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Fenn, S. J.

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SOME EFFECTS OF COPPER CONTAMINATED PIG SLURRY ON EARTHWORMS.

- by -

S.J. Fenn
B.Sc. hons.

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CONTENTS

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Chapter 1 INTRODUCTION

This dissertation is concerned with the application of copper contaminated pig slurry to agricultural land and its effect on earthworm populations. Chapter 1 reviews published information on this topic, chapter 2 presents details of the investigations I carried out at three field sites, chapter 3 describes laboratory experiments set up to examine the rate of copper uptake and elimination by earthworms, and chapter 4 deals with histological work on the localization of copper within earthworms.

1.1 Copper in the soil

From a biological standpoint, copper belongs to a group of eight elements known as trace elements or micronutrients. Although essential to plant and animal growth they are required in only very small quantities, and may be harmful if available forms are present at high concentrations.

Copper in soils may be derived both from the natural weathering of residual or transported parent material, and from a number of anthropogenic sources. The background level of copper resulting from parent material may be very variable, and may often be solely responsible for deficiencies or toxic levels of copper in the soil. Anthropogenically derived copper may be imposed upon the natural background levels and may have a considerably modifying effect on soil concentration, either on a local or more widespread scale. The average copper content of British soils has been quoted as 20mg/kg (Swaine and Mitchell 1960).

The influence of parent material on the copper content of soil is often modified by pedogenic processes, which may lead to the mobilization and redistribution of copper both between adjacent soils and within the soil profile. Factors affecting the relationship between copper in the parent rock and the soil include the degree of weathering; the nature and intensity of soil formation; the drainage; the pH; the redox potential and the amount of organic matter in the soil (Baker 1974). Factors such as climate, topography, vegetation and land use are also important. Soils derived from coarse grained material such as sandstones and from acid igneous rocks such as rhyolites tend to contain smaller amounts of copper than soils derived from fine grained sedimentary rocks such as clays and from basic igneous rocks (Thornton 1979).

The anthropogenic sources of copper include mining and smelting, which can lead to very heavy contamination of soils, mainly as a result of particles carried in fugitive dust or leached from spoil heaps from copper mining. Cannon and Anderson (1971) report a level of 5000mg/kg copper at the soil surface within one mile of a copper smelter at Superior, Arizona. Copper is often associated with other metals such as tin, silver and lead. Mining of these metals may also result in a release of copper.

Urban areas have been found to contain appreciably higher copper levels than rural areas (Purves 1967). Increased levels of copper may be due to a variety of factors including the burning of fossil fuels, dumping of industrial waste, traffic and other industrial activities. The Applied Geochemistry Research group at Imperial College, London, showed in a survey of precipitation from 88 sites on a traverse from southwest to northwest England, that copper deposition ranged from 0.2 to 19.8mg/cm^{-2} per year, with the lower values recorded in rural areas, and the peak values in the vicinity of the industrial cities (Thornton 1974). The total flux of copper in the atmosphere is 75 x 10^{6}kg/yr, 75% of which is estimated to come from anthropogenic sources (Nriagu 1979).

A further source of copper comes from fertilizers, ameliorants and pesticides. The copper content of fertilizer has been found to range from 1 to over 1000mg/kg (Swaine 1962). Bordeaux mixture, consisting of copper sulphate and slaked lime, is often used for controlling downey mildew in vine culture, potato blight and leaf spot in sugar beet. Soil ameliorants containing copper include domestic coal ash, soot, municipal compost and sewage sludge. Copper applied in fungicides and fertilizers is strongly bound in the upper soil horizons (Delas 1963) and accounts for the fact that copper content of many soils decreases down the profile (Mengel and Kirkby 1978).

Sewage sludge and farm animal slurry are frequently applied to the soil as a fertilizer, soil ameliorator, and just as a means of disposal. Sewage sludge contains variable quantities of copper from industrial sources. Heavy application over a considerable period of time can lead to an accumulation of copper in the soil. In industrial areas of England sludge treated soils contained 35mg/kg of E.D.T.A. -

extractable copper compared with 16mg/kg copper in nearby untreated land (Purves 1977). E.D.T.A. is a chelating agent and is often used as a means of determining the level of available copper in the soil.

The application of pig slurry to arable land is thought to be of particular importance in raising the copper levels, and it is towards copper from this source and its effect on earthworms that this study is directed. A fuller introduction to this aspect is given in the next section.

The form in which copper is present, as well as total amounts and concentrations is of great importance when considering its mobility and availability to plants and animals. Copper is strongly held in inorganic and organic exchange sites. It is held as complex combinations by organic colloids. Correlations between the soil organic matter and copper have been noted (Van Rhee 1975). In these complexed forms a large proportion of the total copper content of soils is not available to plants and animals. It has been shown that most of the organic complexes of copper involve phenolic, carboxyl and ~~m~~ydroxyl groups and are fairly weak. Stronger complexes h/ with humic acids have also been demonstrated, in which copper is complexed with phorphyrin groups (Goodman and Cheshire 1973, 1976).

Although copper held on exchange sites is not readily available to plants and animals, cation exchange of Cl^{2+} and $CuOH^{+}$ can take place and is best effected by H^{+} (Mengel and Kirkby 1978). The soil pH has a decided influence on the availability of all the micronutrients. The amount of copper in soil water decreases with increasing pH because of stronger copper absorption (Lindsay 1974). Ordinarily, at pH values higher than 6.5, copper tends to be only slowly available to plants and animals. McLaren and Crawford (1973a) described a method for fractionating soil copper and showed an association between free manganese oxides and copper in the soil, and suggested that the bulk of the available soil copper is in the organically bound fraction.

As well as the pH and organic matter content, the oxidation-reduction status of the soil and its moisture content effect the form in which copper is present. Copper is found in more than one valent state, the lower ones being encouraged by low oxygen supply and high moisture. The changes from one valent state to another are usually brought about by microorganisms and organic matter. In some cases the organisms may obtain their energy directly from the inorganic reaction. In general, high pH values favour oxidation and low values favour reduction. The oxidized states of copper are generally less soluble at pH values commonly found in soils than are the reduced states. The hydroxides of these high valent forms precipitate at low pH values and are extremely insoluble (Brady 1975). The effect of soil moisture on the availability of copper is not clearly understood. Flooded soils often show higher availabilities than well aerated soils. However at high pH values the reverse may occur.

Some organic forms of copper are more readily available than others. Copper complexes of molecular weight <1000 were found to be more available to plants than those with molecular weights exceeding 5000 (Nriagu 1979).

Copper chelate combinations occur when metal cations are bound to organic compounds to form a complex ring structure. In this form the usual ionic characteristics of the metal are lost and the metal is less likely to take part in other reactions in the soil. In this form, copper is protected against precipitation as insoluble hydroxides. Although chelated metals are protected against soil reactions, they are readily assimilated in this form by growing plants. Chelation therefore acts to increase the available pool of cation micronutrients.

Nutrient balance among the trace elements is essential to the plant and animal systems that utilize them, but is greatly complicated by their interactions. For example some of the plant enzyme systems which are dependent upon trace elements require more than one element. Both manganese and molybdenum are needed for the assimilation of nitrates by plants. The utilization of potassium and copper is dependent upon the proper balance between these two nutrients, and copper utilization is favoured by adequate manganese, which in some plants is assimilated only if zinc is present in sufficient amounts (Brady 1974). In many cases an excess or deficiency of copper in an area is accompanied by a similar imbalance in one or more other elements.

1.2 Copper in pig slurry

In livestock farming the term slurry has aquired a fairly precise meaning. It is the urine and dung from animals or the fresh droppings from poultry diluted with varying amounts of water. An indication of the amounts produced and the proportions of dung and urine is shown in table 1.

Table 1

Pigs	Litres per day			
	body wt. kg.	dung	urine	total
Fattening	35	3	2	5
Mature	90	5	4	9

(G.J. Perkin 1979)

Most slurries, as removed from storage and spread on land, consist of dung and urine diluted to 2 or 3 times their volume in water. Slurry comprises undigested food residues, salts, waste products from normal body functions, mucus secreted by the intestinal walls and various nitrogen compounds (which range from protein to urea and ammonia compounds, all of which can be mineralized and nitrified in the soil to nitrate which is a basic plant nutrient. Phosphorus and Potassium are also present in useful quantities, besides lesser amounts of minerals including calcium, magnesium and trace elements (A.D.A.S. 1977). The dry matter content of undiluted dung and urine produced by pigs is 8-10% (A.D.A.S. 1976).

The problem of disposing of pig slurry may be considerable, resulting from the concentration of animal husbandry into fewer and bigger units. The rate of mineralization of pig slurry depends on soil condition, and may give rise to a temporary excess of mineral nitrogen. Furthermore, there is often too little land on the farm to allow pig slurry to be spread at levels which are not detrimental to the soil. An excess of manure may be followed by leaching of nutrients.

Most slurry is spread on grassland. If it contains low levels of toxic metals, and if applied at a suitable time of year and in favourable weather conditions, normal rates of application of up to 4.5m^3 per hectare have generally beneficial effects. However disposing of pig slurry with high copper levels could raise the copper content of the soil to undesirable levels.

Copper is commonly added to pig fattening rations at levels of 200-250mg/kg, to act as a growth promoter. These levels can increase the rate of live weight gain by around 8% (Braude 1975). The practice was initiated twenty years ago on the basis of experimental evidence at that time (Braude 1975). Copper is also added to breeding stock feed as an essential element, but in much smaller amounts to avoid problems of accumulation. The copper is normally fed to pigs as soluble copper sulphate in the diet and occasionally as copper oxide. The copper passes through the gut wall into the bloodstream and varying amounts are removed and stored in the liver. The remainder is discharged mostly in the urine. A large proportion of the copper is excreted. Excretion values of 72-80% have been quoted by Priem and Manton (1980) and 80-95% by Unwin (1977).

Slurries containing highly variable quantities of copper have been recorded, 675mg/kg by Berryman (1971) and 273-1990mg/kg (mean 869mg/kg, 34 samples) by Unwin (1977). The chemical form in which copper is excreted is not known, so it is difficult to assess how much of this copper is available to plants and animals once the slurry has been applied to a field.

When considering the level of copper found in pig slurry it is evident that continuous heavy application of slurry could very easily raise the copper level of the soil to 80mg/kg available copper which is considered by the A.D.A.S. soil scientists to represent the upper threshold level above which problems of toxicity in plants could occur.

Experiments by McGarth et al (1980) showed that after a cumulative slurry application of 1000m^2 per hectare over three years, total copper in the 0-5cm soil horizon (including the vegetation mat) was increased by as much as 38mg/kg. Increases of up to 6mg/kg and 1.7mg/kg were found for the 5-10cm and 10-15cm horizons respectively. E.D.T.A. extractable copper increased similarily to total copper.

