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## **DOCTOR OF PHILOSOPHY**

**The characterisation and control of ochre deposits in land drainage systems.**

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THE CHARACTERISATION AND CONTROL  
OF OCHRE DEPOSITS  
IN  
LAND DRAINAGE SYSTEMS

A thesis for the Degree of PhD

by

I Ap Dewi BSc(Wales)

February 1985



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## 1. SUMMARY

Ochre deposits from sites in England and Wales varied widely in their composition, their appearance, their rate of formation and where they occurred.

It was demonstrated that chemical oxidation could account for the precipitation of iron from drainage water. In sterile samples of drainage water from ochreous sites 80% of the total iron in solution was oxidized within 48 hours. It was also shown that autocatalysis of ferrous iron oxidation by ferric precipitates could occur in drainage water.

Filamentous bacteria were observed by light microscopy in most samples and were assigned to the genus Leptothrix. Gallionella spp were observed in some deposits but their distribution was not widespread. Sphaerotilus spp were not found by light microscopy or isolated in artificial media. The chemolithotroph T.ferrooxidans was found in acidic ochre samples (pH<4.0) and in samples from drainage water of near neutral pH suggesting that it can survive in microenvironments of low pH, contributing to ochre formation over a wide range of drainage water pH. Heterotrophic bacteria capable of growing in artificial media of low pH were isolated, primarily from acidic samples, and the results suggested that they were polysaccharide producing. Complex degrading heterotrophic bacteria were also isolated from ochre using a ferric ammonium citrate medium.

Some deposits, on the basis of chemistry and microbiology, could be described as either pyritic or filamentous ochre. However, the majority of samples fell between these extremes and had various combinations of filamentous bacteria, Thiobacilli and heterotrophic organisms.

A marine antifouling paint containing copper was used to control the growth of sheathed filamentous bacteria in drainage water and other heterotrophic bacteria in artificial media. Since ochre results from the interaction of many factors, chemical and microbial, the use of copper applied as an antifouling paint or incorporated into drainage pipes must be proved effective and economic in relation to ochre prevention in agricultural drainage systems.

## 2. LITERATURE REVIEW

### 2.1 INTRODUCTION

Field drainage can be defined as the removal from agricultural land of surplus water which might otherwise restrict crop growth (Hudson, 1975). In 1977 the Ministry of Agriculture, Fisheries and Food (MAFF) estimated that for over a half of the agricultural land of England and Wales, field drainage was a fundamental necessity for efficient farming. A national survey by MAFF (1977) showed that agricultural production on approximately three million hectares of land was limited by the absence of efficient drainage, while almost another three million hectares depended upon the maintenance of existing drainage systems. Similarly, in many parts of Europe drainage is an urgent priority if production is to be efficient (Eddowes, 1976).

Poor drainage causes standing water in fields, increased susceptibility to poaching by livestock, and risk of wheel slip, rutting and subsequent soil structure damage from machinery. Poor drainage has a marked effect on crop yields through short growing seasons; poor germination and development, stunted root growth, and poor fertilizer response. An increased tendency for weeds, or poor indigenous species, to develop and plant diseases to flourish is also characteristic of wet areas. Animal performance can be affected, not only by poor grass yields and sward quality, but also by problems such as liver fluke, foot-rot and red water which are associated with wet land.

The remedy of these problems by field drainage can be very beneficial. Not only can yields and animal performance be improved but often drainage allows a change in farming systems (Trafford 1977). However, any improvements resulting from drainage should be evaluated in economic terms. It has been shown that drainage can be economically viable and this is frequently substantiated by farmers' experiences (Hunter and Trafford, 1979; Trafford, 1977; MAFF, 1973; Morris and Calvert, 1976).

With any investment, a certain time period is required for the initial expenditure to be repaid. Thus the economic viability of any drainage scheme depends upon the drainage system maintaining its efficiency throughout the repayment period. Trafford (1977) calculated the time needed to repay a loan for drainage. The experiment involved four basic treatments including pipes, subsoiling and moling. The results, given in Table 2.1 show that the repayment period ranged from about three to twelve years. Hunter and Trafford (1979) demonstrated that grant aid was a very important factor governing economic return.

TABLE 2.1 : THE NUMBER OF YEARS REQUIRED TO PAY BACK A LOAN FOR DRAINAGE WORK WITH RESPECT TO TREATMENT

(from Trafford, 1977)

<u>TREATMENT:-</u>	<u>COST AFTER GRANT £/ha</u>	<u>NUMBER OF YEARS TO PAY LOAN BACK</u>
Pipes only at 15 m spacing	154	12.0
Pipes at 60m spacing + subsoiling	103	7.5
Pipes at 60m spacing + moling	103	3.5

These considerations reveal the importance of factors that are likely to effect the efficiency of any drainage system, especially in the repayment period. A reduction in efficiency reduces the benefits of drainage and lengthens the repayment period thus affecting the economic viability of the scheme. Trafford et al (1973) noted that, in the U.K., drainage is only attempted where experience shows that the system is likely to have a life of 5 years or more. They also found that where drain blocking was so severe as to cause drain failure in 6 months to 2 years it was usually uneconomic to attempt drainage.

The efficiency of a drainage system can be reduced by many factors. Incorrect grading, poor design or drain layout, poor overall scheme (i.e. the use or not of secondary treatments), the use or non-use of permeable backfill, and installation when climatic and soil conditions are not correct are all examples of faults that can arise due to the way in which the drains have been installed.

