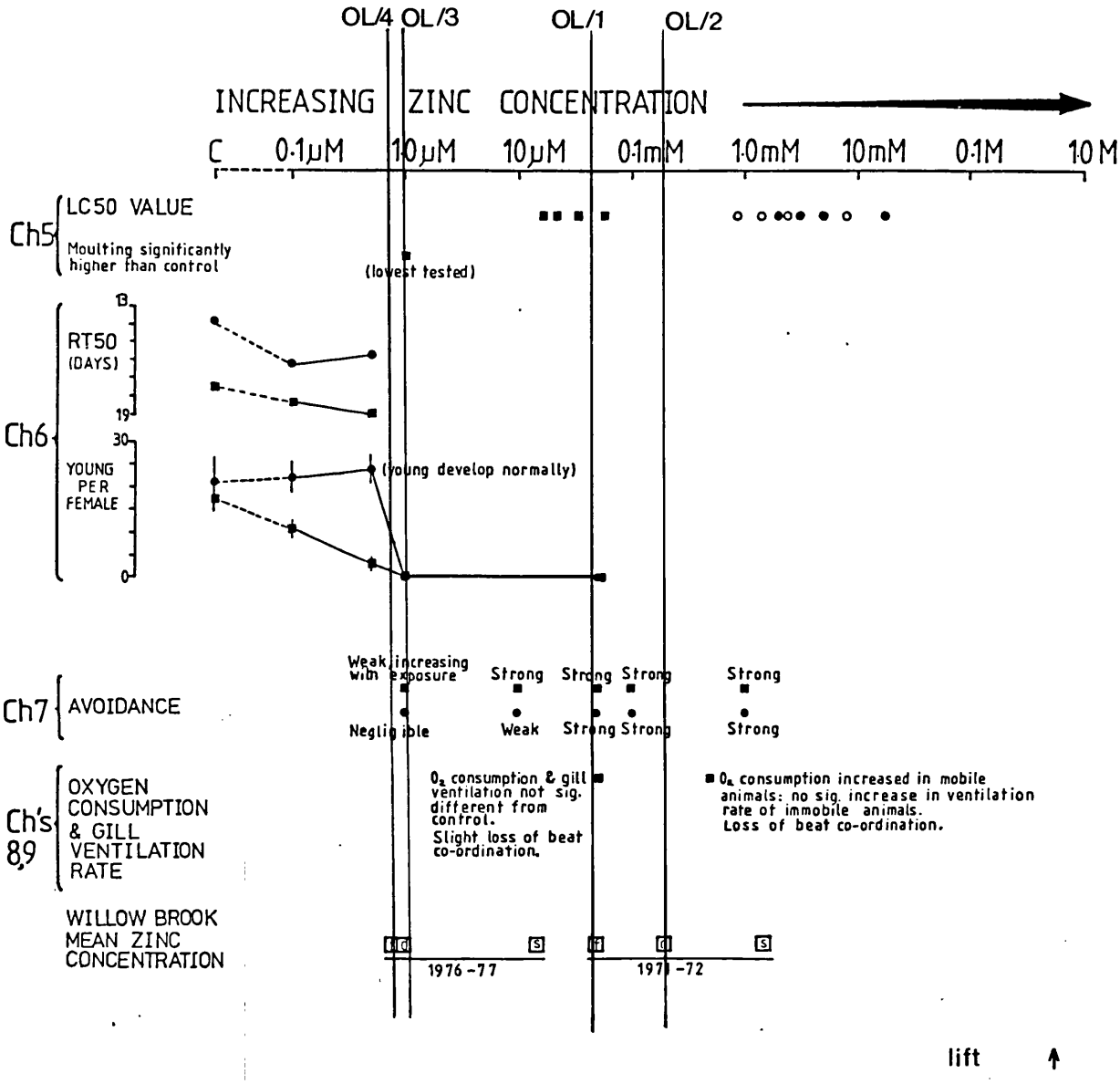


FIG. 10.1

Summary of test results against a logarithmic scale of increasing zinc concentration (C = control medium). The chapter from which the information has been extracted is indicated in the left margin.

o = *C. pseudogracilis* (♂), ● = *C. pseudogracilis* (♀), ■ = *G. pulex*

Mean zinc concentration in Willow Brook is from Harper *et al.*(1979).

f = Fotheringhay, d = Deene outfall weir, s = Source.

An overlay has been provided for ease of comparison between tests. This is referenced in the text as OL/1 to OL/4.

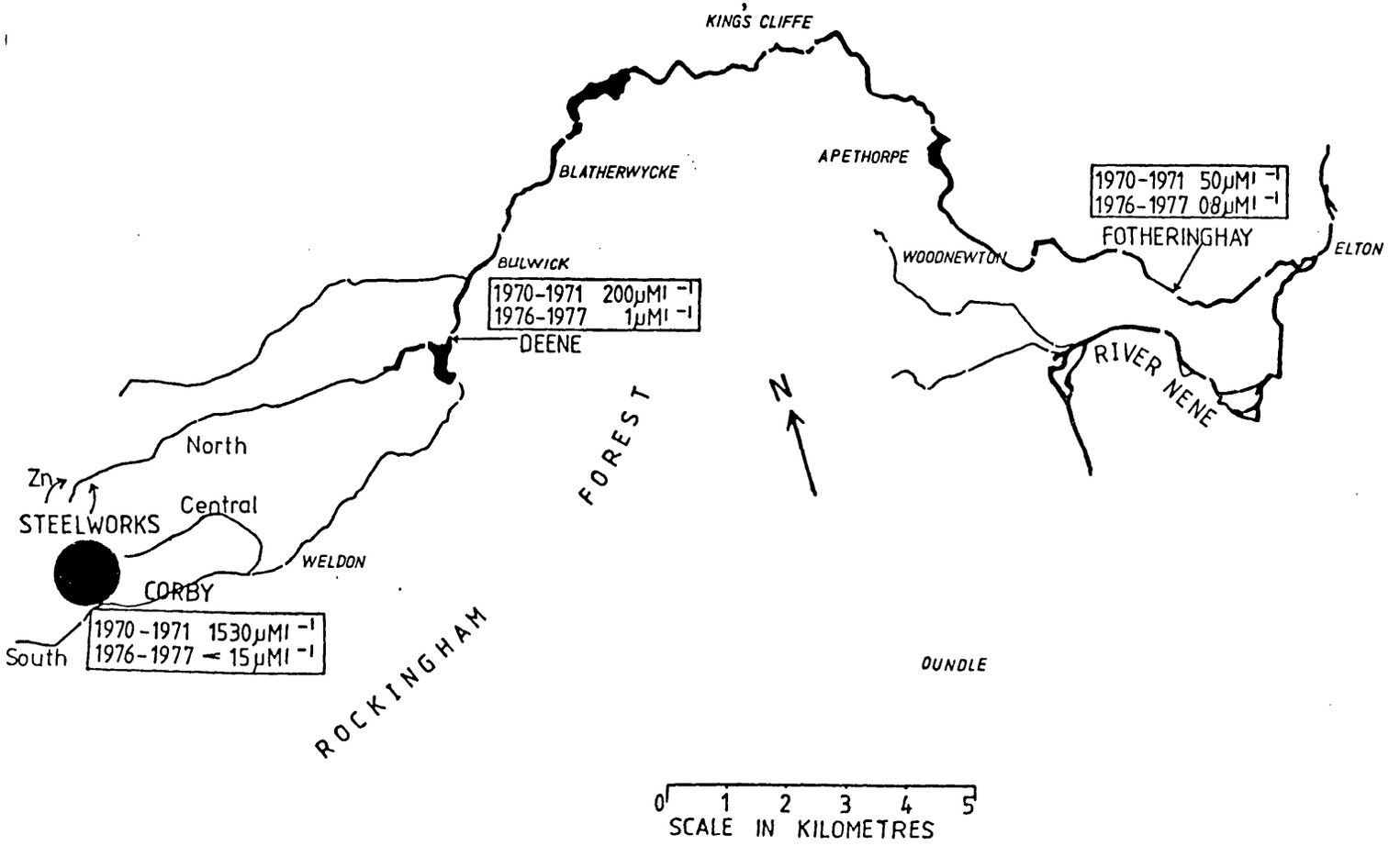


FIG. 10.2

Map of Willow Brook to show position of villages referenced in the text. Mean zinc concentrations at Source, Deene outfall weir, and Fotheringhay are shown next to the appropriate site.

EXTRAPOLATION OF LABORATORY TOXICITY DATA TO THE FIELD SITUATION

The salient results from each category of toxicity test which comprise this study have been summarised on a single diagram against a logarithmic scale of increasing zinc concentration (Fig. 10.1). The mean dissolved zinc concentrations at three well separated sites on Willow Brook, during a heavily polluted period (1970-71), and four years after the implementation of measures to control the amount of zinc in the effluent (1976-77), have been included for comparative purposes. These zinc concentrations, and the sites to which they apply, are shown on a map of Willow Brook (Fig. 10.2). The extent to which the laboratory based tests provide correlative evidence for the observed distribution and abundance of the two species in Willow Brook during these periods can now be assessed with reference to Fig. 10.1 & 10.2.