In another experiment it was shown that 37m^3 of pig slurry containing 240kg nitrogen contained 1.1kg copper. When this volume was spread over a hectare of land, the copper content was sixteen times greater than that taken up by plants (Vetter 1980).

Although plants take up only a few per cent of the additional copper this may be sufficient to cause toxic effects to the plants, and the remainder may be left to accumulate in the soil and become available to the soil fauna. Spreading pig slurry increased the copper content of herbage by between 4 and 100mg/kg for a $115m^2$/ha application three times a year, but the increase was generally less than 15mg/kg. Copper content was found to be strongly influenced by the rate of application.

1.3 Copper and plant-animal interrelationships

There has been an increasing awareness in recent years of the problem of contaminating agricultural soil with an excess of copper, as a result of the application of pig slurry. This has led to greater interest being shown in the long term effects of copper contamination and the need to examine the problem from an ecological point of view. In terrestrial ecosystems there is little understanding of the means and pathways by which copper and other heavy metals are moved through the food chain. Although many studies have been made of the concentrations of a variety of heavy metals in terrestrial habitats the study is complicated by the uneven distribution and the availability of the heavy metals to plants and animals.

The two groups of soil invertebrates which have been studied most extensively are the isopods and gastropods. Both of these use the respiratory pigment haemocyanin of which copper is a component. Isopods have been shown to accumulate e/
large concentrations of copper. In *Porcellio scab~~a~~r* 960-1180mg/kg have been recorded in whole animals, and 14,300mg/kg in the hepatopancreas (Wieser and Makart 1961). Levels recorded for gastropods are much lower. 46±4mg/kg was measured in whole animals of the species *Helix aspersa* (Coughtrey and Martin 1976).

In both isopods and gastropods the copper concentrations vary by a factor of 15-20 between different populations (Nriagu 1979).

Relationships have been shown between the concentration of copper in plant material and that in the invert~~i~~brates e/ feeding on it. The concentration of copper in the soft parts of Helix aspersa reflected the distance of the collecting site from a smelting complex (Coughtrey and Martin 1977). A study of Wieser et al (1976, 1977) showed that isopods living in copper-rich and copper-poor localities in the Austrian tyrol, showed a nearly perfect (r = 0.98) relationship between the average concentrations of copper in litter and the average concentration of copper in the bodies of the isopods feeding on this litter. The concentration factor was approximately 6, that is, on a dry weight basis the concentration of copper in the bodies of the isopods was about 6 times higher than that in their food. The concentration factor for Helix pomatia is 2 (Moser 1978) and for the marine polychete Nereis diversicolor it is less than 1 (Bryan and Hummerstone 1971).

In considering the potential toxicity of copper to invertebrates and plants the powers of adaptation of the organisms must be considered. Populations accustomed to low levels of copper may succumb to concentrations which may yet be harmless to other populations of the same species which have spent their lives in areas of high copper concentration. This was shown by Bryan and Hummerstone (1971) working on Nereis diversicolor. There is some evidence to suggest that the tolerance of elevated levels of copper in the environment is genetically determined. This has been shown in some land plants (Schiller 1974) and in Nereis diversicolor (Bryan and Hummerstone 1971).

1.4 Copper in earthworms

Earthworms play an important part in the terrestrial ecosystem. In moist temperate regions they may contribute up to 80% of the biomass of all the soil organisms (Van Rhee 1976). Their beneficial effects include the improvement of soil structure; the incorporation of dung, plant residues, fertilizers and insecticides; and affects on the availability of soil nitrogen. Elimination of earthworms could lead to a decrease in the rate of release of nutrients in the soil and a gradual deterioration in the soil structure. The importance of Lumbricus terrestris L. was clearly demonstrated by comparing two orchards in one of which Lumbricus terrestris was present whereas in the other earthworms were almost absent due to frequent and heavy spraying with a copper-based fungicide. The orchard with few earthworms had accumulated a surface mat of dead vegetation, 1-4cm thick, which was sharply demarkated from the underlying soil which had a poor crumb structure (Van Rhee 1963, 1967). In grasslands high copper concentrations (260-360mg/kg) proved sufficiently toxic to eradicate worm populations almost entirely (Nielson 1951). Although the application of copper-contaminated pig slurry may not yet have caused soil copper to reach levels which cause total eradication of earthworms, it has been shown that high copper levels are checking reproductive capacity and body growth (Van Rhee 1969, 1974). Decreases in density related to high copper levels, the result of pig slurry applications, have already been noted (Van Rhee 1975).

It appears that at moderate levels of applications of pig slurry, the population density of earthworms increases probably because of the increase in the amount of available food. However, at high levels of application, or as a result of repeated applications at moderate levels, the densities of earthworms have been shown to decrease. Cotton and Curry (1979a) showed that when slurry was applied at 80-100m^3/ha/yr (on average) a moderate increase in both numbers (25-31%) and biomass (23-38%) was recorded in slurry-treated plots. They found invertebrate community structure to be largely unaffected by slurry. A further study by Cotton and Curry (1979b) on the effects of increasing rates of pig slurry application showed that the highest level of application studied, 345m^3/ha/yr, significantly reduced earthworm population density.

The effects of gross pollution arising from the dumping of large quantities of pig slurry in a quarry in Celbridge, Co.Kildare were also studied by Curry and Cotton (1979). In wet weather, slurry frequently overflowed from the quarry, contaminating a strip approximately 10m wide x 100m downhill. There were very few earthworms in the area adjacent to the quarry in April, 12 months after a major spill. The population had substantially recovered by November, but a high proportion of worms adjacent to the quarry were surface dwelling pigmented forms. The typical grassland non-pigmented species (e.g. <u>Allolobophora spp.</u>) remained scarce. Soil copper levels up to 100mg/kg were recorded at the quarry edge but these declined rapidly with increasing distance from the quarry. A corresponding gradient in copper levels present in earthworm bodies was noted.

Van Rhee (1976) also found a clear relationship between the copper content in soils and that in worm bodies. He found a significant correlation ($r = 0.952$ $p = \langle 0.001$) between copper levels in soil samples and earthworms from nine sites. However Ireland (1979) found no relationship between tissue concentration in earthworms and in soil concentrations for copper. His study involved Lumbricus rubellus from three localities in which the copper concentration of the soil varied between 20 and 335mg/kg. The dry weight of worm tissue was found to contain between 11 and 13mg/kg copper at all three sites. The results from this study would seem to imply that copper levels were regulated in Lumbricus rubellus and at a low level.

In studying the relationship between soil copper levels and copper levels in earthworm tissues it is necessary to consider any differences between species of earthworms and in size of the worms. Differences may occur either as a result of physiological or behavioral characteristics of the species or size group. The behavioral characteristics will be especially important if the copper in the soil is concentrated at any particular level, since the depth at which the earthworms live and feed will determine how much contact they have with the copper rich layers of the soil.

1.5 Localization of copper in invertebrates

The means by which heavy metals are stored in animal tissue has received much attention in recent years. Copper may occur in several different forms in living tissue. Coombs' (1974) work on Ostrea edulis showed that it was transported in the form of small soluble organic complexes, mostly containing an amino-acid moiety. It is complexed or detoxified by copper rich proteins such as coeruloplasmin or metallothionein (Winge et al 1975, Noel-Lambot 1976). A very efficient means of accumulating and detoxifying copper has been recognised in the cuprosomes which have been found in terrestrial and freshwater isopods (Wieser and Klinia 1969) and oysters (George 1978). The presence of these membrane-bound vesicles allows copper to reach very high concentrations in some animal tissues. 14,300p pm has been recorded in the cuprosomes of the hepatopancreas in terrestrial isopods (Wieser and Makart 1961). Moser (1978) showed that in Helix pomatia no cuprosomes were present but copper was concentrated in the hepatopancreas, the albumen gland and the tissues of the intestinal tract. About 90% of the copper in the hepatopancreas and albumen gland was so tightly bound that it could be liberated only by ashing, whereas between 55 and 70% of the total copper in the walls of the intestinal tract could be extracted with weak hydrochloric acid. This suggests that in the hepatopancreas and the albumen gland most of the copper is complexed by proteins, but that this means of detoxification is not available in the tissues of the intestinal tract.

In earthworms the chloragogenous tissues have been found to accumulate heavy metals. Ireland (1978) found that the highest concentrations in the chloragosomes were of Ca^{++} followed by fe^{++}, Zn^{++}, Mg^{++}, Pb^{++} and Cu^{++}. Chloragosomes have been shown to display cation-exchange properties and redox activity (Fischer 1973). Fischer (1976) has stated that earthworm chloragosomes contain phosphoric acid, carboxyl, phenolic, hydroxyl and sulphonic acid groups, all of which are constituents of ion exchange compounds. Ireland (1978) also found lead and copper in extracts of earthworm body walls.

1.6 The present study

An attempt was made to study a few aspects of the effect of pig slurry application on earthworms. These included: a) the effect of pig slurry application on soil copper levels in a field situation; b) the relationships between soil copper levels, earthworm densities and the level of copper in worm tissues in natural conditions; c) a determination of the rates of uptake and elimination of copper by worms in experimental conditions and d) the localization of stored copper in earthworms by histological techniques.

Chapter 2 FIELD EXPERIMENTS

2.1 Site selection

Three study areas were selected. Sites were chosen which provided an experimental and control area fairly close to one another to reduce the chances of differences in soil texture and composition occurring. This was considered to be important since differences in the soil could markedly effect the total copper content, its distribution through the soil profile and its availability to worms. The experimental areas were fields or plots where various levels of pig slurry had been applied, and the control areas were those for which no record existed of any pig slurry being applied.

2.2. Site descriptions

Site A Whixley Grange

The Whixley Grange study areas are situated on two adjacent farms which were originally one. The experimental field lies at National Grid Reference SE 592 445 and the control field lies at SE 592 448. Both fields lie at an altitude of between 38-45m above sea level. The experimental field has a slight east facing slope.

The bedrock of the area is new red sandstone and the surface soils of both the experimental and control fields is a sandy loam. The rainfall in the area is 760mm per annum.

Both experimental and control fields are planted with potatoes and have been under cultivation for at least 100 years. No accurate record is available for the amount of pig slurry which has been applied to the experiment field, but it is

Map 1 showing the location of the Whixley Grange study area.