The efficiency of a "correctly laid and designed" system can also be reduced. The blockage of drainage ditches by soil, silt, aquatic weeds and rubbish is a common but easily cured problem (Nicholson, 1953; Blake, 1979). Similarly the accumulation of silt within drain lines can be a serious obstruction. Drain blockage by siltation results from the accumulation of mineral soil particles. The most easily mobilised particles are those in the fine sand range (50µm - 150µm) which can enter the drainage system via the gaps between clay tiles or through the slats and perforations of plastic pipes (Nicholson, 1953; Trafford et al, 1974; Grass et al, 1976).

The roots of trees and agricultural crops have also been identified as potential causes of drain failure. Roots can accumulate in drain lines partly or completely blocking them (Nicholson, 1953; Armstrong, 1976). Hudson et al (1962) included the entry of animals into drain pipes as a cause of blockage but Nolte (1980) noted that tiles in Williams County, Ohio, had been blocked by animals only five times in twenty years.

In some areas drains can be blocked by deposits of gypsum (calcium sulphate) or calcium carbonate (Grass et al, 1976; Grass and Mackenzie, 1972). In 1849 Portman et al described a hard calcareous field drain deposit containing 86% calcium carbonate. Black deposits of manganese may also form in drains by chemical or microbial oxidation of manganese in ground water (Meek et al, 1968; Mackenzie, 1962; Grass et al, 1973; Grass and Mackenzie, 1972; Streutker, 1977; Bloomfield, 1967).

Other deposits that can be encountered in field drains, ditches and drip irrigation systems include those based on iron sulphide. Iron sulphide accumulates as a black sludge and is often seen beneath iron ochre in open ditches (Bloomfield and Coulter, 1973). It has been postulated that the iron sulphide sludges result from bacterial reduction of sulphates. The sulphides produced combine with ferrous ions under suitable reducing conditions to form colloidal ferrous sulphide (Ford et al, 1970; Ford, 1973). According to Bloomfield and Coulter (1973) sulphate is readily reduced under anaerobic conditions at pH values of 5.0 and above. However, Van Beek et al (1982) were uncertain about the role of sulphate reducing bacteria. They found high numbers of these organisms in wells where iron sulphide clogging was evident but were unclear as to whether the bacteria were the cause or result of well clogging.

White or almost colourless sulphur slimes are sometimes associated with the iron sulphide deposits (Spencer et al, 1963). The sulphur slimes have been attributed to the partial oxidation of hydrogen sulphide by bacteria (Ford, 1969; Ford and Tucker, 1974). No evidence had been found by Ford (1969) to show that sulphur slimes present a serious problem in tiles and ditches. However, the blockage of drip irrigation filters by sulphur slimes has been reported (Ford, 1976; Ford and Tucker, 1974, 1975). The bacteria Thiothrix nivea and Beggiatoa spp have been associated with these deposits (Ford and Tucker, 1975; Ford, 1977).

In particular situations other deposits can effectively block a drainage system such as the gelatinous fungal material found by Johnson and Kelso (1980).

It should be noted that drain blockage is complicated further by the simultaneous occurrence of the various drain clogging agents. For example, silt can arise in conjunction with other mineral deposits, organic deposits, and roots, and combinations of many of the latter can also be found.

## 2.2 THE NATURE OF IRON OCHRE

Ochre is a general name used to describe iron based materials blocking agricultural field drains and ditches (Thorburn and Trafford, 1976; Thorburn, 1977). Such deposits were described in the nineteenth century by Mangon (Denison, 1856), Mitchell (1894) and Denton (1883). However, the use of ochre as a pigment was discussed by Pliny in the first century A.D. and prehistoric man used naturally occurring iron deposits in some cave paintings (Kuhn, 1955; Gardner, 1936).

Ochre has been found in drain lines, in the joints and slots of clay and plastic pipes respectively (Grass et al, 1973; Ivarson and Sojak, 1978), in permeable backfill and filter material (Ford, 1969; Field Drainage Experimental Unit (FDEU), 1975; Thorburn, 1977; Dennis and Wickens, 1978; Ivarson and Sojak, 1978), and also within and on the sides of open ditches (Lueschow and Mackenthun, 1952).

Although the deposition of ochre is a widespread problem (Alcock, 1973; Grass, 1969) it is not uniformly severe and may only be serious enough to cause premature failure of a drainage system in localized areas (Trafford et al, 1973). Alcock (1973) noted that the problem is viewed differently in different parts of the U.K. For example, he found that an 'ochre problem site' in Yorkshire may be regarded as a 'serious problem site' in Cambridgeshire.