When levels of dissolved zinc within the effluent discharged to Willow Brook from the Corby Steelworks increased considerably in 1965, a large, well established population of *G. pulex* which had colonised at least as far upstream as King's Cliffe, was totally eradicated (Chapter 2). Comparison between the mean dissolved zinc concentration of 50 µM at the downstream site of Fotheringhay between 1970-71, and the 24 h LC50 value of this species (also approximately 50 µM), clearly emphasises the severity of toxicity (Fig. 10.1, Overlay sheet, line 1 - OL/1).

Under controlled laboratory conditions no individual *G. pulex* survived for longer than 3 days at this zinc concentration (Chapter 5). Physiological tests have indicated that symptoms of zinc stress prior to death may have included loss of pleopod beat co-ordination, although the relative importance of respiratory impairment at acutely toxic zinc levels cannot be predicted without tests of a longer duration (Chapters 8 & 9).

The avoidance response of *G. pulex* was very strong under laboratory conditions at a zinc concentration of 50 μM (Chapter 7), but from the above evidence it must be considered very unlikely that even populations of *G. pulex* from as far downstream as Fotheringhay, could have survived sufficiently long at these concentrations for downstream drift to have carried them clear of the toxicant.

Survival of the field population would have been determined by many more variables than those examined in the laboratory. In addition, the time taken for the concentration of dissolved zinc within the brook to reach these high levels is unknown. These tests are, however, strong evidence that *G. pulex* would have been unable to survive in Willow Brook whilst heavy zinc pollution continued, as a consequence of the acutely lethal action of zinc on the population, rather than any longer term effects on the reproductive cycle, etc.

In marked contrast to the total eradication of *G. pulex* from Willlow Brook, a small population of *C. pseudogracilis* was recorded during a period of heavy zinc pollution in a riffle habitat immediately below the outfall weir from Deene Lake (Chapter 2). The mean zinc concentration at this site was 4 times greater than that associated with the total elimination of *G. pulex* from further downstream at Fotheringhay.

Reference to Fig. 10.1,OL/2 reveals, not surprisingly, that the 1971-72 mean zinc concentration of 200 μM is below that which was acutely lethal in laboratory tests (OL-2). From these results alone it must therefore be concluded that under the prevalent physio-chemical conditions in Willow Brook a zinc concentration of 200 mM was not detrimental to the small population of *C. pseudogracilis*.

Chronic toxicity tests on the reproductive success of *C. pseudogracilis*, showed that a zinc concentration of only 1μM may, under certain physio-chemical conditions, be sufficient to cause reproductive failure in this species (Chapter 6). This sub-acute toxicity test therefore indicates that *C. pseudogracilis* may not have been able to reproduce successfully in Willow Brook during the period when the mean zinc concentration was 200 μM.

On superficial inspection, the results of acute and chronic tests appear to be in direct conflict, and it could be reasonably concluded that the sub-acute tests on reproductive success had grossly over-estimated true field toxicity. Harper, Hancock & Davies (1979), however, in a report of the recovery of Willow Brook from zinc pollution provide the following explanation for the presence of amphipods at Deene outfall weir in 1970, which would appear to resolve this ambiguity.

During the period for which Willow Brook was under surveillance by the Water Pollution Research Laboratory, Deene lake was generally less than 2 m deep with a soft mud bottom. Water from the Northern (zinc polluted) stream had a slower flow rate than the combined Southern and Central streams and initially followed the north bank of the lake (Solbe 1973). Harper, *et al.* (1979) postulated that a significant percentage of the southern arm of the lake remained sufficiently unpolluted to support a reproductive population of *C. pseudogracilis*. The small number of this species found within Willow Brook constituted a transient population, which was maintained by washout over the outfall weir of the lake.

Evidence to support this hypothesis comes from toxicity tests on the avoidance response of this species (Chapter 7). In the laboratory *C. pseudogracilis* can recognise and strongly avoid a 200 μM zinc solution. There was no evidence for a similar avoidance reaction in the field population, which was recorded exclusively from a short stretch of brook immediately below the outfall weir of the lake.

It can therefore be concluded that either the avoidance response was of insufficient magnitude and/or direction to be of survival value in the field environment, or that individuals moving downstream did not encounter sufficiently low zinc concentrations for reproduction to be successfully accomplished. It can be seen from Fig. 10.2 that individuals moving sufficiently far downstream to reach Fotheringhay would still have been exposed to a mean zinc concentration of 50 μM, fifty times higher than that causing total reproductive failure in the laboratory.

Unfortunately no field data exists to substantiate the hypothesis postulated by Harper, *et al.* (op. cit.) that the population of *C. pseudogracilis* below Deene Lake outfall weir was entirely dependent on continual replenishment from Deene Lake, but it is nonetheless entirely consistent with the laboratory data obtained in this study.

In 1972 effluent treatment at the Corby Steelworks was improved and a fall in the level of dissolved zinc in Willow Brook was followed by the recolonisation of its lower reaches by *G. pulex* (Chapter 2). In the Water Authority survey of 1976 *G. pulex* had reappeared as far upstream as King's Cliffe, and had become numerically the most abundant species further downstream at Woodnewton (Chapter 2).

If the lower mean levels of dissolved zinc in Willow Brook over 1976-77 are compared with the results of acute laboratory tests (Chapter 5), it is seen that the mean zinc concentration of the effluent leaving the Steelworks was still relatively high, but zinc concentrations at Deene and Fotheringhay had fallen well below the 96 h LC50 value of both amphipod species (Fig. 10.1, OL/3 & OL/4).

Recolonisation of Willow Brook by *G. pulex* could have been predicted from the results of acute testing, with no requirement for an increase in the tolerance level of the species. A zinc concentration of 1.0 µM, however, caused complete reproductive failure of this species in sub-acute tests (Chapter 6). In addition, an increase in development time and decrease in the mean number of viable young was clearly demonstrated at a zinc concentration of 0.1 µM, eight times lower than that recorded at Fotheringhay during this recovery period.

The implications of these laboratory results concerning the ability of *G. pulex* to recolonise Willow Brook with a mean zinc concentration of 1 µM are therefore two-fold; either they have provided an over-estimation of the true field toxicity of zinc to *G. pulex* , or recolonisation required the acquisition of reproductive tolerance to the elevated zinc concentration.

Without a comparison between the tolerance of G. pulex from Willow Brook, before and after zinc pollution, it is not possible to accurately assess the relative contribution of these two effects, although several studies have established that freshwater crustaceans can acquire tolerance to elevated levels of dissolved metals. Fraser, Parkin & Verspoor (1978) showed that populations of the freshwater isopod *Asellus aquaticus* inhabiting unpolluted water are highly sensitive to lead, but that exposure to the metal allows tolerance to develop in nature. Fraser (1980) also showed that some populations of *A. aquaticus* from polluted water are tolerant to lead, and that non-tolerant animals can be made tolerant by laboratory exposure to low levels of the metal.

Coombs (1980) suggested that the fact that many aquatic organisms can concentrate metals to a very high level and still survive and reproduce indicates that they have evolved tolerance. This has been confirmed by Brown (1977b), who demonstrated that *Asellus meridianus* that were copper and lead tolerant, accumulated metals from both solution and food, but non-tolerant animals could not and died. A comparison between the ability of *G. pulex*

from Willow Brook and from Rutland Water to accumulate zinc from solution may therefore help to establish whether the former population show any indication of zinc tolerance.

A further study of pollution tolerance, more relevant to this study, is that of Hobrough (1973) who suggested that juvenile *G. pulex* were more resistant to pollution (although he failed to specify of what nature!), and that this species can transpose the life history to give more juveniles at the time of the year when pollution effects are maximal. Studies on the population structure of *G. pulex* within Willow Brook would be necessary to investigate this theory.

Since the closure of the Corby Steelworks in 1982 the remaining threat of zinc pollution emanates from the redissolution of zinc from sediments when physio-chemical conditions are favourable. Parallel studies on the amount of zinc bound to the sediments, and its availability to the animals within Willow Brook are necessary to assess whether zinc may still be considered a significant pollutant source.

G. pulex taken from Woodnewton in 1984 were found to have a significantly higher respiration rate than animals from the population in Rutland Reservoir (Chapter 8). Further investigation would be necessary to decide whether this was correlated with the oxygen availability in the natural habitat, or a response to intermittent zinc stress.

C. pseudogracilis was again recorded in the Water Authority survey of 1976, in the typical riffle habitats below Deene lake, and in addition to 1971-72, both immediately upstream and downstream of Blatherwyke lake, although there was no overlap with the population of *G. pulex*. Downstream drift of the Deene population may have occurred naturally, or assisted by a weak avoidance response, although this species showed no significant avoidance of a 1 µM zinc solution in the laboratory.

This increase in the recorded distribution of *C. pseudogracilis* in Willow Brook during the early stages of recovery suggests that the population below Deene outfall weir was now able to reproduce successfully at the mean zinc concentration of 1.0 µM, even though this was not found to be true in the laboratory (Chapter 6) (Fig. 10.1,OL/4). The following explanation is offered to account for this discrepancy: Unlike *G. pulex*, *C. pseudogracilis* is not confined to a definite breeding season (Hynes 1955b). The ability of the species to reproduce successfully up to its critical zinc concentration (Chapter 6), may therefore have favoured the successful production of broods during periods when the dissolved zinc concentration dropped below the mean value. The distribution and abundance of *C. pseudogracilis* in Willow Brook during recovery from zinc pollution can therefore be explained from acute and chronic test results without an assumption of acquired tolerance, but it is possible that exposure to elevated zinc concentrations may have resulted in the acquisition of tolerance, similar to that suggested for *G. pulex*.