--------- control field

_________ experimental field

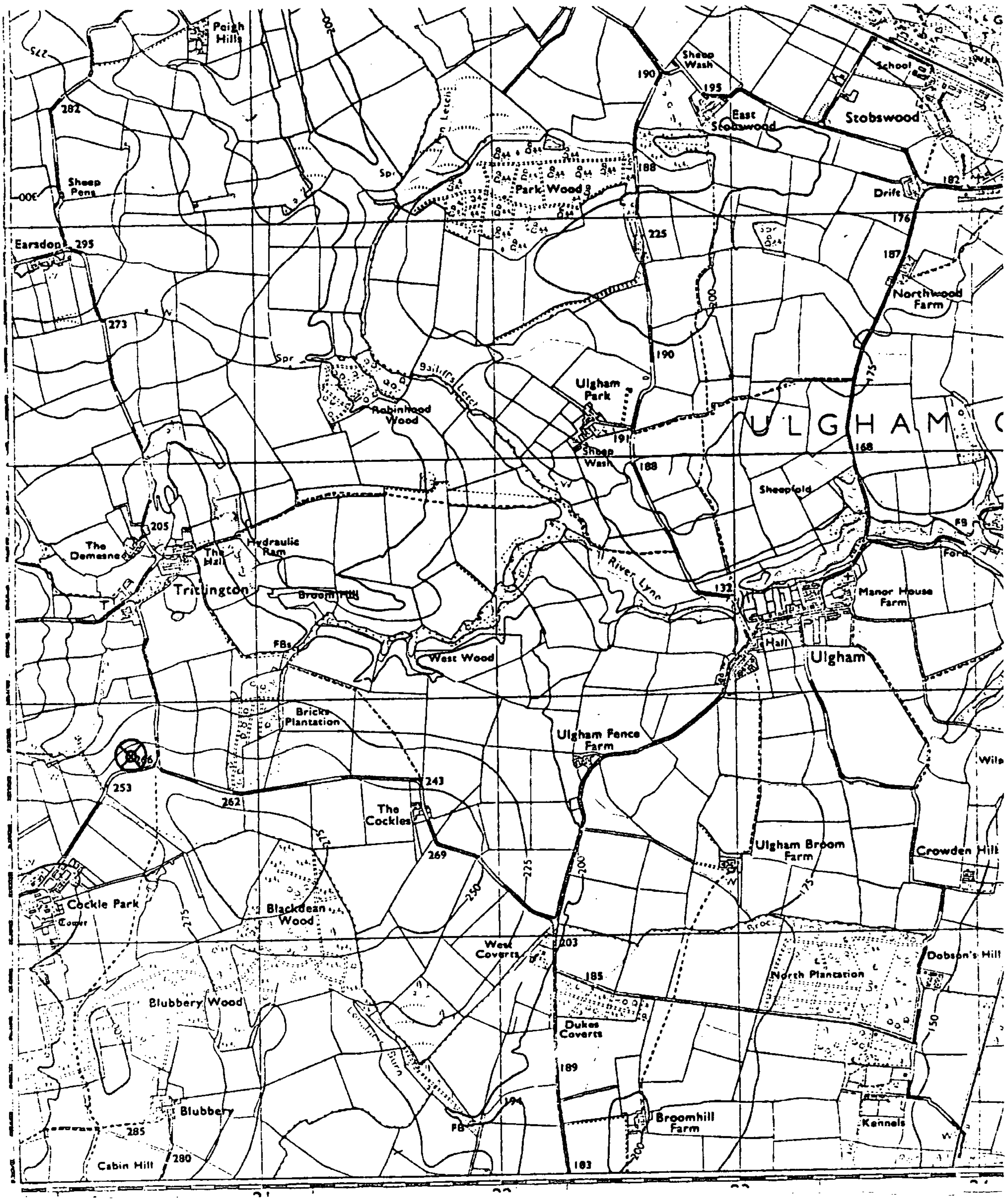
thought that fairly large amounts have been spread. The pig slurry comes from a pig unit on the farm and pig slurry disposal constitutes a problem. It was applied at irregular intervals at the maximum levels which can be applied without causing deoxygenation of the soil. Applications have been made for at least the last five years. The control field has never received pig slurry but it has received applications of farmyard manure.

Site B Cockle Park

The Cockle Park study area is situated at Cockle Park Experimental Farm, National Grid Reference NZ 917 205. The farm lies 30km north of Newcastle upon Tyne and 10km from the east coast, at a mean altitude of 90m above sea level.

The soil is naturally infertile and poorly drained. The parent material is reddish brown boulder till of variable thickness with an average of 6-9m (Arnold et al 1976). The surface soils have a sandly loam texture. Rainfall at Cockle Park is evenly distributed throughout the year, ranging from about 700 to 750mm per annum.

The plots used in this study had been set up to test the effect of pig slurry on hay yield. Permission to sample the soil and earthworms was given by Mark Sheppard, Newcastle University Soil Science Department. The three plots were all 2.5 x 4.0m in size and had all received similar treatment apart from the application of pig slurry. One plot had received a total of 48 litres or $48m^3/ha$ and another 24 litres or $24m^3/ha$, the third had received no pig slurry and acted as a control. The applications were made four times a year and the plots had already received seven applications.



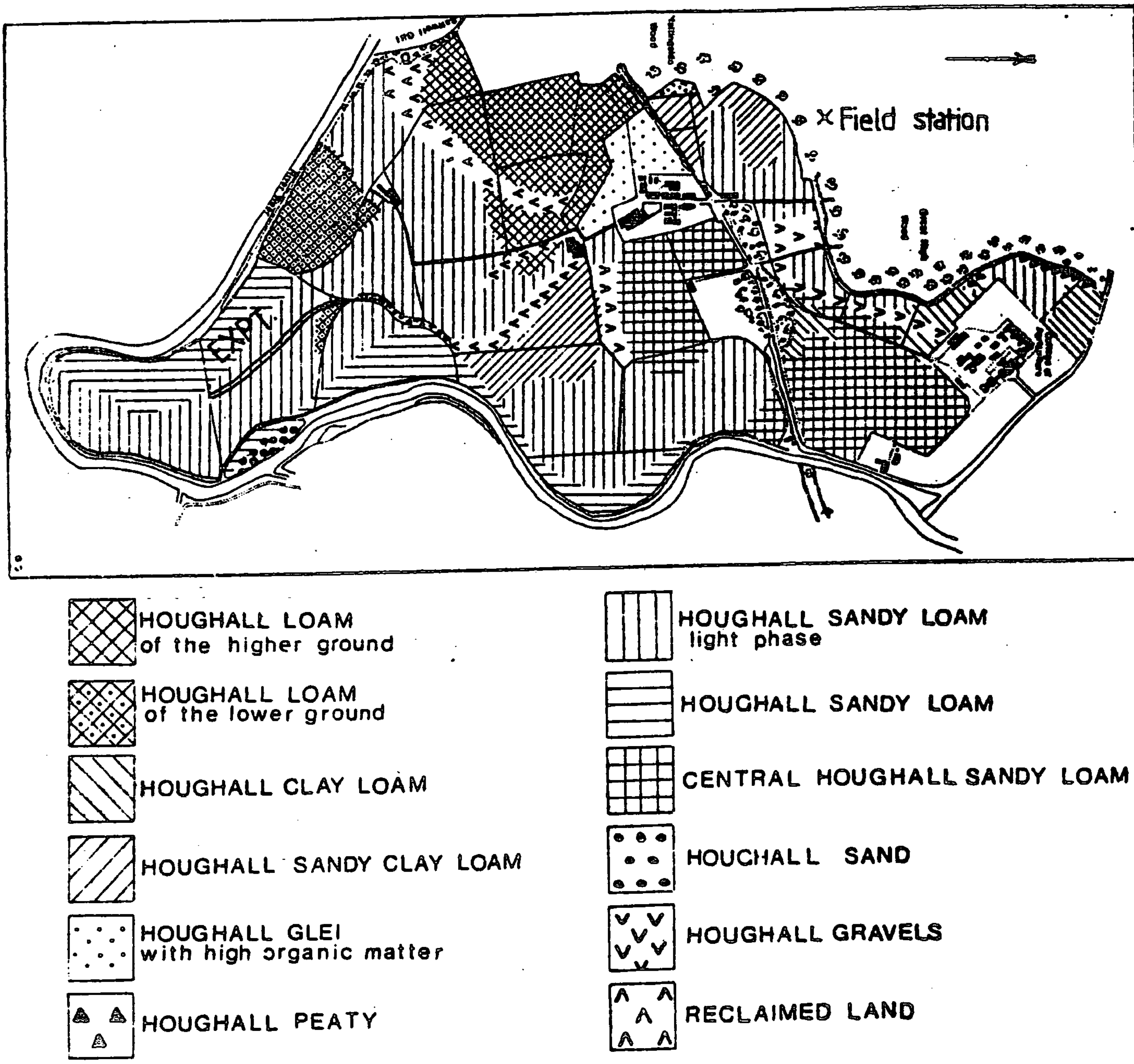
Map 2 showing the location of the Cockle farm study area.

Site C Houghall Farm and Durham University field station

Houghall Farm lies at National Grid Reference NZ 279 403 and Durham University field station lies about ½ mile north-west of it, National Grid Reference NZ 274 405.

The characteristics of the soil at Houghall have been largely determined by the last glaciation and subsequent fluvial modification by the River Wear. The meanders of the river form the boundary of the farm on three sides, south, south-west and east. Most of the area is essentially an old flood plain. The farm lies between 30 and 76m above sea level. The field sampled lies near the lower level. The field station lies on slightly higher ground and slopes towards the east. The topography of the area has been largely determined by the Pleistocene glaciation. The soils are of fluvoglacial origin and are very heterogeneous. The bedrock of the area consists of sandstones, shales and coal seams. The upper soil of both the Houghall Farm field and the field station is sandy loam. The rainfall is evenly distributed throughout the year with an average of 650mm per annum

Both the Houghall Farm site used in the study and the field station are rough pasture. Cattle are grazed at the Houghall field and two horses at the field station. There is no record of the amount of pig slurry applied to the Houghall Farm site but pig slurry disposal is a problem and the field has received frequent slurry applications. The field station has received no slurry.



Map 3 showing the location of the experimental field at Houghall Farm and the control field at Durham University field station.

2.3 Methods

At all three sites, in both experimental and control areas, the earthworm populations were sampled by methods detailed later and the copper levels in the soil and earthworms were tested. pH determinations were carried out on soil from Houghall Farm and Durham University field station and from Cockle Park.

2.3.1 Preparation, digestion and analysis of soils for copper

Soil samples were dried at 80°C for forty-eight hours. The samples were then ground up using a pestle and mortar, and passed through a 0.42mm nylon sieve. 1g of the sieved material was used from each sample. The sieved samples were digested using a wet ashing technique (Thompson and Blanchflower 1972). 30ml of nitric-perchloric acid mixture (4 : 1 by volume, AR grades) was added to each sample in a 30ml flat bottomed glass vial. The vials were fitted into equispaced holes $^3/_4$ inch deep and 1 inch diameter, in a 1 x 1½ x 16 inch aluminium alloy bar. There were 12 holes in each bar and two bars could be heated at the same time. At first, the aluminium alloy bars were heated to about 100°C by electric heating strip elements and left for 1 - 1½ hours while the initial digestion took place. The heat was then increased to about 210°C until the bulk of the acid had been driven off. The heat was then turned down slightly and the samples allowed to dry. The glass vials were removed from the aluminium alloy bar and left to cool. 10ml of 5% HCL was added to each vial and the sample was redissolved.