Grass and Mackenzie (1972) found that the time taken for ochre to develop varied from a few months to 35 years. This variability is reflected in the literature where the times recorded range from several months (Alcock, 1973; Ford, 1969), through 2 or 3 years (Ivarson and Sojak, 1978; FDEU, 1975) to 5 years or more (Meek et al, 1968). Svobodova (1973, 1977) developed a method for calculating the probable time required for tile clogging by iron compounds. She calculated, for example, that for a given drainage system the gaps between tiles would become blocked in 3 months if the soil water

contained 5 mg Fe/l. If the soil water contained 3 mg Fe/l then the gaps between tiles would become blocked in 4-6 years. Kuntze (1982) distinguished between temporary (autochthonous) and permanent (allochthonous) clogging. In the former ochre results from the oxidation of iron derived from the soil and will be produced until the iron content of the soil is exhausted (Thorburn and Trafford, 1976; Thorburn, 1977). The time interval from initial drainage to complete iron oxidation depends on the amount of iron originally present, the physical and chemical nature of the deposits, the degree of soil aeration and the equilibrium pH reached around the iron deposits. After a 'starting up' period immediately after drainage, reaction rates will be at a maximum, gradually declining as the iron deposits are depleted.

In other areas the deposition of iron can continue for a long time and in some cases not diminish at all. This yields a permanent problem according to Kuntze's classification. Such areas would be characterized by an inflow of water, containing iron, from an external source (Haaiker and Wolf, 1965; Linder, 1977).

### 2.2.1 Types of ochre

Ochre is often classified into two types, namely filamentous and pyritic (Thorburn and Trafford, 1976). Both types are associated with a number of bacteria which are discussed in sections 2.3 and 2.4.

#### Filamentous ochre

Filamentous ochre has been described in various ways. It is usually gelatinous and "rag-like", Ivarson and Sojak (1978) compared it to a mare's tail and the present author heard a farmer referring to this form of ochre as a "rope" lengths of which could be pulled from a drain line.



Filamentous ochre normally occurs at near neutral pH but values ranging from 4.0 to 8.3 have been recorded (Bloomfield, 1967; Ivarson and Sojak, 1978; Ford and Tucker, 1975).

This type of ochre is commonly found in wet, peaty soils (Trafford et al, 1973; Ivarson and Sojak, 1978; Ford and Beville, 1970). Usually these soils have small quantities of iron sulphides as compared to soils where pyritic ochre is found (Thorburn and Trafford, 1976). Ochre often forms at the boundary between aerobic and anaerobic zones. At such sites ferrous ions in solution are oxidized and precipitated as ground water passes from one zone to the other (Bloomfield, 1967).

#### Pyritic ochre

This type of ochre is found as a crust, or hard deposit under strongly acid conditions. Under more moderate conditions softer gels and slurries are encountered. Pyritic ochre is associated with relatively large quantities (+ 2%) of pyrite,  $\text{FeS}_2$ , and similar sulphides initially present in the soil (Thorburn and Trafford, 1976). Pyrite is oxidized when the soil is drained producing extreme acidities in the soil and drainage water (Bloomfield, 1972 Trafford et al, 1973). Bloomfield (1967) found pyritic ochre where the drainage water had a pH of 3.2 and contained 145 mg  $\text{SO}_4^{2-}$  per 100 ml. This can be compared with the 2 - 11 mg  $\text{SO}_4^{2-}$  per 100 ml found by Bloomfield at filamentous ochre sites (Bloomfield, 1967).

#### 2.2.2 The composition of ochre

The composition of ochre is highly variable (Denison, 1856). For example, Grass et al (1973) discovered that the iron and manganese content of filamentous ochre varied in the range 35-61% and 0-14% respectively.

Iron is the most important component of the ash of ochre samples with aluminium also forming a <sup>smaller</sup> fraction (Puustjarvi and Juusela, 1952; Petersen, 1966; Johnson, 1979; Thorburn, 1977; Spencer et al, 1963).

The iron of ochre samples is generally in the form of amorphous ferric oxides and hydroxides (Ivarson and Sojak, 1978; Fischer and Ottow, 1972). According to Schwertmann and Taylor (1977), the iron mineral ferrihydrite has been found in particular environments associated with sites such as drainage ditches and small, slow running water courses. These authors noted that this mineral has been described incorrectly in the past as amorphous ferric hydroxide. Given sufficient time, iron deposits can form crystalline compounds such as goethite (Halbach and Ujma, 1978). However, the crystallization of iron deposits in field drains is inhibited by the low concentration of ferrous iron and by the presence of adsorbed ions and organic matter (Johnson and Kelso, 1980; Schwertman and Fischer, 1973).

Spencer et al (1963) noted that a large proportion of filamentous ochre was composed of organic matter. An analysis of filamentous ochre by Ford (1973) showed that the organic matter content ranged from 19% to 51%. Ford (1979) also pinpointed geographical differences in the organic matter contents of ochre; in Florida the carbon content of dried ochre was 20% as compared to under 4% in Southern California.

In addition to organic matter, iron and aluminium, other materials are also present forming the bulk of ochre deposits. These include clays, fine sand, vegetation detritus, compounds of manganese, calcium, magnesium etc, as well as bacterial fibres, and other organic material of microbial origin (Thorburn, 1977).

A noteworthy feature of iron ochre, in the field, is its very high water content (Schwertman and Fischer, 1973; Dennis and Wickens, 1977).

### 2.3 THE MICROBIOLOGY OF FILAMENTOUS OCHRE

The association between bacteria and ochre is often traced back to the work of Winogradsky (1888) who reported the presence of an iron oxidizing bacterium in spring water. The microbial origin of ochre is also evident

in the writings of Pliny who described ochre as a "slime".