In 1983 a further invertebrate survey of Willow Brook by the Water Authority recorded the continued recolonisation by *G. pulex* as far upstream as Bulwick Mill (A.W.A. Unpublished data), but there was no evidence of *C. pseudogracilis* at any point throughout the brook.

The results of this laboratory toxicity study have shown that the adult *C. pseudogracilis* has a much higher tolerance to dissolved zinc than does *G. pulex*, and might therefore be expected to do better than the native species in zinc polluted environments. Indeed, this species was taken by the author from the Grand Union Canal in 1982, at a site immediately downstream of the Round Oak Steelworks (Brierley Hill). The presence of Ephemeroptera nymphs indicated an acceptable level of organic pollution for *G. pulex* but this species was absent.

The competitive advantage of *G. pulex* over *C. pseudogracilis* in a simple, zinc free laboratory system has been demonstrated in this study (Appendix 2). Whilst there may be many other factors which determine the distribution of the two species it is interesting that the author has been unable to find any report of a fast flowing unpolluted stream supporting co-existing populations of *C. pseudogracilis* and *G. pulex*. The disappearance of *C. pseudogracilis* from Willow Brook as recovery from zinc pollution has continued, and the coincidental increase in the range of *G. pulex* may reflect the superiority of the native species in a typical stream habitat where zinc is no longer a significant pollutant.

In summary, the results of both acute and chronic toxicity tests have been relatively consistent with the observed effects of zinc on the two amphipod species in Willow Brook, and reasons have been suggested for any differences. The contents of this study should therefore provide a useful starting point from which to develop a greater understanding of the effects of zinc on related freshwater systems.

DEVELOPMENT OF THIS TOXICITY STUDY

The possibility of certain metals having adverse effects on organisms was recognised prior to the 1940's but it was not until 1940-50 that the effects of chemicals and wastes on non-human organisms such as fish became of interest (Buikema *et al.* 1982). Since the late 1950's the acute toxicity test has been the foundation of studies to monitor pollution effects. A large proportion of toxicity data in the literature relates to the lethal effects of single toxicants on one or more animal species. This often appears to have little application to the field, and failure to standardise test procedures renders this work of limited comparative value with similar studies.

This study was designed with several primary objectives. Possibly the most important consideration in planning the study format was to maintain a relatively simplistic approach. This was highly desirable from a practical point of view since inexpensive equipment could be used and test results obtained within a limited time period. Furthermore, by restricting the number of variable parameters in each test, their interpretation had less ambiguity, although relevance to the field situation was necessarily sacrificed.

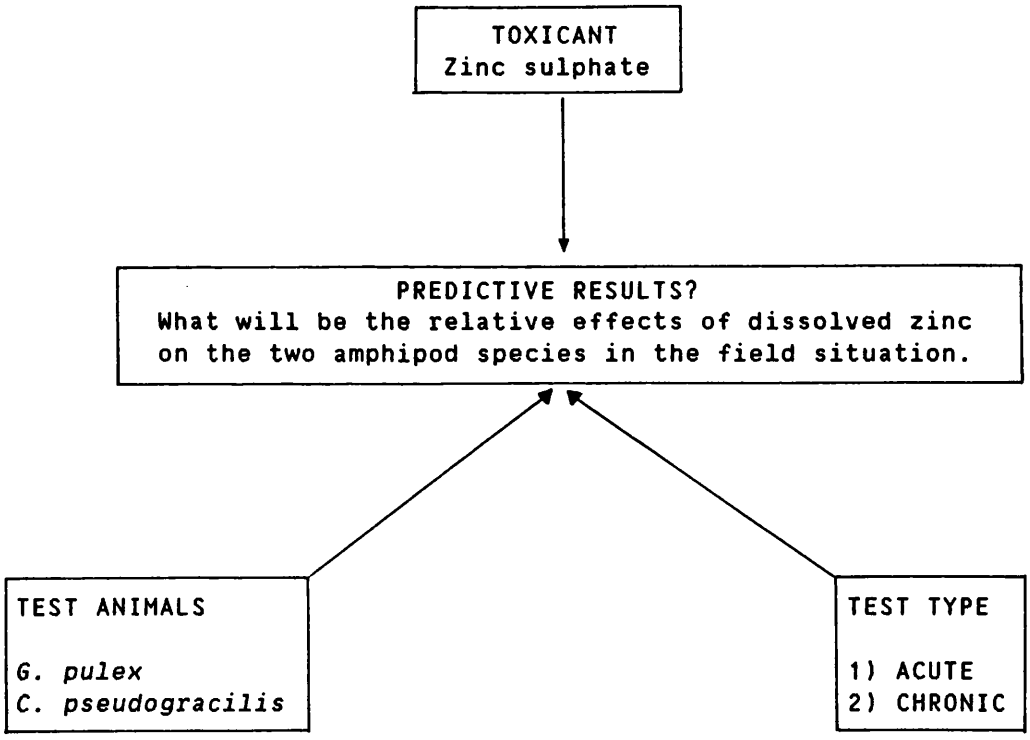
In order for the tests to retain as much application to the field situation as possible, the test species were obtained from a very similar physico-chemical environment and all tests were conducted with this zinc-free water as diluent. This was considered more important than using an artificial test medium since a standard diluent for freshwater toxicity studies has yet to be established, and the results would therefore lack both field application and comparative value.

Another important feature of the study was to incorporate sufficient acute and chronic tests to comprehensively describe the concentration range over which zinc was toxic to each species. The study was initiated with an analysis of acute zinc toxicity in order to provide a clear, unequivocal measure of toxicity on which to base sub-acute tests of longer duration, and assess the value of tests for which the effective zinc concentrations could not be predicted. The main value of the acute toxicity test was that it clearly demonstrated a large difference in the short term tolerance level of *G. pulex* and *C. pseudogracilis*.

Of more potential field value was the investigation of reproductive success at zinc concentrations below those causing mortality of the adult. In contrast to the acute tests, these experiments revealed that the highest zinc concentration tolerated for any reproduction was the same in both amphipod species. The combined results of these two approaches provided a clear evaluation of the difference in zinc concentration between that which is almost immediately fatal, and that which will only affect the ability to reproduce.

The third series of tests were concerned with behavioural responses to zinc and in contrast to the previous experiments the effective concentrations and the type of response which would be obtained were unknown. The avoidance response was found to be a good indication of sublethal toxicity for both amphipod species but changes in activity within a homogeneous zinc solution were only detected at highly toxic zinc concentrations. Similarly, tests on the susceptibility of the respiratory system of *G. pulex* to zinc yielded no positive results at realistic sub-acute concentrations, although the potential of these tests as indicators of sub-acute zinc toxicity could not be fully exploited in the time available.

A final consideration in the study design was to provide a basis from which to develop a more complex test regime, in which field application assumed priority, with less emphasis on limitation of expense and time. This approach, which has been outlined in the introduction to the study (Chapter 1) is re-illustrated diagrammatically as follows;



Two components of this approach can be recognised as having the potential for further development. First, the form of the toxicant may be varied and additional toxicants considered. Secondly, the test procedure may be expanded to consider different levels of biological organisation in addition to the individual animal, with which this study has concerned.

Metal speciation

Zinc was administered in this study as the soluble sulphate salt, since this ion is widely distributed in nature, and is generally present in unpolluted fresh waters in concentrations ranging from 0.0765 μM to 0.1530μM (Bachmann 1963). Furthermore, the solutions were quick and easy to prepare and may closely resemble the form in which zinc enters natural waters as a mine or smelter pollutant (Chapter 4).

Recently, there has been increasing awareness that not all metal species have the same toxicity to biological material (Ravera 1982). A metal is introduced into the aquatic environment in a given physical and chemical form which influences its distribution and fate. In addition, several environmental factors such as pH, dissolved oxygen concentration, hardness, and presence of chelating substances may modify the original species after release into natural waters (Chapter 1). Since the influence exerted by the metal on the organism varies with its physico-chemical form, knowledge of the heavy metal species present in the natural environment is highly desirable in an ecotoxicological study.

At the present time, difficulties in experimental technique prevent a routine consideration of metal speciation within biological toxicity studies. Separation of the various metal forms is technically difficult and several analytical aspects are unknown (Ravera op. cit.). Since the total concentration of the metal is generally low, quantifying different species may be difficult, requiring collaboration between physical chemist and biologist. Finally, if the metal species are successfully identified there is no guarantee that they will remain in stable equilibrium. Metal species may change prior to identification, or after presentation to the test species (Ravera op. cit.). The technical problems associated with metal speciation would appear to put the accurate determination of toxic metal species beyond the scope of most biological studies, although this aspect must remain an important future consideration in studies of this nature.

Sedimentary zinc

It is generally in the dissociated ionic form, Zn++, that zinc is considered most toxic as a freshwater pollutant (Lloyd 1960; Skidmore 1965), and it is for this reason that only the toxicity of dissolved zinc has been considered in this study. Wentsel, McIntosh & Anderson (1977) and Wentsel, McIntosh & Atchison (1977) discovered, however, that chironomid larvae avoided sediments contaminated with zinc, chromium and cadmium. Studies on the toxicity of sedimentary zinc would therefore be useful to assess the contribution of this potential source of metal towards overall toxicity, although two experimental difficulties are associated with this approach. First, a constant amount of metal must be retained in a solid form if the test is to be quantified. A necessity to maintain other physico-chemical variables acceptable to the test species may therefore impose a limit on the range of test concentrations over which this condition can be satisfied. Secondly, unless all the zinc remains in solid form, the contribution of the soluble fraction towards toxicity must be evaluated separately. If a stable equilibrium between dissolved and solid fractions is not achieved, the results cannot be reliably interpreted.