The copper analysis was carried out on a Perkin-Elmer 403 or a Pye Unicorn SP9 atomic absorption spectrophotometer, depending on availability. Cross checks between the two showed that the reading obtained were comparable. Before aspiration the redissolved samples were transferred to centrifuge tubes and centrifuged at 3,800 R.P.M. for ten minutes. The samples were then aspirated directly from the centrifuge tubes.

2.3.2 Preparation, digestion and analysis of earthworms

The earthworms were washed free of adhering soil particles with distilled water, and placed individually in pastic petri dishes containing a piece of filter paper moistened with distilled water. The mature worms were identified using the Lumbricidae Key by B.M. Gerard (1964). But due to difficulty in identifying immature species, these were grouped as either immature *Lumbricus spp.* or immature non-*Lumbricus spp*. The petri dishes containing the worms were left in an 8°C room for four days to allow the contents of the earthworm guts to be expelled. After four days starvation the worms were killed by freezing and stored for periods ranging from 1-10 days.

Before digestion, the worms were removed from the freezer and allowed to thaw. Worms with a wet weight greater than 0.3g were analysed individually. Smaller worms were grouped to form samples of similar size and of the same classification groups. This was done to avoid the inaccuracies associated with detecting very low levels of copper. The samples were placed in 30ml flat bottomed glass vials and dried at 105°C for 24 hours in a muffle furnace. The dry weight of worm in each glass vial was determined and the worms were digested in the same way as the soils. However, greater vigilance was required during the worm digestions because frothing occurred with the larger samples. Frothing was reduced by adding the acid mixture to the worms in the glass vials and leaving it cold overnight prior to heating. This, together with regular shaking, reduced the frothing and allowed the digestion to proceed effectively. The time needed for the initial hot acid digestion varied with the size of the worm. Completion was indicated by a clear pale mixture in which all frothing had ceased. The worm digests were centrifuged before aspiration in the atomic absorption spectrophotometer only if some particulate material was noted.

2.3.3 Preparation, digestion and analysis of pig slurry

Samples of both low copper sow slurry and high copper fattening pig slurry from Houghall Farm and Cockle Park were analysed for copper content. The slurry was weighed wet in a beaker and then dried at 80°C for five days in a muffle furnace. The beaker containing the sample was weighed again to determine the dry matter content of the slurry. A small amount of the dry sample was ground up with a pestle and mortar, and 0.5g was transferred to a flat bottomed glass vial and digested and analysed in the same manner as the soil.

2.3.4 Earthworm sampling

Hand sorting was used to sample the earthworm population densities at all three sites. This method was considered to give the most reliable results and, although time-consuming, it could be carried out in the field and did not require additional equipment.

Hand sorting involves the taking of a known volume of soil from the area to be sampled, often using an auger or quadrat, and carefully sorting through it to find any worms that are present. Nelson and Satchell (1962) tried to test the efficiency of hand sorting by introducing known numbers of worms into soil. 93% of all worms were recovered. They found that smaller worms and dark coloured worms were often missed. They concluded that hand sorting was satisfactory only for worms of more than 0.2g live weight.

The methods available for sampling earthworms include, soil washing (Morris 1922, Landell 1936), vibration methods (Reynolds 1973), chemical methods (Evans and Guild 1947), heat extraction (Edwards and Lofty 1977) electrical methods (Doeksen 1950, Edwards and Lofty 1975) and hand sorting. Several studies have been made on the relative efficiencies of the various methods, and it seems that in most cases hand sorting has been shown to be the most efficient (Svendson 1955, Bouché 1969, Raw 1959, Nordström and Rundgren 1972). However the size and species of worms to be sampled, the soil characteristics, the season of the year and the efficiency of the sorter will all influence the reliability of the results obtained.

2.3.5 pH soil measurements

pH determinations were carried out at Houghall Farm, Durham University field station and Cockle Park. Soil samples for pH determinations were taken from about 2cm below the surface. This was below the surface mat of vegetation and organic material, and was considered to be the most relevant depth. pH determinations were made immediately on returning to the lab since storage and drying are known to effect the pH (Allen 1974). About 5g of soil was placed in a 25ml beaker which was half filled with distilled water. This was stirred and left for 15 minutes during which time the pH meter was calibrated using buffer solutions. After 15 minutes the sample was stirred again and the pH was determined using a Pye Unicorn Model 291 MK 2 pH meter.

2.3.6 Experiment 1 The effects of continuous applications of pig slurry on the copper content of soil and earthworms

Two aspects of the effect of pig slurry application at three different field sites were studied: firstly, the effect of pig slurry application on soil copper levels in a field situation, and secondly, the relationship between soil copper levels, the level of copper in worm tissues in natural conditions, and earthworm numbers and biomass.

Site A Whixley Grange

Sampling was carried out using a $1/100^2$ auger. Preliminary attempts to sample the experimental field showed that the upper layers of the soil were very dry and few earthworms were found in a $1/100m^2$ core. For this reason the top layer of soil was cleared to a spades depth (approximately 30cm) and then a sample was taken using the auger.

The field to be sampled was planted with potatoes and had a slight slope. To ensure that the whole field was sampled equally it was divided into four parts and within each quarter

10 samples were taken at random from between the rows of potatoes. The cores were hand sorted immediately and any worms found were collected. A small sample of soil was taken 2cm below the surface from each core for copper analysis, the rest was returned to the field.

The control field was smaller than the experimental field and only 20 cores were taken. The field was divided into four equal parts and five cores were taken at random from each.

The control field had not been farrowed and the top soil was not as loose and dry as in the experimental field. The top soil was therefore not cleared away before sampling with the auger. Again the cores were hand sorted immediately and a small soil sample was taken for copper analysis from 2cm below the surface. Any worms found were collected. Very low numbers of worms were found using the sampling method described so a hole was dug using a spade and more worms were collected for copper analysis at both experimental and control sites.

On returning to the lab the worms were washed, identified and weighed. Then, together with the soil samples, they were prepared and analysed for copper as described earlier. Only 20 of the 40 samples collected from the experimental field were used for analysis so that the results would be comparable with those from the control field. The 20 experimental samples were selected randomly.

Site B Cockle Park

Three plots were sampled at Cockle Park. Plot A to which 48 tons/hectare of fattening pig slurry had been applied four times a year for seven applications. Plot B to which 24 litres of slurry had been applied four times a year for seven applications and Plot C which acted as a control plot since no slurry had been applied.

The size of the plots at Cockle Park was 2.5 x 4.0 meters and other experiments were being carried out on the central 1 meter strips. It was therefore necessary to confine the sampling to 75cm on either side of the strip. For this reason only 10 samples were taken from each plot. Five cores were taken randomly from both sides of the central strip in each plot using a $1/100m^2$ auger. Due to the low numbers of earthworms found, cores were taken to a depth of 30cm. The cores were hand sorted immediately and a small soil sample was taken from 2cm below the surface for copper analysis and pH determinations. Any earthworms found were collected along with a little soil. After they had been returned to the lab, the earthworms were washed, weighed and identified, pH determinations were made on the soil samples. Both worms and soil samples were then prepared and analysed for copper.

Site C Houghall Farm and Durham University field station

A $1/100m^2$ auger was used to sample both the experimental field at Houghall Farm and the control field at the field station. 20 cores were taken at random from the field at Houghall. The field station field lay on a slope so 10 samples were taken randomly from the upper half of the slope and 10 from the lower. The cores were hand sorted. Soil samples were taken at 2cm depth and any earthworms collected. In the lab the worms were washed, identified and weighed, and pH values were determined for the soil samples. Both worms and soil samples were prepared and analysed for copper.

2.3.7 Experiment 2 The effect of a single application of slurry on the copper content of soil

In this experiment an attempt was made to determine whether one application of pig slurry could significantly increase the soil copper concentration.

The field used for this experiment was a silage field at Houghall Farm. Two sets of samples were taken, one before the application of slurry and a second one week after slurrying. (The field had received applications of slurry in earlier years, so copper levels could be expected to be relatively high.) For each set of samples 20 cores were taken along a straight line which approximately bisected the field. Two markers were selected one at each side the field, and random distances were paced out between them using random numbers. It was necessary to use a line transect since the farm manager had agreed to apply pure pig slurry to a strip of the field about six meters wide, while the rest received mixed slurry. The cores were taken to 10cm depth. Soil samples were cut from each core at 2cm and 8cm below the surface. The cores were then sorted in the field and any earthworms found were collected. In the lab the worms were washed, identified and weighed, pH determinations were made for both the 2cm and 8cm soil samples. Both worms and soil were prepared and analysed for copper.

2.4 Results and discussion

2.4.1 Experiment 1. The effects of continuous applications of pig slurry on the copper content of soil and earthworms

Earthworms and soil from experimental and control areas at three sites were analysed for copper. The three sites were also sampled to give an estimate of earthworm density and biomass.

Site A Whixley Grange

Table 2 shows the copper levels found in both the earthworms and the soil. Some of the earthworms used for the copper analysis were obtained by digging, since insufficient numbers were obtained from the cores which were taken. The earthworms used covered a range of sizes. The pig slurry from this site was not analysed.

Table 2 Copper levels in mg/kg in soil and worms from Whixley Grange

field	Items analysed					
	Soil			Worms		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
Expt.	16.9	±0.96	20	19.62	±1.95	8
Cont.	8.8	±0.16	20	11.13	±0.86	10

Students t test was used to show that the difference in the soil copper concentrations of the experimental and control field was highly significant >0.0005 (t = 8.37). The difference between the copper concentrations in the earthworm bodies from the two fields was also highly significant at >0.005 (t = 6.50).

The concentration factors are shown in table 3.

Table 3 The copper concentration factors between worms and soil at Whixley Grange

field	Mean conc. of Cu in mg/kg		Ratio of $\frac{\text{Cu in worms}}{\text{Cu in soil}}$
	Soil	Worms	
Expt.	16.9	19.62	1.16
Cont.	8.8	11.13	1.28

The concentration factors at both the experimental and control field are similar which may imply that there is a direct relationship between soil copper levels and worm copper levels at Whixley Grange.

The numbers of earthworms found at this site using the sampling techniques described earlier was very low. The numbers and species found in the ten cores from each plot found are shown in table 4.