Subsequently numerous authors have found bacteria in filamentous ochre and have associated them with its formation. The most commonly reported genera are Gallionella, Sphaerotilus, Leptothrix, Crenothrix, Cladothrix, and Thiothrix (Thorburn and Trafford, 1976; Trafford et al, 1973; Spencer et al, 1963; Ford, 1969; Ford and Beville, 1970; Ford and Tucker, 1975; Ford, 1979 A and B; Ford, 1977; Ivarson and Sojak, 1978).

### 2.3.1 THE FILAMENTOUS BACTERIA

#### 2.3.1.1 Definition and history

Filamentous bacteria can be defined as organisms composed of chains or filaments of cells within a thin organic sheath. Iron and manganese may be deposited on the sheath (Pringsheim, 1949; Farquhar and Boyle, 1971). The most commonly studied filamentous bacteria are those belonging to the Sphaerotilus - Leptothrix group but other organisms, including Streptothrix, Lieskeella, Phragmidiothrix, Crenothrix and Clonothrix, can also show filamentous growth in sheaths. It should be noted that organisms such as Bacillus spp, Beggiatoa spp, Vitreoscilla spp and Thiothrix spp can also form filaments or chains of cells but without a sheath (Farquhar and Boyle, 1971; Godhino-Orlandi, 1980)

These bacteria can occur in a wide range of habitats. They have been found in streams and water supply pipes where the concentration of dissolved nutrients is low and also in polluted rivers and streams and in sewage treatment plants (Fenchel and Blackburn, 1979).

The early work on the filamentous bacteria was discussed by Pringsheim (1949). Sphaerotilus natans was first discovered by Kutzing in 1833 and Leptothrix discophorus by Kutzing in 1843. The latter produced a red aqueous deposit containing iron. Cohn (1870) discovered an iron bacterium Crenothrix polyspora and in 1875 described Cladothrix dichotoma. In 1888

Winogradsky reported the results of cultural experiments and suggested that Leptothrix could derive energy from the oxidation of ferrous ions. Molisch (1892, 1910) opposed this view on the basis that Leptothrix could thrive without added iron in the growth medium used. Other authors, up to 1949, agreed with either Winogradsky or Molisch regarding the relationship between the filamentous bacteria and iron. This question is discussed in section 2.3.1.6.

#### 2.3.1.2 Taxonomy

Many of the sheathed filamentous bacteria have not been isolated in pure culture and therefore descriptions of them are based on observations in the natural environment. This has led to much confusion concerning the classification of these bacteria, since it is known that their appearance can vary considerably with environmental conditions (Phaup, 1968; Eikelboom, 1975). Thus some of the different genera may only represent different growth forms in certain environments (Fenchel and Blackburn, 1979; Mulder and Van Veen, 1963).

Another problem is that organisms isolated in the laboratory can be subject to changes and mutations with consequent loss of morphological and physiological features. For example, they can lose the capacity to synthesize a sheath or to oxidize manganese (Van Veen et al, 1978; Farquhar and Boyle, 1971; Phaup, 1968; Stokes, 1954). The media used to isolate the filamentous bacteria can also affect their growth form. Van Veen et al (1978) noted that non filamentous colonies of S. natans were encouraged by media high in sugars and especially peptone.

The taxonomy of the filamentous bacteria is summarized in Figure 2.1 which is abstracted from Bergey's Manual. Attention is also drawn to the detailed key produced by Godhino-Orlandi (1980).

Figure 2.1 : THE SHEATHED BACTERIA

From Bergey's Manual 8th edition.  
(Buchanan and Gibbons, 1974)

I Single cells, motile by means of a polar flagellum  
or subpolar flagella.

A. Sheath rarely encrusted with iron and not  
encrusted with manganese oxides

Sphaerotilus

B. Sheath encrusted with iron or manganese  
oxides

Leptothrix

II Single cells not motile by flagella

A. Sheaths not attached

1. Sheaths not encrusted with metal  
oxides

Streptothrix

2. Sheaths may be encrusted with metal  
oxides

Lieskeella

B. Sheaths attached

1. Sheath not encrusted with metal oxides

Phragmidiothrix

2. Sheath encrusted with metal oxides

a. filaments may be swollen at  
tip Crenothrix

b. filaments taper at  
tip Clonothrix

The main area of dispute concerning taxonomy is within the Sphaerotilus-Leptothrix group. Pringsheim (1949) believed that Leptothrix ochracea and Cladothrix were modifications or "forms" of S.natans. Cladothrix was described as a form characterized by false branching and L.ochracea as an older growth form of S.natans consisting of yellowish tubes containing only a few living cells. The synonymy of Cladothrix and Sphaerotilus is generally accepted. Cladothrix dichotoma is a variant of S.natans grown under unfavourable conditions where the tendency for false branching is greater (Mulder and Van Veen, 1963). Some authors have also adopted Pringsheim's classification with regard to Leptothrix spp. For example, Romano (1974) classified Leptothrix spp as a species within the Sphaerotilus genus, namely S.discophorus. The latter was characterized by an ability to oxidize manganese and accumulate iron. According to Romano (1974) S.natans had no ability to oxidize manganese and had little tendency to deposit iron on its sheath. This classification was adopted by Ivarson and Sojak (1978) in their work on Canadian ochre deposits where Leptothrix, Cladothrix and Sphaerotilus were considered to be the same organism.