Mixed toxicants

In most instances of field toxicity it is rare that a single substance can be isolated as the only toxicant. Certain types of waste discharges ie. pharmaceutical manufacture wastes, consist of a complex mixture, frequently of organic nature (Murphy 1980). The zinc polluted Willow Brook described in this study was also known to receive small quantities of phenols and ammonia which may have contributed towards toxicity at certain times of the year (Chapter 2). In order to limit the complexity of the study, and facilitate interpretation of the result these toxicants were not treated as significant components of overall toxicity towards *G. pulex* and *C. pseudogracilis*.

The possibility that sub-lethal concentrations of secondary toxicants may combine additively with that of the main toxicant to have a lethal effect should be an important consideration in the development of a study of this nature. In a comprehensive review of the joint toxicity of metals, Sprague (1970) outlined a scheme for expressing the combined effects of several pollutants in which the toxicities of the individual components are summed as a fraction of the incipient LC50, and the total toxicity of the mixture expressed as a single value.

Towards a multi-disciplinary approach

Sindermann (1979) emphasised the need for toxicity studies to take a multi-disciplinary form, incorporating field experiments as part of the total experimental design. This aim has been only partially realised in this present toxicity study, since a comprehensive multi-disciplinary approach was beyond the scope of the study.

Four levels of organisation can be identified at which acute and chronic toxicity testing can be conducted according to an integrated multi-disciplinary regime;

1) Cell
2) Organ/tissue
3) Individual animal/Population
4) Community/Ecosystem

The first two levels of organisation involving mainly biochemical and histochemical techniques are primarily of value in determining the causes of toxicity, and may therefore assist in explaining the effects on life functions at the next highest level, eg. growth, respiration and behaviour.

Two investigations of this nature with direct application to the present study would be a histological determination of the effect of various zinc concentrations on gill structure, and the location of stored zinc within the body tissues.

The third level of organisation concentrating on the response of whole animals to the toxicant forms the basis of this study. The tests performed at this level of organisation are of a physiological or behavioural nature, and are designed to determine the effect of zinc on populations of the test species. The results must therefore be extrapolated in order to predict responses at higher levels of biological organisation.

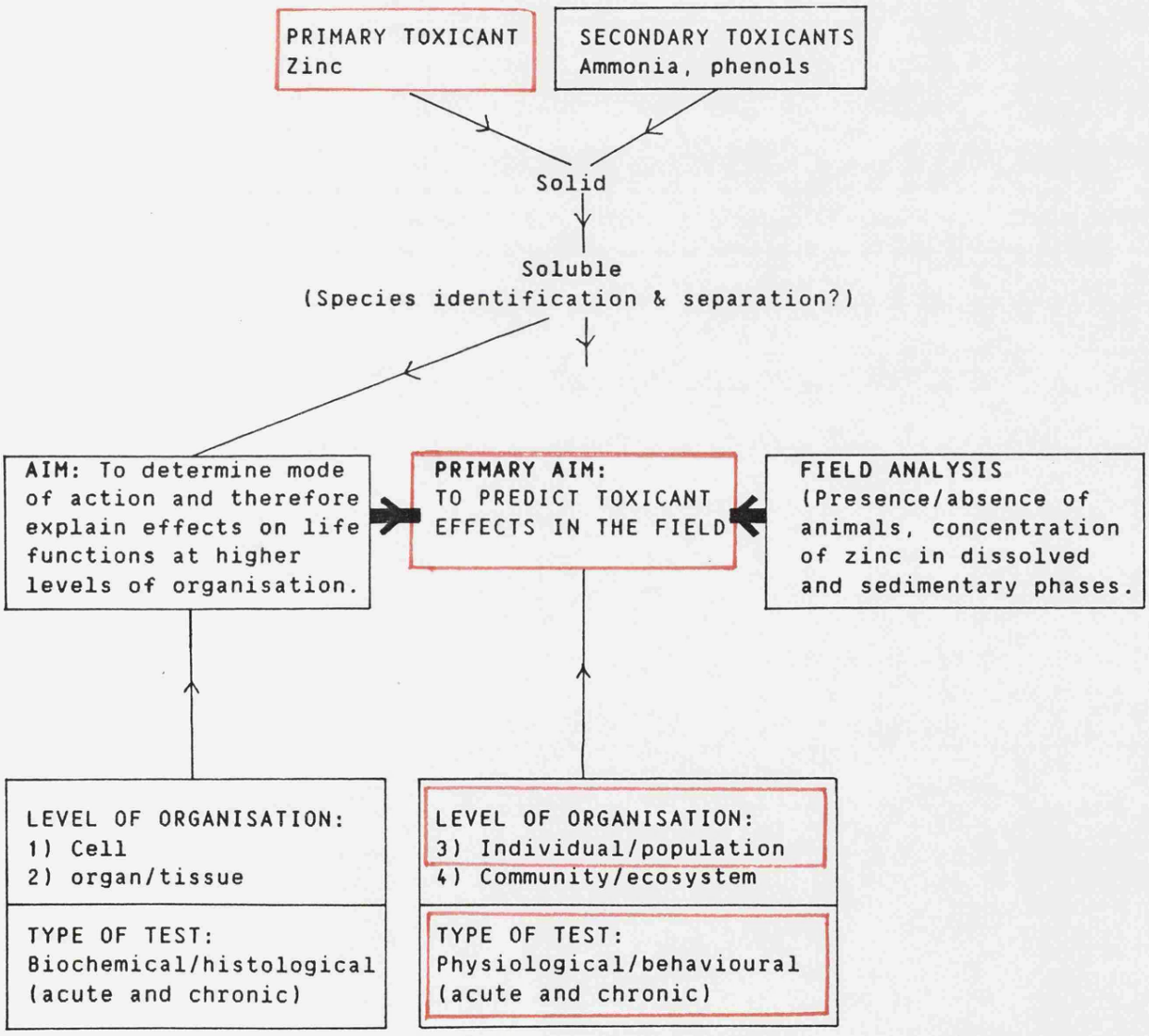
According to Sprague (1971), single species tests such as those used in this study have proved remarkably effective to estimate the responses at high levels of biological organisation, and despite considerable theoretical deficiencies in their use, are presently the major and only reliable way of estimating damage from pollution be excess quantities of a trace metal. Their development has consequently far outstripped the development and determination of toxicological responses at higher levels of biological organisation.

Cairns (1983) presents a less optimistic view, that in the absence of any scientifically justifiable evidence to indicate the degree of reliability with which one may use single species tests to predict responses at higher levels of biological organisation, the most pressing need in the field of toxicity testing is for the development of parallel tests at higher levels of organisation.

The fourth level of biological organisation to be considered in toxicity testing is consequently that of the community and ecosystem. Martin (1973) strongly supported the development of whole community bioassays accounting for predator pressure, environmental variability, and competition.

Sindermann (1979) pointed out, however, that 'micro-environment studies often have horrendous logistic problems and fail to control enough variables for the 'purist'. This type of approach would therefore seem the logical progression from a study of this nature in which basic toxic limits had been established.

The development of the present study outlined above can be summarised in the following diagram;



Components of the original study are outlined in red.

SUMMARY

1) A laboratory based toxicity study was conducted into the effects of dissolved zinc when present above essential trace levels, on two species of morphologically similar amphipod crustaceans, *Gammarus pulex* (L.), and *Crangonyx pseudogracilis* (Bousfield).

2) The ultimate aim of the study was to assess the extent to which transient zinc pollution may have dictated the distribution and abundance of *G. pulex* and *C. pseudogracilis* in Willow Brook, a small Northamptonshire river.

3) Rutland reservoir was an ideal source of test animals, being similar to Willow Brook in chemical and physical characteristics, but having no history of trace metal or organic pollution.

4) *G. pulex* had a much lower acute tolerance to zinc than did *C. pseudogracilis*. The later species also showed a marked difference in zinc tolerance between the sexes.

5) The ability of both species to produce viable broods was greatly impaired at zinc concentrations well below those which were acutely lethal to the adult. Complete reproductive failure occurred at the same zinc concentration in both species. In *C. pseudogracilis*, an increase in brood development time was the only indication of toxic stress below this concentration, whilst *G. pulex* suffered a clear-cut reduction in brood size at a zinc concentration ten times lower than that causing total reproductive failure.

6) Adults of both species showed a strong avoidance of zinc concentrations well below those which were acutely lethal. *G. pulex* was able to detect and avoid zinc at a concentration at least ten times weaker than that which evoked a response from *C. pseudogracilis*. Lethal concentrations of zinc produced a variety of modifications to the 'normal' activity pattern of both species but no significant change in activity was recorded at sub-lethal concentrations.

7) The effect of zinc on the respiratory system of G. pulex was examined. In those concentrations which were lethal to the animal within several hours, an immediate increase in oxygen consumption was obtained. No change in oxygen consumption was observed at zinc concentrations approximating to the 24 h LC50 value of the species. Loss in ventilatory rhythm was detected at all acute zinc concentrations tested.