Table 4 The numbers and species of earthworms found in samples from Whixley Grange

Species	Field	
	Expt.	Cont.
A.chlorotica	1	0
L.rubellus	1	0
Imm. *Lumbricus spp.*	2	1
Imm. non-*Lumbricus spp.*	4	2

The numbers were considered to be too low to give a meaningful estimate of density. Worm densities in agricultural land are usually low due to disturbance, lack of cover and in some cases damage to the earthworms by farm machinery. However, it is thought that the exceptionally low numbers were due to the season and weather conditions under which the sampling was done. The samples were taken in July which is a time when earthworm activity is usually reduced. The ground was very dry due to a spell of warm dry weather and it is thought that many of the worms may have been quiescent, and some species may have burrowed to a depth below that of sampling, to avoid the dry conditions in the upper soil horizons.

Allolobophora rosea, *Allolobophora caliginosa* and *Allolobophora chlorotica*, although usually found within 10cm of the surface move to deeper soil in hot dry periods Gerard (1967). Hopp (1947) found that in summer most species became inactive and again went deeper in the soil.

Worm burrows were in evidence and their abundance suggested that there were more worms present than was indicated by the sampling method.

Site B Cockle Park

Slurry was applied to the two experimental plots four times a year. Seven applications had already been made. Copper analysis was done for a sample of third application slurry which had been kept in a deep freeze since the time of application. The copper concentration was 600mg/kg. The dry weight of the pig slurry varied between applications but there was little difference between that applied to the plot receiving 48m^3/ha and that receiving 24m^3/ha. The average dry weight of the seven applications was 3.5% for both plots.

The copper levels in the soils and worms from Cockle Park are shown in table 5.

Table 5 Copper levels in mg/kg in soil and worms from Cockle Park

Slurry application	Items analysed					
	Soil			Worms		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
48m^3/ha	21.77	±1.97	10	23.51	±1.69	21
24m^3/ha	19.58	±1.23	10	14.98	±2.21	13
Control	11.39	±0.08	10	7.8	±0.53	5

The copper levels in both the soil and worms can be seen to reflect the level of slurry application. Students t test was used to show that the difference in the copper concentration of the soil from the plot which had received 48m^3/ha of pig slurry and from the control, was highly significant at >0.0005 ($t = 5.26$). The difference in the soil copper concentration from the plot which had received 24m^3/ha and the control was also highly significant at >0.0005 ($t = 6.66$). However the difference between the soil copper level in the plots receiving 48m^3/ha and 24m^3/ha was not significant ($t = 0.94$). When students t test was used to test whether the difference in mean copper levels in the worm tissues were significant, slightly different results were obtained. The copper levels in the worms from the plot receiving $48m^3$/ha were significantly different from the control at 0.0005 ($t = 4.42$) but the copper levels in the worms from the plot treated with $24m^3$/ha were not significantly different from the control ($t = 1.97$). The copper levels in the worms from the plots treated with $48m^3$/ha and $24m^3$/ha were however significantly different from each other at >0.001 ($t = 3.65$).

The concentration factors for the three plots are shown in table 6.

Table 6 The concentration factors between worms and soil at Cockle Park

Pig slurry application	Mean conc. of cu in mg/kg		Ratio of $\frac{\text{cu in worms}}{\text{cu in soil}}$
	Soil	Worms	
$48m^3$/ha	21.77	23.51	1.07
$24m^3$/ha	19.58	14.98	0.77
Control	11.39	7.8	0.68

Although slightly lower than the concentration factors found at Whixley Grange, those from Cockle Park are not greatly different. The differences could be due to differences in the two soil types, which may alter the form and availability of copper in the soil.

The pH values found for the three plots were 5.8, 6.1 and 5.7 for the plots receiving $48m^3$/ha, $24m^3$/ha and no pig slurry respectively.

The number, biomass and species of worms for each plot are shown in table 7.

Table 7 The number, biomass and species of worms found in samples from each of the plots at Cockle Park

Species	Pig slurryapplication		
	$48m^3/ha$	$24m^3/ha$	Cont.
Imm.Lumbricus spp.	7	10	1
Imm.A. rosea	43	0	0
L. rubellus	1	0	0
Mat.A. rosea	11	1	0
Mat.A.chlorotica	1	0	0
Imm.non-Lumbricus spp.	1	14	12
Density estimate	$640/m^2$	$260/m^2$	$130/m^2$
Biomass estimate	22.02mg	15.42mg	5.97mg

Estimates for density and biomass were calculated by multiplying the numbers and biomass of worms found in the 10 cores sampled ($10/100m^2$) by 10 to give a value for $1m^2$. No estimates of variance are available since the worms from the 10 cores were bulked. The results show higher density and biomass in the plots which have received slurry and the highest numbers in the plot which had received the highest application of slurry. This may have been a result of the thicker growth of vegetation on the slurry-treated plots. The density and height of vegetation reflected the level of application of pig slurry. The vegetation growth, as well as being an indication of nutritional content of the soil helps to retain moisture in the upper regions of the soil. It is evident that at the levels found at Cockle Park, copper is not having a detrimental effect on earthworms. Zajonc (1970) recorded increases in earthworm populations after the application of inorganic fertilizers, probably through the indirect effect of increasing the plant biomass. Waters (1955) drew attention to a relationship between grass yield and earthworms. He concluded that earthworms under highly productive pastures benefit from a greater production of dead root debris, rather than from the higher input of animal dung, fertilizers or shoot debris.

Site C Houghall Farm and Durham University field station

Table 8 shows the copper concentration found in the soil and earthworms from this site.

Table 8 Copper levels in mg/kg in soil and worms from Houghall Farm and Durham University field station

Field	Items analysed					
	Soil			Worms		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
Expt.	62.93	±3.15	11	19.36	±0.64	11
Cont.	19.39	±1.35	9	14.60	±0.94	8

Analysis of the copper levels in the soil at Houghall Farm gave, by comparison with those from Whixley Grange and Cockle Park, high readings. This could be a direct result of the regular application of pig slurry over a number of years. No details were available of the amount of slurry applied to the study field, but a very rough estimate can be made. Houghall Farm has about 3000 pigs each year (pers. comm. J. Chapman) which produce approximately 3 litres of slurry each day. This gives a total of approximately 3285m^3 of slurry which is spread over 16.4 hectares of grassland to give an application rate of 200m^3/ha/yr. Both the sow slurry and fattening pig slurry from Houghall Farm were analysed. They gave values of 300mg/kg and 700mg/kg respectively. The dry weight of a sample of sow slurry was 8.6% and the dry weight of fattening pig slurry was 7.7%. However the dry weight values are very variable and the degree of mixing in the slurry tank before application will greatly influence the dry weight that is applied to a field since the solid material sinks to the bottom of the tank. At Houghall Farm the pig slurry was usually applied as a mixture of sow and fattening pig slurry, often together with cattle slurry.

The concentration factors for copper are given in table 9.

Table 9 <u>The copper concentration factors between worms and copper at Houghall Farm and the field station</u>

Field	Mean cu conc in mg/kg		Ratio of $\frac{\text{cu in worms}}{\text{cu in soil}}$
	Soil	Worms	
Expt.	62.93	19.36	0.31
Cont.	19.39	14.60	0.75

The level of copper in worms from the experimental field appears to be rather low considering the relatively high copper level in the soil. It may be that the worms are taking up copper to a certain concentration above which they excrete it, or it could be that much of the copper at Houghall Farm is in a form which is unavailable to the worms.

In a comparison of invertebrate detrtivores Williamson (1979) found that the concentration factors for lead, zinc and cadmium were extremely variable, and were affected by season and size or age of the animal.(He studied the snail Cepea hortensis, and the woodlice Oniscus asellus and Philoscia muscorum.) Additional causes for the variability of concentration factors are changes in the availability of metals in senescent plant material. Nriagu (1979) states that in soil invertebrates, the accumulation of copper is directly proportional to weight, or even to a higher power of the weight, of the animals. However when the concentration of copper in earthworms was plotted against the average weight of worm for one sample in experiment A (chapter 3) there was found to be no significant relationship between the copper concentration and weight of worm. It is therefore considered acceptable to determine average copper concentrations for worms of varying sizes. Mortimer (1977) found no significant difference between small and large Nereis diversicolor when he compared the copper content of worms from 10 different size classes.

Van Rhee (1976) looked at the relationship between copper content of soil and worm numbers. He looked at soils ranging from 109.7 ppm dry weight to 6.7 ppm dry weight and found no relationship. He suggested that the lack of correlation may be due in part to variations in soil type or soil management measures. Also he suggested that most of the copper accumulation appeared not yet to have reached levels which could damage worm populations. He did however find a significant correlation between copper content of soils and that in worm bodies.

The pH values from Houghall Farm and the field station were found to be 6.4 and 7.1 respectively.

The numbers, species and biomass of the worms from Houghall Farm and the field station are shown in table 10.

Table 10 The number, biomass and species of worms found in samples from Houghall Farm and the field station

Species	Field	
	Expt.	Cont.
Imm. *Lumbricus spp.*	12	3
Imm. non-*Lumbricus spp.*	18	2
Mat. *L. rubellus*	3	1
Mat. *L.terrestris*	2	0
Density estimate	$175/m^2$	$30/m^2$
Biomass estimate	1.82mg	0.293

This site also showed a higher density of worms in the experimental field. It seems that even at 62.93mg/kg copper the worms were not adversely affected by the copper level in the soil.

Cotton and Curry (1979) found in plots at the Agricultural Institute, Johnstown caste, Ireland, that plots treated with 80-100t/ha/yr of pig slurry had on average 31.3% more earthworms and 37% more biomass than the control. They also found very little change in the proportional representation of species between treatments. They did however record an effect on spatial distribution. A greater degree of earthworm aggregation was found in slurry treated plots, which may be due to the uneven spread of slurry.

It is thought that most earthworm populations are food limited and that an additional food source would be a great benefit (Satchell 1967).

From these experiments it seems that there is a relationship between the copper level in the soil and that in the earthworms, but that it is variable. The variability is probably a result of soil type affecting the availability of copper to the earthworms.

It seems that at the three sites studied the copper applied to the soil in pig slurry had not raised the soil copper levels high enough to have a detrimental affect on the earthworm populations. The earthworms were actually reaching higher densities in the fields which had received pig slurry applications, probably as a result of the beneficial effects of pig slurry on the vegetation.

2.4.2 Experiment 2. The effects of a single application of slurry on the copper content of soil

The average copper levels found in the soil before an application of pig slurry was made and one week after slurry application are shown in table 11.