Other authors, however, have classified Sphaerotilus and Leptothrix as different genera on the basis of the latter's ability to oxidize manganese compounds (Mulder and Van Veen, 1963; Van Veen et al, 1978). Additionally, Mulder and Van Veen (1963) found that L.ochracea had short sheaths, mostly devoid of cells as opposed to the long, partly filled sheaths of S.natans. These authors were also unable to reisolate S.natans from crude cultures of L.ochracea.

The evidence concerning the taxonomic position of Sphaerotilus and Leptothrix species was discussed by Van Veen et al (1978). The similarities between the two organisms include the formation of a sheath, a requirement for vitamin B<sub>12</sub> and the formation of poly-β-hydroxybutyrate as a reserve material. Evidence was also given to show that sufficient

differences existed between them to warrant separate genera. These differences are listed below.

1. An ability to oxidize Mn(II) is only seen in Leptothrix spp.
2. S.natans can respond very well to increased organic nutrients. It is a typical wastewater organism rarely found in natural waters. In contrast, most Leptothrix sp hardly respond to added nutrients and are typically found in uncontaminated natural waters. This is supported by Collins' (1964) survey of the organisms present in samples from different classes of industrial problems. The predominant organism in effluents was Sphaerotilus, Leptothrix spp, on the other hand, were found in pipeline systems.
3. Cells of S.natans are much larger than those of Leptothrix spp.
4. Leptothrix spp except L.Lopholea possess only one polar flagellum whilst the cells of the latter, in common with S.natans, are motile by a tuft of subpolar flagella.
5. False branching only occurs in S.natans and L.Lopholea.
6. Holdfasts are found in S.natans and L.Lopholea but not in other Leptothrix spp.
7. Under the electron microscope sheaths of S.natans appear smooth whilst Leptothrix spp produce a "netlike structure".

Eikelboom (1975) noted that the taxonomic position of the majority of filamentous organisms occurring in activated sludge is unknown. Many Gram-negative, sheath-forming bacteria belong to the Sphaerolitus-Leptothrix group but Eikelboom concluded that many filamentous bacteria mentioned in Bergey's Manual (see figure 2.1) are so ill-defined that a critical examination of the genera is urgently warranted. Lewin (1970)

suggested the abandonment of the order Chlamydobacteriales and this suggestion was supported by Van Veen et al (1978). The Chlamydobacteriales are composed of three families, Chlamydobacteriaceae, Peloplocaceae and Crenotrichaceae. Lewin suggested that those organisms of the Chlamydobacteriaceae, including Sphaerotilus spp and Leptothrix spp, which reproduce and disperse by motile cells with polar tufts of flagella could be relocated among the Pseudomonadales. The remaining genus in this family, Toxothrix, and all members of the Peloplocaceae and Crenotrichaceae might conveniently be set among the Flexibacterales.

#### 2.3.1.3 Sphaerotilus spp

Organisms of the genera Sphaerotilus are gram negative and individual filaments are composed of ellipsoidal or rod shaped, non-sporulating, bacterial cells within a colourless sheath. False branching is sometimes observed (Waitz and Lackey 1959; Dondero et al, 1961; Phaup, 1968; Ehrlich, 1981; Thornburn and Trafford, 1976). Two species of Sphaerotilus are usually described, S.natans and S.discophorus. Some authors have classified Leptothrix spp as S.discophorus. This was discussed in section 2.3.1.2. This classification is not adopted here and Leptothrix spp are described as a separate genera in section 2.3.1.4. Phaup (1968) also noted a third, minute form, of Sphaerotilus at low temperature but the possibility that this type was a strain of S.natans was noted.

In young cultures of S.natans all cells are contained in sheaths in single rows. In older cultures free swimming and non-motile cells may be seen. According to Phaup (1968) the cell size is about 1.2  $\mu\text{m}$  wide and 3-8  $\mu\text{m}$  long. These cells are motile by a bundle of intertwined flagella which may give the appearance of a single flagellum. In liquid cultures the cells become attached to solid surfaces by means of a holdfast. Sheath synthesis proceeds from the holdfast end of the filament and presumably develops synchronously with cell division; (Lackey and Wattie, 1940).



Yoshikawa and Takiguchi (1979) found that in a continuous flow apparatus the concentration of nutrients in the growth medium affected the attached growth of S.natans. At low nutrient concentrations the growing organism was attached to the inside of the growth vessel but at high concentrations single, unattached, cells or short chains were observed. Phaup (1968) noted that calcium was required for attached growth of S.natans in a continuous flow apparatus. Free swimming cells can also adhere to existing sheaths and give rise to false branching. This occurs more frequently in poor media. False branching may also arise as a result of a sheath rupturing thus allowing cells to protrude from the break while growth also continues in the original direction (Phaup, 1968).

The available information concerning the composition and formation of the sheath in Sphaerotilus filaments has been summarized by Phaup (1968) and Van Veen et al (1978). The sheath is a polysaccharide - protein - lipid complex. Romano and Peloquin (1963) found that the sheath contained 36% reducing sugars, 11% hexosamines, 27% protein, 5.2% lipid and 0.5% phosphorus in the dry matter. No muramic or teichoic acid were detected. The sheath is surrounded by a polysaccharide slime layer, the amount of which varies with the growth medium. Waitz and Lackey (1959) noted that the gelatinous nature of the sheath was responsible for the formation of masses of gelatinous stranded material.