8) This laboratory toxicity data was found to be entirely consistent with the known distribution and abundance of *G. pulex* and *C. pseudogracilis* in Willow Brook, during zinc polluted and recovery phases.

9) Ways in which this toxicty study could be developed to have greater application to specific field situations were discussed.

APPENDIX 1.

(A) PREPARATION OF ZINC CHLORIDE SOLUTIONS.

METHOD

1) 1 litre of zinc chloride solution with a notional concentration of 100 mM was prepared from 0.45 μm filtered Rutland reservoir water, and zinc chloride (Analar grade).
2) After a standing period of 1 h at $18\pm1^{o}C$ any precipitate formed was collected on a 0.45 μm millipore filter and redissolved in 5 cm^3 of 2.5% nitric acid. Deionised water was added to give a total volume of 100 cm^3.
3) The filtrate was used to prepare zinc solutions of notional concentration 1-100 mM. After 24 h at $18\pm1^{o}C$ each concentration was filtered (as in step 2) to remove any further precipitation.
4) A sample of filtrate at each notional concentration was acidified to prevent further precipitation and diluted to 0.1 mM with deionised water.
5) Samples were analysed by Atomic Absorption Spectrophotometry (Varian Techtron AA-6), to determine the actual concentration of the solution, and precipitates.

RESULTS

The actual concentration of each zinc solution was found to be only approximately 60% the notional value (Fig. A1.1).

This difference was too great to be explained by either the expected experimental error in preparing the initial solutions, or the subsequent precipitation of comparatively small amounts of zinc from each solution. It was therefore concluded that due to the extremely deliquescent nature of this salt it was impossible to weigh out accurately, small amounts of zinc chloride to prepare solutions of known concentration.

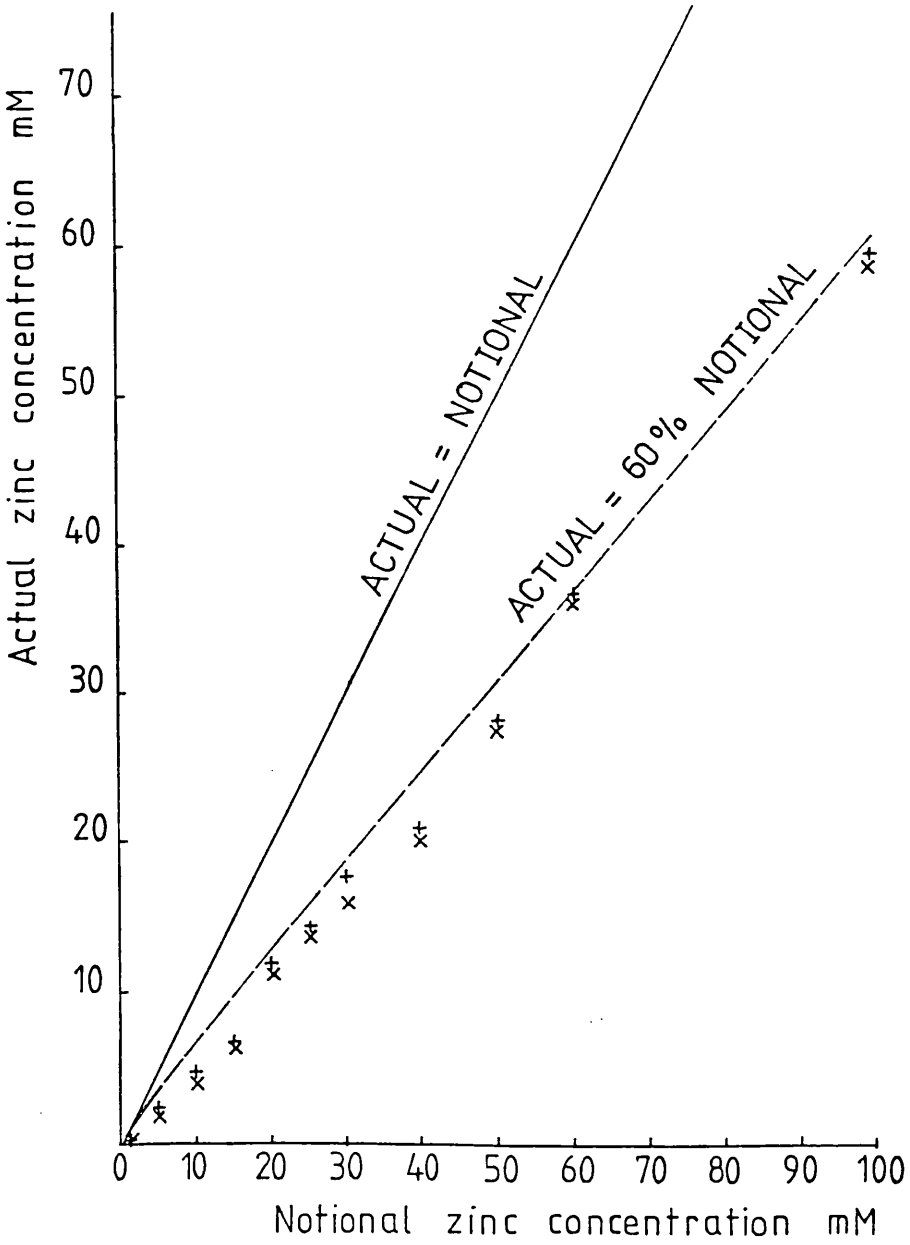


FIG. A1.1.
Plot of actual concentration v notional concentration for $ZnCl_2$ solutions.

+ = Actual concentration of solution + amount lost due to precipitation
x = Actual concentration of solution

(B) pH OF ZINC SULPHATE SOLUTIONS

METHOD

Zinc sulphate solutions between 0.5 µM and 50.0 µM were prepared by addition of $ZnSO_4.7H_2O$ (Analar grade), to the appropriate amount of millipore filtered Rutland Reservoir water. After vigorous shaking for 5 min the pH of duplicate samples was determined using an EIL 7050 pH meter. The mean results are presented as a graph of pH v zinc concentration.

RESULTS

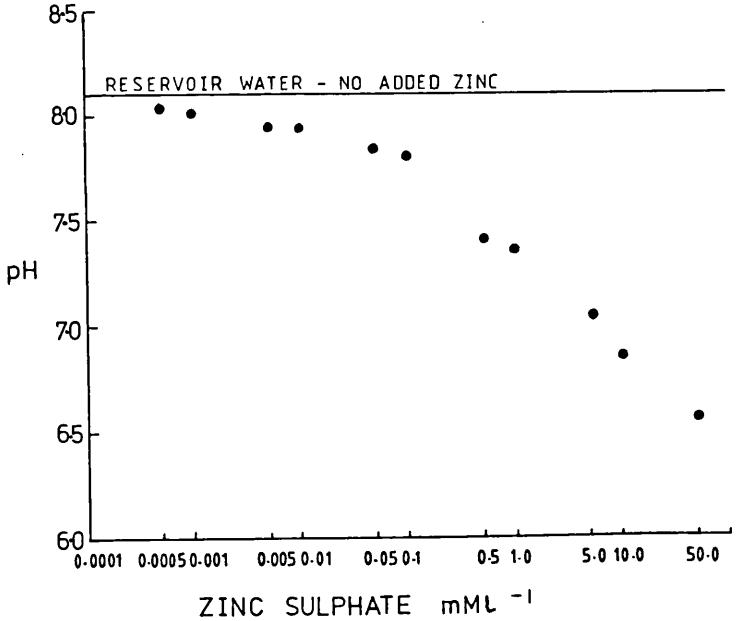


FIG. A1.2
Graph of pH v zinc sulphate concentration.
Natural pH of Rutland Reservoir water ≡ 8.15

The pH of zinc sulphate solution increased with increasing strength, but even at the highest test concentration of 50 mM it did not fall below 6.5. This is physiologically acceptable to both species (Chapter 5), and therefore it was not necessary to buffer any zinc solution used.

(C) ANALYSIS OF ZINC SULPHATE SOLUTIONS

Zinc sulphate solutions were analysed by Atomic Adsorption Spectrophotemetry to determine the actual concentration of the test solutions. The results are presented in Table A1.1.