Table 11 Soil copper levels before and after an application of pig slurry

Time of sampling	Depth of soil extraction	Mean cu cont. mg/kg	S.E.	n
pre-slurry application	2cm 8cm	70.6 84.7	±5.29 ±7.03	20 20
1 week after slurry application	2cm 8cm	74.2 86.7	±5.55 ±5.97	20 20

Students t test was used to test for significance between the average values obtained. No significant differences were found. First the 2cm and 8cm samples were compared to determine whether copper applied in the pig slurry remained in the surface layers of the soil, or was leached downwards, $t = 1.59$. Since no significant difference was found the 2cm copper readings and the 8cm copper readings were combined in a comparison of the pre-slurry sample and the one week after slurry sample. Since the samples were of greater size ($n = 40$) a d test was used. Again there was no significance ($d = 0.64$). It therefore appears that one application of slurry does not significantly increase the copper level. However the concentration of copper in slurry may vary depending on the dry weight. It is not known in what form copper is present in pig slurry or what proportion of it is associated with the organic matter. This may vary with the age of the slurry.

Chapter 3 LABORATORY EXPERIMENTS

The rate of uptake and elimination of copper by earthworms was investigated under controlled conditions. In this way a continuous experiment could be carried out on previously determined species and numbers of worms. The copper concentration could be adjusted and evenly distributed throughout the soil.

3.1 Methods

Samples of pig slurry from both the sows and fattening pigs at Houghall Farm were taken and analysed for copper as described in section 2.3.3.

3.1.2 Collection of earthworms

The earthworms for the laboratory experiments were collected from Durham University field station. Formalin was used to extract the earthworms since this was thought to be the most efficient method, considering the large numbers of worms that were required. Survival of the worms was good if they were washed in fresh water as soon as they were picked up. First the herbage over the area to be treated was clipped as short as possible using a pair of shears (approximately $50cm^2$). 40ml of 40% formalin was then emptied into a 10 litre bucket of fresh water. A second bucket was half-filled with fresh water. About half a bucket of formalin solution was poured onto each $50cm^2$ area of clipped herbage. The first worms began to appear after about 1 minute. They were picked up as soon as they appeared, swirled round in a bucket of fresh water, and then placed in a collecting jar with a little soil and grass.

On returning to the lab the worms were transferred to a larger container filled with field station soil and were stored at 8°C for up to three days until a sufficient number of worms had been collected for each experiment.

3.1.3 Experiment A. Rate of copper uptake

In this experiment the rate of uptake of copper by earthworms from soil contaminated with copper sulphate and pig slurry was determined.

Sufficient soil was dug from Durham University field station to fill 20 plastic plant pots with a diameter of 26.5cm and a depth of 23.0cm. The turf on the soil was discarded so that all the soil used in the experiment could be thoroughly mixed.

200mg/kg was selected as a fairly high level of copper in soil, which was not sufficiently high to kill the worms. Because of the difficulty in determining the exact weight of copper sulphate required to raise the copper level in field station soil to 200mg/kg an estimate was made of 4g in 6000g of soil. 40g of $CuSO_4 5H_2O$ was dissolved in 10 litres of fattening pig slurry and left overnight. Then 10 pots of soil (60kg) and the 10 litres of fattening pig slurry combined with $CuSO_4 5H_2O$ were all thoroughly mixed in a large plastic bin. When tested, the copper level in the mixed soil was found to be 285mg/kg. This was considered to be too high so further mixing was necessary. The prepared soil was combined with field station soil in a ratio of 5 : 3. When thoroughly mixed the soil was analysed as described in section 2.3.1 and found to give a copper concentration of approximately 200mg/kg. The soil was divided evenly between the 10 pots and left for three days.

A further 60kg of soil were mixed with 10 litres of sow pig slurry, to give soil with a copper concentration of approximately 20mg/kg. This was divided between 10 pots and left for three days. These were to act as controls. 420 earthworms were selected from those previously collected and twenty worms were added to each pot. The worms in each pot included:

15 immature _Lumbricus spp._
2 _Lumbricus rubellus_
1 mature _Lumbricus terrestris_
2 immature non-_Lumbricus spp._

The remaining 20 worms were starved, digested and analysed as described in chapter 1. The pots were all covered with a square of sheeting and had another square tied round the bottom to prevent the worms escaping. They were kept in an open frame outside Durham University green houses.

At weekly intervals one experimental pot and one control pot were randomly selected and the contents of each were hand sorted separately on a white enamel tray to remove all the worms. The worms were starved, digested and analysed for copper as previously described. Five samples of soil from each pot were also analysed. This was continued for 10 weeks until all the pots had been emptied.

3.1.4 Experiment B. Rate of copper elimination

The rate of elimination of copper from worms which had previously been maintained in soil contaminated with copper sulphate and pig slurry was examined. Enough soil was dug from the field station to fill nine plastic plant pots (54kg). The soil was thoroughly mixed in a large bin with 22.5g of $CuSO_4 5H_2O$ (2.5g $CuSO_4 5H_2O$ in 6000g soil) dissolved in 9 litres of fattening pig slurry. When tested this was found to give a copper concentration of approximately 200mg/kg. The soil was divided between nine pots and left for three days. 270 of the previously collected earthworms were selected and 30 were added to each of the pots. The worms in each pot included:

25 immature Lumbricus spp.
1 Lumbricus rubellus
1 mature Lumbricus terrestris
3 immature non-Lumbricus spp.

The pots were all covered top and bottom with squares of sheeting to prevent the worms escaping and left for three weeks.

After three weeks the copper contaminated pots containing the worms were emptied and the soil was hand sorted to remove all the worms. 240 of the worms were then distributed equally between eight pots containing sorted uncontaminated field station soil. The pots were covered top and bottom with sheeting. The remaining 30 worms were prepared, digested and analysed for copper. At weekly intervals one of the pots was selected randomly and the soil was hand sorted to remove the worms which were then prepared, digested and analysed for copper as described in section 2.3.2. This was continued for eight weeks.

The third to tenth week experimental pots in experiment A, where contaminated worms were maintained in contaminated soil acted as a control.

3.1.5 Experiment C. Rate of copper uptake from slurry contaminated soil

An attempt was made to raise the level of copper in soil to a level of 200mg/kg using pig slurry. The soil used was taken from Houghall Farm field which had been previously treated with pig slurry. Because of the large volume of pig slurry required it was decided to use only five pots of soil. The pig slurry used was taken from the fattening pigs at Houghall Farm. It was found to contain 700mg/kg copper dry weight and consisted of 20% dry weight. Because of the liquid character of the pig slurry only 2 litres were added to each pot at a time. This was repeated at weekly intervals. In sunny weather the pots were left uncovered to allow the slurry to dry. After eight litres of slurry had been applied the pots smelled unpleasant and were covered in dung flies. Analysis showed the copper level to be approximately 60mg/kg. A further week was allowed to pass. Due to the shortage of time it was decided that the worms should be introduced at this stage. 20 worms collected from Houghall Farm were introduced into each pot after each had been moistened with a little water. A further 20 worms were analysed for copper. After one week one of the pots was selected and hand sorted for worms. However all the worms had died and no further sorting was carried out.

3.2 Results and discussion

3.2.1 Experiment A. Rate of copper uptake

In this experiment the rate of copper uptake by earthworms from soil contaminated with copper sulphate and pig slurry was studied. The copper concentration in soil and earthworms, sampled at weekly intervals from the experimental and control plots, is shown in table 12, and in figure 1. From the graph it appears that the relationship between copper concentration in the worms and time is curvilinear. (The curve was fitted by eye.) The uptake of copper is rapid at first but falls off, and after about four weeks no further increase in the copper concentration of the earthworm tissues can be detected. The control worms did not take up significant amounts of copper at any time during the experiment.

It is evident that earthworms regulate the concentration of copper in their tissues since the level in the experimental worms stabilized at a level well below that present in the soil. The maximum average concentration for a sample of worms was 50.7mg (In expt B) but the highest level recorded in an individual was 69.82mg/kg in an immature Lumbricus spp.

The weights used to calculate the mean copper concentration included all the worms in a sample, regardless of size or species. This was considered acceptable because no relationship was found between the weight of worm and the copper concentration in it when such pairs of values were plotted against each other. Also, no significant differences were found between the concentrations in the different species of worms used. (This was tested using a Wilcoxon matched pairs test.)

From figure 1, the copper concentrations in the samples from week 4-10 appear to have reached an equilibrium level. The mean level in these samples was therefore used to calculate the copper concentration factors for the experimental and control pots.

Table 12a The copper concentration factors for the experimental and control pots in experiment A

Sample	Mean cu conc. in mg/kg		Ratio of $\frac{\text{cu in worms}}{\text{cu in soil}}$
	Soil	Worms	
Expt.	194.2	47.2	0.24
Cont.	19.24	14.4	0.75

When both the lab and field experiments are considered earthworm copper levels varied between 11.13 and 47.5mg/kg. At the low soil copper levels found at Whixley Grange, the worms concentrated copper in their tissues above the levels found in the soil. However at higher soil copper levels the worms must have actively excreted copper or resisted its uptake to maintain the level below about 50mg/kg.

3.2.2 Experiment B. The rate of copper elimination

In this experiment the rate of elimination of copper was investigated. The worms used had been kept in soil contaminated with $CuSO_45H_2O$ and pig slurry (200mg/kg copper) for three weeks, before being transferred to fresh field station soil with a copper concentration of approximately 20mg/kg. The copper levels in the soil and earthworms sampled are given in table 13 and figure 2. The data are presented along with data for weeks 3-10 from experiment A. These worms from the experiment A are considered to represent contaminated worms maintained in contaminated soil and are used as a control for experiment B. From figure 2 it can be seen that copper was lost very rapidly (within a week) from contaminated earthworms returned to relatively clean soil containing low levels of copper. The copper concentration in earthworm tissues dropped to an equilibrium level similar to that of worms which had always been maintained at copper levels of approximately 20mg/kg (control worms in experiment A). In this experiment no attempt was made to determine the rate of copper elimination from worms which had been kept in highly copper contaminated soil for a long period of time. The worms used had only three weeks to acquire the relatively high copper concentrations seen in the week 0 experimental worms. It may be that after longer periods of exposure to high copper concentrations the copper in earthworms takes a chemically different, less labile form, or other physiological changes may occur which alter the rate of elimination. If earthworms are taken from a highly contaminated area they may have undergone some degree of adaptation to a high tissue copper concentration, as has been found for Nereis diversicolor in estuarine muds with high Cu concentrations.

3.2.3 Experiment C. A comparison of the rate of copper uptake from copper sulphate contaminated soil and pig slurry contaminated soil

It is evident that in experiment C it was some constituent of pig slurry other than copper that killed the worms since they died before the copper had time to accumulate in the tissues.

Gisinger (1961) mentioned particularly benzoic acid and phenols produced as breakdown products in stored urine, also ammonia, methane and sulphide arising from the fermentation of faeces, as substances occurring in animal wastes that are toxic to animal and plant life to varying degrees.

Since no results were obtained from this experiment, no comparison was possible between the rate of copper uptake by earthworms from soil contaminated with copper sulphate plus pig slurry and pure pig slurry. However experiments by McGarth et al (1980) indicate that the initial rate of uptake of copper is at least as great from slurry derived copper as from copper salts added to the soil to give levels of 100-200mg/kg copper. Their results also suggest that uptake of copper from slurry decreases with time even more rapidly than from copper salts and was not significant after 12 months.