Van Veen et al (1978) considered the function of the sheath; in slow running water, low in nutrients, it enables the bacteria to attach themselves to solid surfaces and protects them against parasites and predators. Venosa (1975) demonstrated that the parasite Bdellovibrio bacteriovorus was unable to penetrate the sheath of S.natans although it caused lysis <sup>in</sup> swarm cells of Sphaerotilus. However, the sheath is not necessary for cell propagation since sheathless mutants can grow and divide.

Sphaerotilus spp have an optimum pH around neutrality with a range from 6.0 to 8.0 or 10.0 (Phaup, 1968; Lackey and Wattie, 1940). Chang et al (1979) noted a marked fall in respiration rate below pH 6.6. These organisms can tolerate temperatures between 5°C and 35°C and have an optimum of 30°C (Stokes, 1954). They are obligate aerobes but can grow at extremely low oxygen tensions. Van Veen et al (1978) showed that final cell yield was not enhanced in strongly aerated culture solutions as compared to stationary cultures. However, improved aeration did result in increased growth rates.

Sphaerotilus spp will utilize a variety of organic acids, sugars and alcohols as carbon and energy sources. The organisms grow well in most complex nitrogenous laboratory media when supplemented with carbon sources (Lackey and Wattie, 1940). The organisms can also utilize inorganic nitrogen sources especially when pH is controlled (Van Veen et al, 1978). pH adjustments are needed to counteract shifts that occur after the uptake of  $\text{NH}_4^+$  or  $\text{NO}_3^-$ . Sphaerotilus spp require a basal mineral salts supplement of sodium, potassium, calcium, magnesium, sulphate, phosphate and chloride. Lackey and Wattie (1940) found a nutrient threshold of 5.0 µg/ml for  $\text{NaNO}_3$ ,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgSO}_4$  and  $\text{CaCl}_2$  in a medium also containing glucose. Omitting any one of these salts at this concentration prevented growth. It has been found that supplementing media with cyanocobalamin or methionine, from which vitamin B<sub>12</sub> can be synthesized, results in increased growth of S.natans (Waitz and Lackey, 1959; Mulder and Van Veen, 1963).

Waitz and Lackey (1959) found that Sphaerotilus natans grew well in the presence of hydrogen sulphide with an associated formation of intracellular deposits of sulphur. It was argued that Sphaerotilus natans could be classified as a sulphur bacterium on that basis.

According to Lackey and Wattie (1940) the broad adaptability of S.natans in terms of its nutrient requirements is an "important factor in making it a successful inhabitant of rivers and sewage disposal plants".

#### 2.3.1.4 Leptothrix spp

As discussed earlier (2.3.1.2) Leptothrix spp have been classed with Sphaerotilus as S.discophorus by some authors. Here it is assumed that they are a different species although this view is not held by all authors quoted in this section.

Two main forms of Leptothrix spp are usually considered (Mulder and Van Veen, 1963, Mulder, 1964).

1. Leptothrix discophora - appears as an uneven golden brown sheath which may be very thick from encrusted manganese and iron oxides. These have the smallest cells amongst isolated strains in the Sphaerotilus - Leptothrix group (Van Veen et al, 1978). Motile cells may be free swimming or surrounded by a thin sheath. This bacterium grows poorly in running soil extracts containing iron and manganese. Under these conditions the sheath becomes covered with a thick, dark brown, fluffy layer of iron and manganese. The oxides can increase the diameter of the bare sheath 10-25 fold up to about 20-25  $\mu\text{m}$ .

2. Leptothrix ochracea - according to Van Veen et al (1978) this is the most common "iron-storing" ensheathed bacterium. The accumulation and sedimentation of these organisms can be linked to the formation of bog iron ore. It is composed of a smooth, colourless or yellow-brown sheath containing ferric compounds. The sheath usually contains no living cells. Colourless sheaths have also been observed. These contain either long chains of large rods (1 - 1.2  $\mu\text{m}$  wide and 3-4  $\mu\text{m}$  long) or fungus-like threads in which septa are hardly visible.

Mulder (1964) studied L.ochracea in an enrichment culture, in slowly running artificial ditch water, and found that more than 99% of the sheaths were empty and yellow. Some sheaths did contain cells but these migrated out of the sheath. Pringsheim (1949) claimed that this tendency for cells to become flagellated swimmers, leaving the sheath, was more pronounced with L.ochracea than with S.natans. Van Veen et al (1978) described the formation of large masses of empty cells by L.ochracea within a relatively short time. This was demonstrated by a microscopic study of growing crude cultures. It was observed that ferric compounds were deposited on the sheath after the cells had left them.

In addition to the two types above Van Veen et al (1978) also identified three other strains of Leptothrix.

3. Leptothrix lopholea - This organism is very similar to S.natans in that it forms holdfasts, develops a bundle of subpolar flagella and shows false branching. However, L.lopholea has smaller cell diameters and can oxidize manganese.

4. Leptothrix pseudo-ochracea (Mulder and Van Veen, 1963). The cells of this organism are very slender as compared to other Leptothrix spp. The cells are very motile, by means of a single, thin, polar flagellum.

5. Leptothrix cholodnii - L.cholodnii can be isolated from non polluted, polluted waters and activated sludge. In the presence of manganese the sheaths become covered, in an irregular distribution, with granular manganic oxides. In ferrous ion containing water the sheaths become covered with a moderately thick, dark brown layer of ferric hydroxide.