1	2		3	4	5	6
TIME (h)	[REAL] (mM)		[MEAN OF DUPLICATE] (mM)	[NOTIONAL] (mM)	[MEAN OF TEST]	% DEV OF [MEAN] FROM [INITIAL]
0	46.803	-	(46.803)			
24	46.880	46.344	46.612	50.000	46.707	0.20
0	18.783	18.660	18.722			
24	18.324	18.691	18.507			
48	18.354	17.834	18.094			
72	18.110	18.140	18.125			
96	18.324	19.027	18.676	20.000	18.425	1.59
0	8.688	8.902	8.795			
24	8.443	8.535	8.489			
48	8.795	8.474	8.634			
72	8.902	8.642	8.772			
96	8.810	8.718	8.764	10.000	8.691	1.18
0	6.272	6.517	6.394			
24	5.996	6.058	6.027			
48	5.976	5.956	5.966			
72	5.905	6.017	5.961			
96	5.803	6.241	6.022	7.500	6.074	5.00
0	4.130	4.183	4.156			
24	3.916	3.984	3.950			
48	3.931	3.961	3.949			
72	3.877	4.007	3.942			
96	3.839	3.908	3.873	45.000	3.974	4.38
0	3.051	2.986	3.019			
24	2.998	3.028	3.013			
48	3.097	3.151	3.124			
72	2.837	3.090	2.964			
96	3.032	2.986	3.009	4.000	3.026	0.24
* 0	2.157	2.172	2.164			
24	2.115	2.126	2.121			
48	2.038	2.034	2.036			
72	2.038	2.992	2.015			
96	2.088	2.046	2.067	3.000	2.081	3.84
* 0	1.081	1.003	1.042			
24	0.916	1.074	0.995			
48	0.982	0.976	0.979			
72	0.976	0.948	0.962	2.000	0.995	4.56
* 0	0.410	0.486	0.448			
24	0.439	0.466	0.452			
48	0.455	0.459	0.457			
72	0.443	0.449	0.446	1.000	0.454	1.34
* 0	0.095	-	(0.095)			
24	0.095	-	(0.095)			
48	0.097	-	(0.097)			
72	0.101	-	(0.101)	0.100	0.097	2.10
0	0.000	0.000	0.000	0.000	0.000	-
24	0.000	0.000	0.000	0.000	0.000	-

TABLE A1.1
Explanation of column contents over page

TABLE A1.1 : Explanation of column contents

Column	Contents
1	Time of sampling (h)
2	Result of analysis on duplicate or single samples (mM)
3	Mean of duplicate samples where appropriate (mM)
4	Notional/intended zinc concentration (mM)
5	Mean of the analysed zinc concentration averaged over the duration of sampling
6	Percentage deviation of mean concentration (column 5) from initial concentration at time = 0

* indicates that in initial experiments this value exceeded 5%. This result was obtained by changing the respective zinc solution at 24 h intervals.

APPENDIX 2.

INTERSPECIFIC COMPETITION BETWEEN *GAMMARUS PULEX* AND *CRANGONYX PSEUDOGRACILIS* UNDER OPTIMUM PHYSIO-CHEMICAL CONDITIONS.

INTRODUCTION

C. pseudogracilis has, since its introduction into Britain during the first half of this century, spread rapidly throughout the canal system, and other navigable waterways. In contrast to *G. pulex* it is thought to favour slow flowing or still water environments (Bousfield 1958; Holland 1976), although co-existence of the two species has been reported in both rivers and reservoirs (Holland 1976; Bullock, Clark & Ison 1982).

The following observations, were made whilst sampling Rutland reservoir, and during the subsequent maintenance of mixed laboratory cultures. These suggest that co-existence between *G. pulex* and *C. pseudogracilis* is at least partially dependent on their spatial separation within a common environment.

1) Average densities of both amphipod populations, calculated from grab sampling in North and South Arms of the reservoir, simultaneously exceeded 200 animals m^{-2} on only two monthly occasions in 1981-1982. Statistical analysis of the comparative number of each species within a single grab sample containing a total of greater than 10 amphipods showed that in 7/10 samples on the first occasion, and 5/11 samples on the second, the sample contained significantly more individuals of one species (Bullock Unpublished data).

2) Edge sampling of large stones within 1 m of the surface produced almost exclusively *G. pulex*, whilst hand netting in *Cladophera* sp., and *Elodea* sp. beds gave predominantly *C. pseudogracilis*.

3) Under laboratory conditions *G. pulex* exhibits strong cannibalistic tendencies towards weak or newly moulted individuals (Clegg 1952; Hobrough 1973). This is far less marked but also true of *C. pseudogracilis* (pers. obs.). In mixed cultures, *G.pulex* has been observed to catch and devour *C. pseudogracilis*, but the reverse has not been recorded.

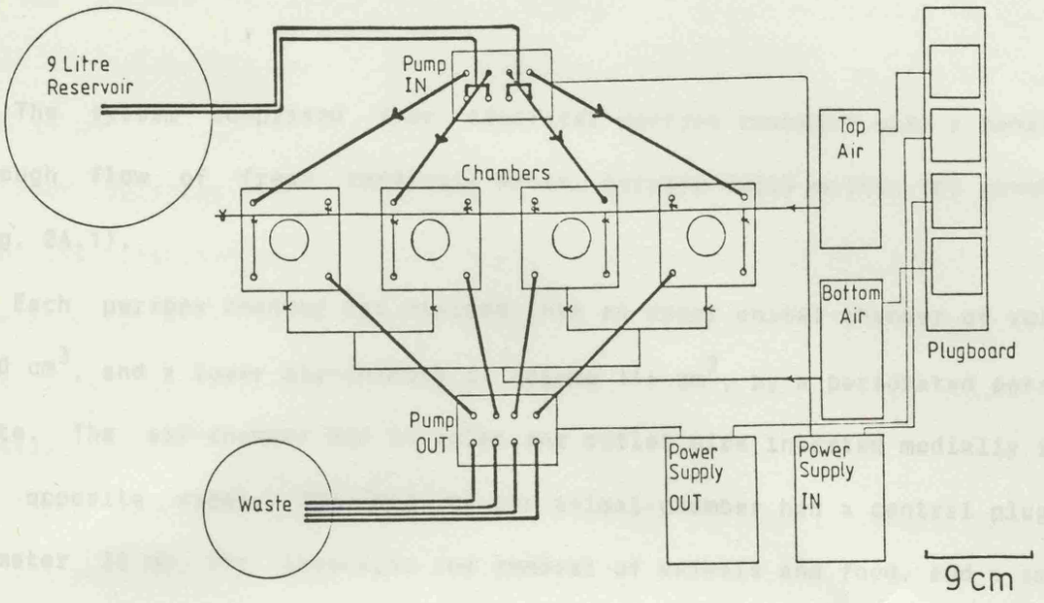
From these observations the following predictions were made.

1) Under optimum conditions both species would show good survival in mono-specific culture, although the cannibalistic tendencies of *G. pulex* may result in attacks on smaller individuals.
2) In mixed culture with no spatial separation *G. pulex* would predate *C. pseudogracilis* even in the presence of excess vegetable food.

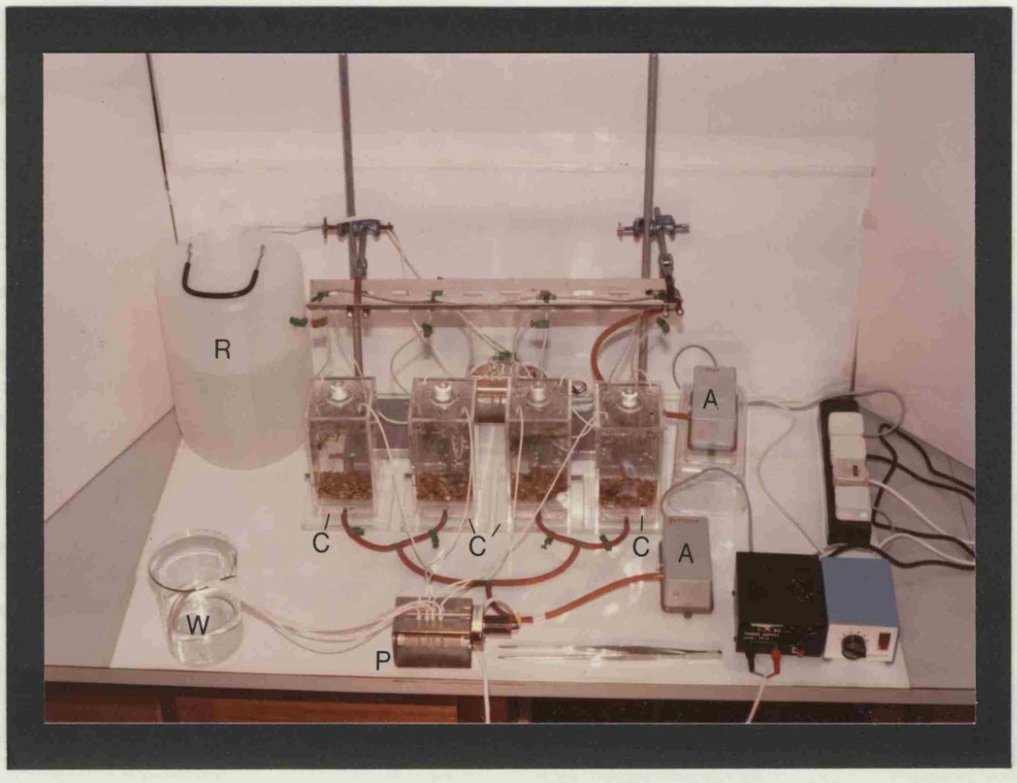
The following experiment was devised to investigate these predictions.

RATIONALE

The principle of the experiment was the creation of a very simple freshwater environment within 1 litre perspex chambers, into which individuals of either or both species could be placed and observed for a limited time period. The chambers were continuously aerated and food supplied regularly in excess.



a)



b)

FIG. 2A.1
Experimental system
a) Diagrammatic representation of the system
b) Photograph of the apparatus in operation,
P = pump, R = Reservoir, W = Waste, C = Chambers
A = Air pumps.

EXPERIMENTAL DESIGN

The system comprised four identical perspex chambers with a constant through flow of fresh reservoir water, aerated fully within the chambers (Fig. 2A.1).

Each perspex chamber was divided into an upper animal-chamber of volume 1000 cm^3, and a lower air-chamber of volume 144 cm^3, by a perforated perspex plate. The air-chamber had an inlet and outlet pipe inserted medially into two opposite sides. The top of the animal-chamber had a central plug of diameter 30 mm for insertion and removal of animals and food, and a small hole in each corner providing for 2 airlines, water inlet and water outlet. The perforated base of the animal-chamber was covered in gravel of assorted size and a large sprig of *Elodea* sp. provided.