3.3 General discussion

From both the laboratory and field experiments described in this study, it appears that earthworms normally maintain an average level of at least 11mg/kg of copper in their tissues, but not more than 50,7mg/kg. Copper is an essential element and it could therefore be expected that a minimum concentration was necessary for their survival. Although, contrary to this study, Ireland (1979) suggests that copper is regulated within very narrow concentration limits and earthworms do not concentrate it, he found levels of 11-13 ppm copper in earthworms from soils with copper levels ranging from 20 to 335 ppm copper. The discrepancy between the two studies may be the result of differences in the soil characteristics. The levels of other trace elements in the soil may also influence the worm's ability to excrete or take up copper. In this study it was found that copper was concentrated at levels of up to an average of 50.7mg/kg which did not cause death, but above this level the copper concentration was efficiently regulated. Ireland (1979) stresses the importance of heavy metal interactions as a factor affecting the uptake of individual heavy metals. He found the concentration of soil calcium and copper to influence heavy metal accumulation. Absorption of lead is markedly reduced by increased dietary copper (Petering 1974) and metabolic studies have shown that high levels of molybdenum interfere with the retention of copper reserves in the liver (Brogan et al 1973). Dolar and Keeney (1970) suggested that since Cu, Zn and Mn are available to plants as divalent cations, antagonism or synergism in their uptake by plants is likely to occur. They showed that interactions between Cu, Zn and Mn in the same chemical fraction influenced their individual uptake. If the importance of other trace elements in copper regulation is to be evaluated it is essential that the mechanisms of uptake and excretion are properly understood. At present sufficient knowledge of the mechanisms is lacking.

From this study there is no evidence that, at any of the soil levels studied, copper is having a detrimental effect on earthworm populations. However caution is needed in interpreting these results. The chemical form, and therefore availability, of the copper is unknown, and although the earthworm populations appeared to be undamaged, continued application of copper in pig slurry must lead to an accumulation in the soil (or dissipation into adjacent environments) which may eventually reach a critical threshold level of available copper. Also soil conditions need not remain constant and any alteration such as a change in management practices may alter the proportion of available copper.

Although no detrimental effects on earthworm populations were noted in this study, there was a definite relationship between soil copper levels and copper levels in the earthworms' bodies. This could have a considerable effect on animals' feeding on earthworms. Earthworms are food items for many birds as well as for certain mammals. It is possible that birds feeding in pastures on copper contaminated worms, for extended periods, could acquire toxic copper levels. More work on changes in concentrations of heavy metals between different trophic levels is required when considering the possible effects on the biota. The copper found in animal bodies is usually in a more available form than that found in soil and plants. Changes in binding constants and availability of coppers occurs as it is transferred from producers to consumers. The copper that a herbivorous animal resorbs from its gut contents and assimilates into its tissues constitutes a new and more easily available source of copper in the ecosystem (Nriagu 1979). In addition the digestive enzymes of herbivores can alter the availability even of the fractions of copper that pass through the gut and are voided with the faeces. Since herbivorous animals injest and digest much more food than they assimilate, the amount of copper flowing through the guts of primary consumers is probably not negligible and may considerably alter the availability of copper to other plants and animals.

TABLE 12 Copper concentrations of weekly samples of worms and soil in experiment A showing rates of uptake

Week	Copper concentration mg/kg											
	Expt.						Cont.					
	Soil			Worms			Soil			Worms		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
0	188.8	±3.06	5	17.3	±2.79	7	18.5	±0.7	5	17.3	±2.79	7
1	187.4	±2.79	5	39.3	±5.62	9	20.3	±1.09	5	13.0	±1.25	9
2	197.8	±3.54	5	40.9	±4.0	11	19.3	±0.52	5	12.9	±1.22	10
3	195.8	±3.73	5	48.9	±6.44	12	18.8	±0.47	5	12.9	±2.10	14
4	189.0	±2.47	5	50.7	±3.58	15	18.7	±0.67	5	13.4	±1.17	15
5	193.8	±3.26	5	44.1	±2.97	19	19.8	±1.03	5	18.2	±1.86	13
6	201.6	±3.26	5	49.9	±2.26	20	19.3	±0.27	5	14.9	±1.56	17
7	194.6	±3.50	5	46.1	±3.29	16	19.5	±0.25	5	13.8	±2.24	11
8	193.0	±4.34	5	43.9	±1.43	18	19.6	±1.26	5	11.4	±1.21	9
9	197.5	±1.4	5	48.7	±2.46	15	18.7	±0.53	5	12.7	±0.96	10
10	197.8	±3.4	5	49.3	±4.23	12	19.1	±1.34	5	16.4	±0.6	10

TABLE 13 Copper concentrations of weekly samples of worms and soil in Experiment B, showing rates of elimination

Week	Copper concentration mg/kg											
	Expt.						Cont.					
	Soil			Worms			Soil			Worms		
	x̄	S.E.	n	x	S.E.	n	x̄	S.E.	n	x̄	S.E.	n
0	196	±1.9	5	44.8	±3.80	25						
1	19.8	±2.6	5	16.0	±0.74	22	195.8	±3.73	5	48.9	±6.44	12
2	20.1	±2.4	5	13.1	±0.89	22	189.0	±2.47	5	50.7	±3.58	15
3	17.5	±1.8	5	13.0	±1.04	20	193.8	±3.26	5	44.1	±2.97	19
4	18.9	±3.2	5	16.0	±2.09	20	201.6	±3.26	5	49.9	±2.26	20
5	19.5	±2.4	5	13.3	±1.27	24	194.6	±3.50	5	46.1	±3.29	16
6	18.9	±2.8	5	15.7	±0.89	19	193.0	±4.34	5	43.9	±1.43	18
7	19.3	±3.1	5	17.0	±2.57	7	197.5	±1.4	5	48.7	±2.46	15
8	19.2	±4.3	5	17.2	±1.48	18	197.8	±3.4	5	49.3	±4.23	12

Fig.1 Rate of copper uptake.

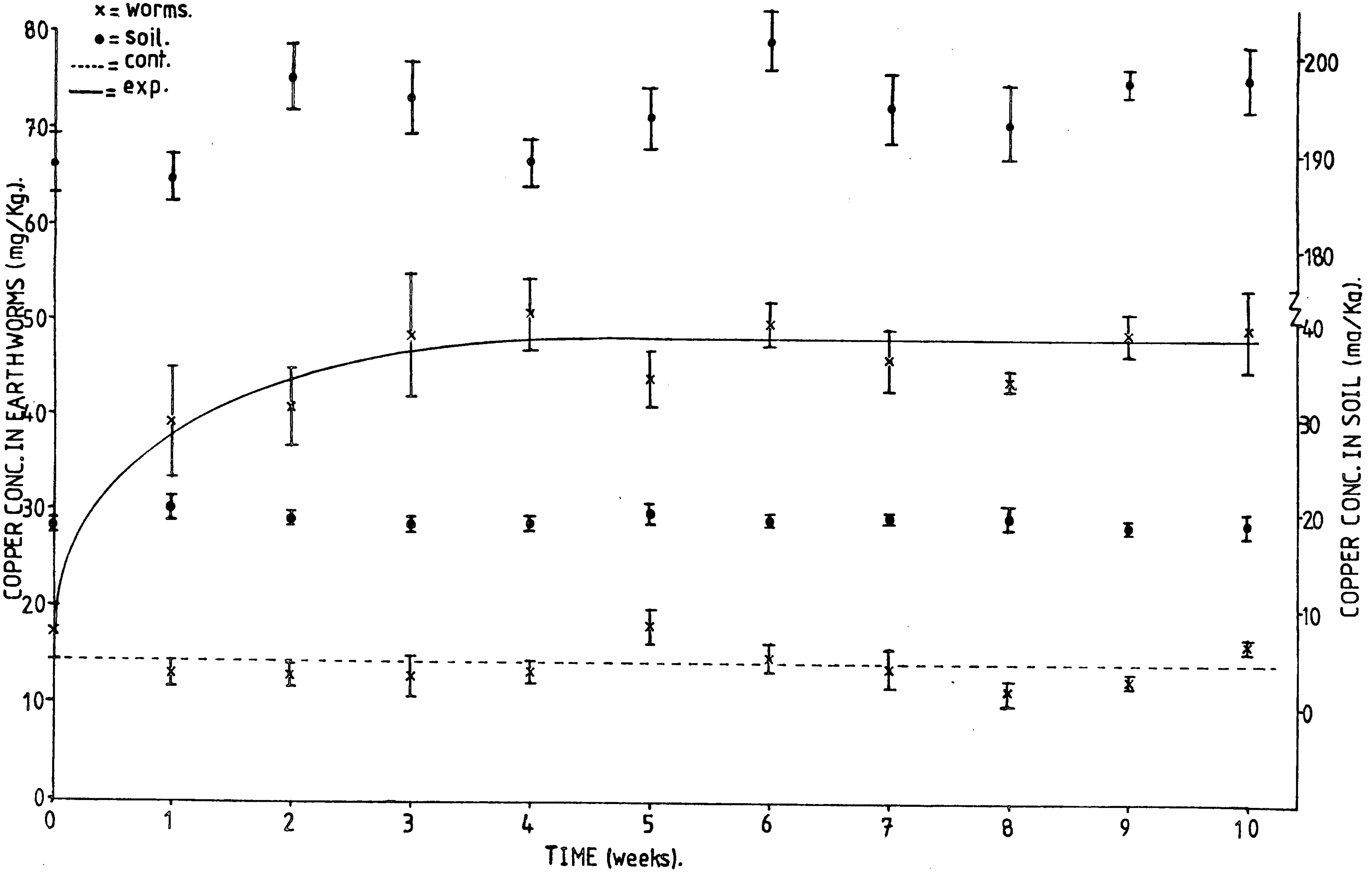
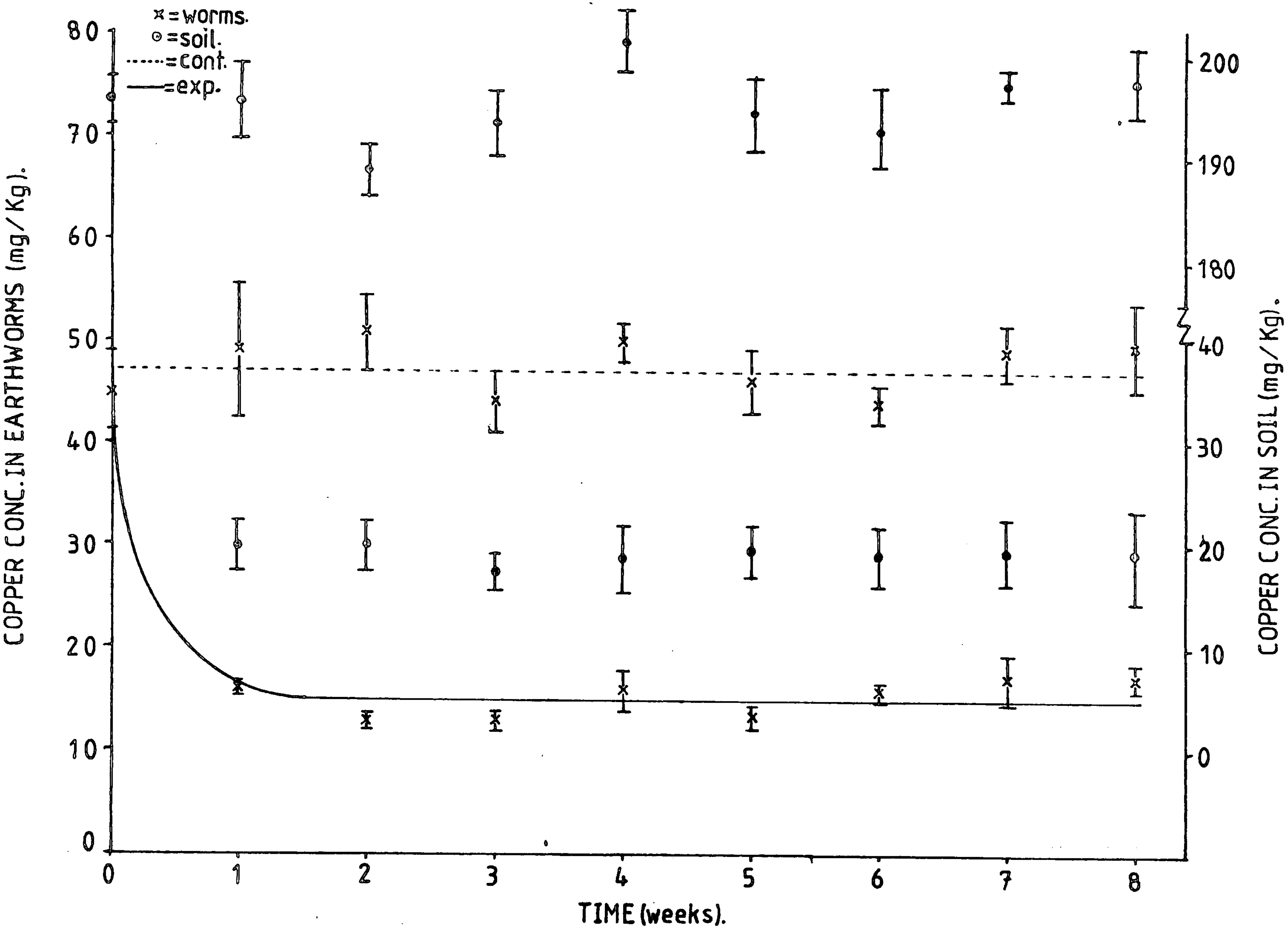