The isolation of Leptothrix spp has proved very difficult (Mulder, 1964; Pringsheim, 1949; Mulder and Van Veen, 1963). One possible reason is that the number of living cells in environments containing many filaments

is probably low. Since Leptothrix spp have not been isolated and cultured extensively there is only limited evidence on their nutrition. Leptothrix spp are assumed to grow in conditions where organic matter levels are low. These organisms do not respond to increased levels of organic nutrients. In their pH optima and their need for vitamin B<sub>12</sub> they are similar to Sphaerotilus spp. Van Veen et al (1978) referred to experiments with a S.discophorus strain which they assumed was equivalent to Leptothrix cholodnii. This work suggested that amino acids can inhibit the growth of Leptothrix spp. For examples, 10 g/l of casamino acids completely suppressed growth. Leptothrix spp may tolerate up to 2 g/l of peptone but tryptone and trypticase inhibit growth at this concentration.

#### 2.3.1.5 Other sheathed filamentous bacteria

Many of the sheathed bacteria have not been isolated in pure culture and therefore very little information is available about them. A short description of those mentioned in Bergey's Manual 8th edition (1974) is given below.

1. Streptothrix spp - consist of thin rods in a hardly visible sheath of 0.5 - 0.8  $\mu$ m diameter. Iron and manganese are not deposited on the sheaths. Eikelboom (1975) referred to Streptothrix hyaline as Haliscomenobacter hydrossis. The latter was very similar to Toxothrix spp and also to Peloploca and Penonema subtilissum. H.hydrossis was described as small, unbranched, straight filaments protruding like needles from floccs. Occasionally the filaments were entwined in bundles.

2. Lieskeela spp are unattached sheaths that may be encrusted with metal oxides. Ehrlich (1981) described Lieskeela as filaments of rod shaped cells with rounded ends. Two filaments wound around each other are surrounded by a common slime capsule. The organism exhibits creeping motility. Lieskeela spp have not been cultivated and Godinho-Orlandi (1980) noted that the filaments are very unstable.

3. Phragmidiothrix consists of attached sheaths that are not encrusted with metal oxides and are not branched. Cell width may be variable along the filament and thus its diameter varies from 3 - 6  $\mu\text{m}$ . The sheath is delicate and colourless (Bergey's Manual; Farquhar and Boyle, 1971; Godhino-Orlandi, 1980).

4. Crenothrix - Pringsheim (1949) believed that Crenothrix was a "sessile" organism covering solid surfaces. Young threads look much like Leptothrix spp and only well developed ones can be recognised as Crenothrix. It forms filaments up to 1 cm long which are attached to surfaces. The filaments may be swollen at the free end, are thin, and may become encrusted with iron or manganese oxides at their base. The cells vary from cylindrical to disk shaped. The cells may leave the sheath but motile swarmers are not formed. Crenothrix has not been grown on artificial media in pure culture. According to Kuntze (1982) Crenothrix is a local variety of Sphaerotilus or Leptothrix being an adaptation to strongly flowing water containing iron. (Ehrlich, 1981; Bergey's Manual, 1974; Farquhar and Boyle, 1971; Godhino-Orlandi, 1980).

5. Clonothrix - Clonothrix spp form filaments up to 1.5 cm long. In one species they taper towards the top. The filaments may be attached to a surface. They have a distinct sheath that may be encrusted with iron or manganese oxides. According to Godhino-Orlandi (1980) the older parts of the filaments are encrusted with metal oxides while the younger filaments are more slender, colourless and hardly distinct. The cells within the sheath are cylindrical. During reproduction the cells leave from a broken sheath. Clonothrix has not been cultured in artificial media. Kuntze (1982) regarded Clonothrix as a variety of Sphaerotilus or Leptothrix.

### 2.3.1.6 The relationship between filamentous bacteria and iron

The relationship between the filamentous bacteria and iron is frequently discussed in the literature. Complete autotrophy is seldom assumed but the role of iron and the possible derivation of energy from its oxidation has been considered.

Pringsheim (1949) concluded that filamentous bacteria could derive energy from ferrous ion oxidation and also utilize organic compounds as an energy source. Similarly, Thorburn and Trafford (1976) believed that the filamentous bacteria could obtain energy from several sources including the oxidation of Fe(II).

The major problem associated with work on the role of iron in the nutrition of these bacteria is rapid chemical oxidation of Fe(II) at the neutral pH conditions required by the organisms (Van Veen et al, 1978). Farquhar and Boyle (1970) described an "iron oxidizing" test where ferrous sulphate was added to a sample of bacterial culture. This test was used to demonstrate the ability of sheathed microorganisms to oxidize Fe(II) and deposit iron compounds within their sheath. Van Veen (1973) found this technique to be insufficient due to the chemical oxidation of Fe(II). It has been suggested that chemical oxidation is the predominant mechanism by which iron is accumulated by filamentous bacteria. Phaup (1968) was able to demonstrate iron deposition on sheaths of heat-killed cultures of S.natans. Similarly, if S.natans is cultivated in the presence of chelated ferric complexes the sheath becomes covered with ferric hydroxide (Van Veen et al, 1978). Ford and Beville (1970) suggested that chemically precipitated iron can be trapped by the sheaths because of their "sticky" nature. The accumulation of chemically precipitated iron compounds is also suggested by the results of Waitz and Lackey (1959) who found that iron was not essential, except in trace quantities, for the growth of Sphaerotilus spp. They found that ferric chloride at 2 µg/l had no effect on the growth of Sphaerotilus and at 25 µg/l growth was apparent but reduced. The toxic