Water contained in a 9 litre reservoir was pumped through silicone tubing (0.5 mm i.d.), and down a 20 µl micro-sampling pipette (Corning) into the bottom of the animal-chamber. A shortened micro-sampling pipette connected to an outward pump, removed water from the top of the diagonally opposite side of the chamber at a slightly faster rate thereby regulating chamber volume. Full replacement of the water within the chamber was achieved every 12 h. Air was pumped into the base of the chamber to support the water column maintaining an aerobic environment within the gravel on the bottom of the animal-chamber. A secondary air supply from two micro-pipettes inserted at diagonally opposite corners of the chamber maintained full oxygenation within the water column.

PROCEDURE

Both single and mixed species cultures were established with a total number of 40 amphipods in each. *C. pseudogracilis* was subdivided by sex, and

in the absence of marked sexual dimorphism *G. pulex* was separated by size into juveniles less than 5 mm in length and mature adults of greater length than 10 mm.

Four cultures were maintained simultaneously for 14 days during which time the distribution of animals within the chambers and the number of dead were recorded daily. Bemax (wheat germ) was provided as a food source. After this time the cultures were dismantled and the number of live individuals counted.

Three replicate experiments were performed for single species cultures and six replicate experiments for mixed species cultures, to give a total of 60 animals tested from each sex and size class under both conditions.

RESULTS

REPLICATE	SPECIES	NUMBER OF ANIMALS							
		MALE		FEMALE		>10mm		<5mm	
		I	F	I	F	I	F	I	F
1a)	C. pseudogracilis	20	18	20	19				
1b)		20	13	20	16				
1c)		20	19	20	16				
TOTAL =		60	50	60	51				
2a)	G. pulex					20	15	20	8
2b)						20	18	20	7
2c)						20	11	20	1
TOTAL =						60	44	60	16
3a)	Both species	10	0	10	0	10	7	10	7
3b)		10	0	10	0	10	9	10	7
3c)		10	2	10	0	10	7	10	6
3d)		10	0	10	0	10	7	10	8
3e)		10	0	10	0	10	10	10	6
3f)		10	5	10	0	10	8	10	2
TOTAL =		60	7	60	0	60	48	60	36

TABLE 2A.1.
A comparison between the initial (I) and final (F) number of animals in both single species and mixed species culture.

Single species culture: *C. pseudogracilis* only.

During the first week of the experiment both sexes remained almost exclusively on the gravel bottom, after which time they became active on the *Elodea* sp. sprig within the water column. Movement was mainly by crawling with little swimming activity. Both sexes had a high percentage survival in all 3 replicate tests giving a mean total survival of 84.1%

Single species culture: *G. pulex* only.

G. pulex was active on the gravel bottom, *Elodea* sp. and swimming within the water column for the duration of the experiment. The larger size class were very conspicuous, but the smaller animals tended to crawl under the surface gravel. The *Elodea* sp. sprig was devoured rapidly and needed replacing every 2-3 days. No small dead animals were observed but larger dead individuals remained for 1-2 days before being devoured.

The survival rate was markedly different for the two size classes. In the larger size class, 73% of the total number survived, whilst in the smaller size class less than 30% survived. The visible absence of small dead individuals throughout the test strongly indicated that they were cannibalised either immediately on death or after active predation by the larger individuals.

Mixed culture.

As soon as the cultures were established *C. pseudogracilis* burrowed beneath the gravel surface and after two days no individual of this species was visible. In contrast *G. pulex* remained active on the *Elodea* sp. and gravel substratum. No dead individuals of *C. pseudogracilis*, or the smaller size class of *G. pulex* were seen, although several dead individuals of the larger size class remained untouched.

On several occasions large *G. pulex* were observed to catch female *C. pseudogracilis* and devour them. This predatory behaviour is reflected in the final species composition for the mixed cultures. Survival within the larger size class of *G. pulex* was almost the same as in mono-culture, but the smaller individuals of the species survived much better. In contrast *C. pseudogracilis* showed a much lower survival rate with a total of only 11.6% males at the end of the experiment. The total elimination of female *C. pseudogracilis* may be the result of their larger size and therefore the inability to hide within the gravel substratum, where they would be inaccessible to predation by *G. pulex*.

CONCLUSION

The results of this experiment strongly support the predictions made in the introduction, which were formulated from the hypothesis that under favourable physio-chemical conditions *G. pulex* will actively predate *C. pseudogracilis* unless spatial separation is possible. The absence of *C. pseudogracilis* from shallow stream habitats occupied by *G. pulex* can therefore be at least partially explained as an inability under these conditions, to achieve adequate spatial separation.

APPENDIX 3

BASIC PROGRAM TO CALCULATE RESPIRATION RATE

```
10 REM      $$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
15 REM      This program calculates the respiration rate of a group of animals
20 REM      in ul of oxygen/mg wt (wet & dry)/hr, from % saturation data
25 REM      obtained from a flow through respirometer designed by Mary Hardwick.
30 REM      The respirometer consists of two rank electrodes connected to a
35 REM      flatbed recorder. The data is obtained in the form of two traces
40 REM      one representing the control saturation (100%) and the second
45 REM      recording the % desaturation of the control by the test animals.
50 REM      This data is most conveniently handled by measuring the saturation
55 REM      of the animal trace at 5 minute intervals throughout the experiment
60 REM      and correcting for minor fluctuations in the control. This series of
65 REM      five minute saturations monitoring the oxygen consumption of the
70 REM      test animals is the form in which data is input into this program
75 REM      In this series of experiments the tests were of 7 hours duration,
80 REM      and zinc solution of known concentration was introduced at the start
85 REM      of the 4th hour.
90 REM      $$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$$
95 REM
100 REM      -----------------------------------------------------------------
105 REM      Program written by   Mary Hardwick, Leicester University
110 REM                           25.VI.1984.
115 REM
120 REM
125 REM      The single subroutine called prints a line of dashes between each
130 REM      five minute record for clarity
135 REM
140 REM     -----------------------------------------------------------------
145 REM
150 :
155 REM    ..................................................................
160 REM    Set up the appropriate number of loops
165 REM    ..................................................................
170 PRINT "Enter the number of animals for which the calculation is required"
175 INPUT N
180 FOR II = 1 TO N
185 :
190 :
195 REM   ...................................................................
200 REM  reset variables used in the heading information here
205 REM   ...................................................................
210 S = 0
215 L = 0
220 A = 0
225 :
230 :
235 REM   ...................................................................
240 REM   Enter here the experimental information to head the results sheet
245 REM   ...................................................................
250 INPUT "enter conc of zinc solution in uM";X
255 LPRINT "ZINC CONCENTRATION (uM) = ";X
260 LPRINT
265 :
270 INPUT "antennae present or absent (Y/N) ";Q$
275 IF Q$ ="Y" THEN 290
280 LPRINT "Antennae absent"
285 GOTO 295
290 LPRINT "antennae present"
295 :
300 INPUT " Number of animals";A
305 LPRINT "NUMBER OF ANIMALS = ";A
310 :
315 LPRINT "INDIVIDUAL LENGTHS (mm) = "
```

```
325    INPUT "length = ";L
330    LPRINT L
335    S = S + L
340 NEXT KK
345 :
350 LPRINT "MEAN LENGTH = ";S/A;" mm"
355 LPRINT
360 :
365 INPUT" WEIGHT OF ANIMALS = ";W
370 LPRINT "TOTAL WEIGHT OF ANIMALS (mg) = ";W
375 LPRINT "MEAN WEIGHT = ";W/A;" mg"
380 :
385 LPRINT: LPRINT: LPRINT
390 :
395 :
400 REM  ..........................................................
405 REM  Write out the headings for the results sheet.
410 REM  ..........................................................
415 LPRINT"TIME";TAB(7);"O2 STN";TAB(17);"O2 SOL";TAB(28);"FLOW";TAB(36);"TOT  O2";TAB(46);"O2 USED";TAB(59);"RATE";TAB(73);"RATE"
420 LPRINT"mins";TAB(10);"%";TAB(18);"ul/L";TAB(28);"ul/hr";TAB(37);"ul/hr";TAB(47);"ul/hr";TAB(57);"ul/mg.w/hr";TAB(70);"ul/mg.d/hr
425 :
430 GOSUB 935
435 :
440 :
445 REM  ..........................................................
450 REM  The first line of data to be printed consists of the initial
455 REM  saturation at the start of the experiment (should be approx 100%)
460 REM  the flow rate, upper and lower oxygen solubilities, total available
465 REM  oxygen and the amount of oxygen used in ul/mg/hr for wet and dry
470 REM  weights of the animal.
475 REM  ..........................................................
480 INPUT "FIRST SATURATION";SAT
485 INPUT "FLOW RATE L/HR  ";FL
490 :
495 :
500 REM  ..........................................................
505 REM  The oxygen solubility is obtained in air saturated freshwater from
510 REM  an approprite nonogram (Hitchman 1973), as ppm (mg/L). In this case
515 REM  the temperature used was 18 +/- 1 degree C, and therefore the values
520 REM  corresponding to 17 & 19 degrees C have been taken for upper and lower
525 REM  limits to be obtained. This is converted to ul/L using the relationship
530 REM  1 mg oxygen/L = 1.428 mg oxygen/L, to give the values SOL & HIG.
535 REM  The total available oxygen is then obtained by multiplying these values
540 REM by the flow rate in L/hr; LX and UX.
545 REM  ..........................................................
550 LET SOL = (9.35/1.428)*1000
555 LET HIG = (9.70/1.428)*1000
560 LX = FL * SOL
565 UX = FL * HIG
570 :
575 IF SAT = 100 GOTO 680
580 :
585 :
590 REM  ..........................................................
595 REM  If the initial saturation is not 100% then calculate the total oxygen
600 REM  used (US & LS) as total available oxygen multiplied by the % fall in
605 REM  saturation. To obtain the weight specific rate (mg) (UR & LR) divide
610 REM  through by the total wet weight of animals . The water content of
615 REM  Gammarus pulex is 77.09 +/- 0.8, therefore to obtain the respiration
620 REM  rate per mg dry weight the appropriate correction is made giving LD &
625 REM  LD; the upper and lower respiration rates in ul/mg.dry wt/hr.
630 REM  ..........................................................
635 US = ((100 - SAT)/100) * UX
640 LS = ((100 - SAT)/100) *LX
645 UR = US/W
```

```
655 UD = UR * (1/0.2211)
660 LD = LR * (1/0.2371)
665 :
670 GOTO 735
675 :
680  US =0
685  LS =0
690  UR =0
695  LR =0
700  UD = 0
705  LD = 0
710 :
715 :
720 REM  ..........................................................................
725 REM  Print out these values in the appropriate columns
730 REM  ..........................................................................
735 LPRINT"0";TAB(7);SAT;TAB(17);SOL;TAB(25);FL;TAB(36);LX;TAB(46);LS;TAB(59);LR;TAB(70);LD
740 LPRINT TAB(17);HIG;TAB(36);UX;TAB(46);US;TAB(59);UR;TAB(70);UD
745 :
750 GOSUB 935
755 :
760 :
765 REM  ..........................................................................
770 REM  Now enter in the rest of the saturation values for each 5 minute time
775 REM  interval. Set K to increment to the total number of hours for which
780 REM  the experiment is to run. In the following calculations only the
785 REM  % saturation, oxygen used, and the wet & dry respiration rates are
790 REM  given.
795 REM  ..........................................................................
800 FOR K = 1 TO 7
805    FOR I = 5 TO 60 STEP 5
810       INPUT "ENTER SATURATION ";NS
815       US = ((100 - NS)/100) * UX
820       LS = ((100 - NS)/100) * LX
825       UR = US/W
830       LR = LS/W
835       UD = UR * (1/0.2211)
840       LD = LR * (1/0.2371)
845       LPRINT I;TAB(7);NS;TAB(46);LS;TAB(59);LR;TAB(70);LD
850       LPRINT TAB(46);US;TAB(59);UR;TAB(70);UD
855       GOSUB 935
860    NEXT I
865    LPRINT: LPRINT: LPRINT
870 NEXT K
875 :
880 :
885 NEXT II
890 :
895 END
900 :
905 :
910 :
915 REM  ..........................................................................
920 REM  Subroutine to print lines between each set of data
925 REM  ..........................................................................
930 :
935 LPRINT"-------------------------------------------------------------------------------"
940 RETURN
```