Fig 2. Rate of copper elimination.



Chapter 4 LOCALIZATION OF COPPER

An attempt was made to determine where in earthworm bodies copper is stored. This was done using histological techniques. Due to the time-consuming nature of the methods used, only mature Lumbricus terrestris were examined.

The compound used to stain for copper was rubeanic acid (dithiooxamide). It was first successfully used for histochemical localization of copper by Okamoto (1938, 1939). It gives a greenish black colour with copper salts. Feigh (1954) regarded this as due to the formation of an inner complex salt of the di-imido form of rubeanic acid. Rubeanic acid has a sensitivity of 0.006y for demonstrating naturally-occurring copper.

4.1 Methods

Six mature Lumbricus terrestris were introduced into a plastic plant pot containing soil contaminated with copper sulphate and pig slurry to give a copper concentration of approximately 200mg/kg (see section 3.2). The worms were left in the pot for five weeks at 8°C. The pot was covered top and bottom with sheeting to prevent the worms escaping.

The worms were then removed from the pot and placed individually in petri-dishes with a piece of filter paper moistened with distilled water. They were starved for four days at 8°C. The worms were killed by dropping them into liquid nitrogen. They were then serially sectioned using a cold knife and cold microtome. The sections cut were 20μ thick. Five sections after every 50 were placed on a microscope slide and immersed in 0.1% rubeanic acid in 70% ethanol for 10 minutes. 2g of sodium acetate (analytical reagent grade) was added to the 1 litre of 0.1% rubeanic acid solution and the slides were left in the solution for 48 hours. The slides were transferred to 70% ethanol for 1 hour and then into absolute ethanol for three minutes and Xylene for three minutes. They were mounted in Canada balsam.

The slides were examined under a microscope for a black stain and sections showing the stain were photographed. The above procedure was then repeated using worms that had been taken directly from Durham University field station.

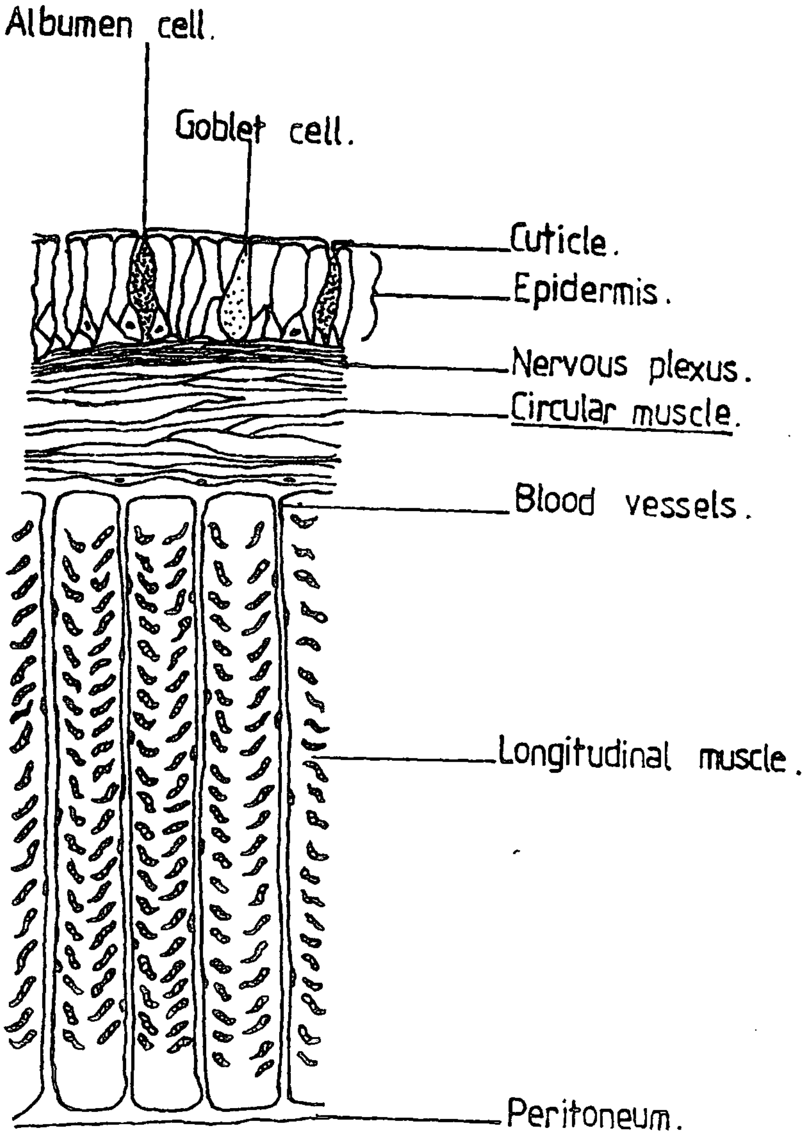
4.2 Results and discussion

The slides prepared from worms which had been maintained in copper contaminated soil (200mg/kg copper) showed black staining, indicative of the presence of copper, in the circular muscle. This is shown in plate 1. The region which was stained is also shown diagrammatically in figure 3. The slides from worms which had been taken directly from the field station did not stain for copper. Although in the sections examined copper was stored in the circular muscle of the worm, the stain used gives no information on the chemical form in which the copper was present.

From the experiments described in chapters 1 and 2, and because copper is an essential element, it is thought to be likely that some copper was present in the worms from the field station. Probably the stain did not react sufficiently with the expected low levels of copper present to give a detectable black colouration. There is therefore no information on the localization of copper in worms which are not heavily contamined with copper.

Other studies (Ireland and Richards 1977) report copper to be located in the intestinal region of Lumbricus terrestris and Lumbricus rubellus from sewage beds on Keele University campus, in particular in the chloragogenous tissue. However, Ireland (1975) also found copper in body wall homogenate although at a lower concentration than in the chloragogenous tissue. The proportion of the copper stored in the body wall and chloragogenous tissue may vary with increasing copper loads in earthworm bodies.

Fig 3.



Transverse section of a portion of earthworm body wall showing circular muscle where staining occurred. (after Grove and Newell 1962)

Plate 1: Photograph of a section of body wall from Lumbricus terrestris. The circular muscle showing staining is indicated by the arrow.

Plate 1.

SUMMARY

1. The effect of repeated applications of pig slurry on the copper concentration of soils and worms and on worm population densities was examined in a field situation at three sites.

2. A relationship was found between soil copper levels and copper concentrations in earthworm bodies. The concentration factors were variable, probably as a result of differences in soil characteristics between the three sites.

3. Areas receiving pig slurry which showed soil copper levels of between 16.9mg/kg and 62.93mg/kg had higher earthworm population densities than control sites receiving no pig slurry. This was probably due to increased vegetation growth resulting from the additional nutrients applied in pig slurry.

4. A single application of pig slurry did not raise soil copper levels significantly.

5. Laboratory experiments were set up to determine the rates of uptake and elimination of copper from contaminated soil.

6. When transferred from soil with approximately 20mg/kg to soil contaminated with copper sulphate and pig slurry (approximately 200mg/kg copper), earthworms showed an increase in the copper concentration in their tissues. When copper concentration in worms was plotted against time, the rate of copper uptake was shown to be rapid at first but then levelled off. Equilibrium was reached after about three weeks.

7. When earthworms were transferred from copper sulphate contaminated soil (approximately 200mg/kg copper) to relatively uncontaminated soil (approximately 20mg/kg copper), rate of copper elimination was rapid. Equilibirum was reached after approximately one week.

8. Attempts to determine the rate of copper uptake from soil contaminated with pure pig slurry resulted in the death of all the worms used, due to some toxic constituent of pig slurry apart from copper.

9. Staining with Rubeanic acid indicated that in highly copper contaminated worms copper was stored in the circular muscle tissue.

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