EXAMPLE :

ZINC CONCENTRATION (uM) = 100

Antennae absent
NUMBER OF ANIMALS = 10
INDIVIDUAL LENGTHS (mm) =
7
7
7
7
7
8
8
9
9
10
MEAN LENGTH = 7.9 mm

TOTAL WEIGHT OF ANIMALS (mg) = 150
MEAN WEIGHT = 15 mg

TIME mins	O2 STN %	O2 SOL ul/L	FLOW L/hr	TOT O2 ul/hr	O2 USED ul/hr	RATE ul/mg.w/hr	RATE ul/mg.d/hr
0	100	6547.62	7.08E-03	46.3572	0	0	0
		6792.72		48.0924	0	0	0
5	81				8.80786	.0587191	.247655
					9.13755	.0609171	.275518
10	78				10.1986	.0679905	.286759
					10.5803	.0705356	.319021
15	67				15.2979	.101986	.430138
					15.8705	.105803	.478532
20	65				16.225	.108167	.456207
					16.8324	.112216	.507534
25	64				16.6886	.111257	.469242
					17.3133	.115422	.522035
30	65				16.225	.108167	.456207
					16.8324	.112216	.507534
35	62				17.6157	.117438	.49531
					18.2751	.121834	.551037
40	64				16.6886	.111257	.469242
					17.3133	.115422	.522035
45	63				17.1521	.114348	.482276
					17.7942	.118628	.536536
50	65				16.225	.108167	.456207
					16.8324	.112216	.507534
55	64.5				16.4568	.109712	.462724
					17.0728	.113819	.514734
60	61.8				17.7084	.118056	.497917
					18.3713	.122475	.553937

APPENDIX 1$

MICROFICHED COMPUTER PROGRAMS FOR CHAPTER 5

FILE NAME	DESCRIPTION
HWKVE5	Fortran program which computes the probit values for a set of data, and evaluates the best fit regression line through those points. Four graphs of probit value against log zinc concentration are then plotted per A4 page. This program is suitable for use with any mortality data in order to obtain LC50 values, but the graphing routine is specific to *G. pulex* and must be amended when the critical concentration range differs to that of this species.
HWKC5	Command file to run HWKVE5 interactively on a Cyber 77, producing graphical output on a calcomp plotter.
HWKLVL	Fortran program to plot a graph of male LC50 v female LC50 for *C. pseudogracilis*, and draw the best fit regression through the points.
HWKLD50	Fortran progran to plot the values of LC50 against time and draw the best fit curve through the points.
HWKLD51	Modification of HWKLD50 to print more than one line on the graph.

APPENDIX 2$

MICROFICHED COMPUTER PROGRAMS AND DATA FILES FOR CHAPTER 7

FILE NAME	DESCRIPTION
HWKPBH HWKMER	Fortran programs to merge two 15 min digitised data files into a single file containing X and Y co-ordinates only.
HWKCYB	Fortram program to plot unmerged data as a graph on the Cyber.
HWKGR5	Fortran program to plot original data for *G. pulex* for all 3 parameters (Distance travelled, time spent resting, number of turns).
HWKGRT	Control program to run HWKGR5.
HWKGR4	Fortran program to plots original data for *C. pseudogracilis* for all 3 parameters.
HWKGRS	Control program to run HWKGR4.
	Fortran programs to;
HWKGR6	1) Plot distance travelled with control modification.
HWKGR3	2) Plot time resting with control modification.
HWKGR7	3) Plot number of turns with control modification.
HWKGR8	4) Plot distance travelled as mean of 10 lines.
HWKGR9	5) Plot time resting as mean of 10 lines.
HWKG10	6) Plot number of turns as mean of 10 lines.
HWKGRC	Control program to run above plotting routines.

DATA FILES FOR CRANGONYX PSEUDOGRACILIS:

T30CR1 - T30CR10	(10 FILES)
T1CR1 - T1CR10	(10 FILES)
T01CR1 - T01CR10	(10 FILES)
CCRM1 - CCRM8	(8 FILES)
CCR1 - CCR10	(10 FILES)

DATA FILES FOR GAMMARUS PULEX:

T30GA1 - T30GA10		(10 FILES)
T1GA1 - T1GA10		(10 FILES)
T01GA1 - T01GA10		(10 FILES)
CGAM1 - CGAM8		(8 FILES)
CGA1 - CGA10		(10 FILES)

APPENDIX 3$

MICROFICHE COMPUTER PROGRAMS AND DATA FILES FOR CHAPTER 8

FILE NAME	DESCRIPTION
HWKOXY	Fortran program which processes data files to produce graphs of percentage oxygen saturation v time. Data files are then reprocesses to plot oxygen consumption against time. Optional features are described within the program.
HWKR	Control program to run HWKOXY.
	Data files consist of 4 items per line; Time, oxygen saturation, upper respiration rate, and lower respiration rate.
R500A1 R500A2 R500A3 R500A4 R500A6 R500N5	Test solution 500 µM zinc sulphate.
R100A1 R100A2 R100A3 R100A4 R100A6 R100N5	Test solution 100 µM zinc sulphate.
R50A1 R50A2 R50A3 R50A4 R50A5 R50N6	Test solution 50 µM zinc sulphate.

APPENDIX 4$

MICROFICHED COMPUTER PROGRAMS AND DATA FILES FOR CHAPTER 9

FILE NAME	DESCRIPTION
HWKVENT	Fortran program to plot raw ventilation data as pleopod beats against time and record half beats.
HWKVEN	Control program to run HWKVENT.
HWKREG3	Fortran program to calculate the number of beats per minute over each of twelve five minute records.
HWKREG	Control program to run HWKREG3.
	Data files;
HWK5001 HWK5002 HWK5003	Test solution 500 µM zinc sulphate, animals with no antennae.
HWK5004 HWK5008	Test solution 500 µM zinc sulphate, animals antennae tips missing.
HWK5005 HWK5006 HWK5007	Test solution 500 µM zinc sulphate, animals antennae.
HWK109 HWK1010 HWK1011 HWK1012	Test solution 100 µM zinc sulphate, animals antennae.
HWK5013 HWK5014 HWK5015 HWK5016	Test solution 50 µM zinc sulphate, animals antennae.

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