effects of large quantities of iron on Sphaerotilus were also demonstrated by Chang et al (1979). For example, at pH 6.0 20 µg/l of iron, as iron citrate, inhibited the maximum respiration rate of S.natans by over 88% Van Veen et al (1978) noted that the effect of iron on S.natans can be reduced by complexing agents. For example, it is possible to grow the bacterium in a nutrient medium containing 1.8 g/l  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in the presence of 0.25 g/l quinic acid. The growth of Sphaerotilus spp in the absence of iron and their susceptibility to relatively low iron concentrations suggests that these organisms accumulate chemically precipitated iron without deriving energy from its oxidation.

When Leptothrix was first isolated it was thought to be autotrophic.

Van Veen et al (1978) suggested that autotrophy may exist in L.ochracea since natural habitats of the organism contain few living cells amongst large quantities of iron containing empty sheaths. However these authors pointed out that cells and newly-formed sheaths are not impregnated with iron suggesting that they do not require iron in order to grow.

Additionally, it was found that the amount of iron in relation to cell dry weight is low in Leptothrix spp. A typical ratio of cell dry weight to Fe(III) of 1:4 was found in laboratory cultures of Leptothrix. This can be compared to the same ratio determined for the autotrophic, iron oxidizing T.ferrooxidans which ranges from 1:200 to 1:500.

Work on the oxidation of metal ions by Leptothrix spp has concentrated on manganese. The advantage of manganese is that it does not oxidise rapidly in bacterial cultures at the pH values associated with filamentous bacteria. The concensus of opinion is that the precipitation of Mn(II) is enzymically mediated. Heat treatment or enzyme inhibitors can prevent Mn(II) oxidation (Van Veen et al, 1978). Van Veen (1972) suggested that a protein was released from the cells of S.discophorus, which is probably equivalent to Leptothrix spp (see section 2.3.1.2). This compound combined with Mn(II) and precipitated as a manganic oxide-protein complex. It is possible that Leptothrix spp may capture some



energy from Mn(II) oxidation but Van Veen et al (1978) noted several reasons why the derivation of energy from manganese oxidation by Leptothrix spp is unlikely. The energy released by the oxidation of Mn(II) to Mn(IV) is low and the maximum concentration of manganese tolerated by Leptothrix spp is also very low as compared to, for example, the ferrous ion concentrations tolerated by T. ferrooxidans.

Johnson and Stokes (1966) attributed the oxidation of manganese by S. discophorus (Probably Leptothrix spp, 2.3.1.2) to an inducible enzyme(s). They found that organisms grown in the presence of  $MnSO_4$  were able to oxidize it to  $MnO_2$ . This ability was not found in cells grown in the absence of  $MnSO_4$ . Van Veen (1972) questioned this conclusion, suggesting that Johnson and Stokes had not recognised the inhibitory effect of phosphate on Mn(II) oxidation. Van Veen (1972) showed that phosphate could inhibit manganese oxidation but only in cells that had no manganese oxides on their sheaths. Thus cells grown in the absence of Mn(II), having no manganic precipitates, would not oxidise manganese. According to Van Veen this is due to phosphate inhibition rather than an inducible enzyme system.

It is not known whether the enzyme system responsible for manganese oxidation also operates for iron oxidation (Van Veen et al, 1978). Rogers and Anderson (1976A) found that the growth rate of S. discophorus (L. cholodnii or L. discophora according to Van Veen et al, 1978) was independent of iron concentration in the medium. There was no correlation between iron concentration and final cell protein yield. These results suggested that iron deposition is caused by a component of the organism's sheath. Rogers and Anderson (1976A) also showed that blocking protein synthesis did not prevent continued iron precipitation. Once iron deposition was initiated protein synthesis was not required for further oxidation.

Despite the lack of evidence to suggest that Leptothrix spp gain energy from iron oxidation Jones (1975) did show that the numbers of L.ochracea in fresh water were proportional to total iron concentration. Counts obtained from samples with less than 100 µg/l of iron were very low but they rose sharply with iron concentration up to about 1000 µg/l. These results suggest that some positive interaction exists between Leptothrix spp and iron, even though they do not use iron as an energy source.

Therefore, in conclusion, filamentous bacteria can precipitate and accumulate ferric and manganic compounds. In the case of Sphaerotilus spp the presence of these metals does not appear to stimulate growth. In fact raising iron concentration will eventually reduce its growth. The oxidation of manganese by Leptothrix spp is related to an enzyme system but there is no evidence that energy is derived by this oxidation. The mechanism of iron oxidation by Leptothrix spp is uncertain and could be chemical, enzyme mediated or caused by a component of the sheath.

It appears that neither Sphaerotilus spp nor Leptothrix spp derive energy from the oxidation of Fe(II or Mn(II)). However, the close association between these bacteria and iron and manganese in nature cannot be ignored (Petersen, 1966). Fenchel and Blackburn (1979) concluded that "while there is now strong evidence to show that the sheathed bacteria are not chemolithotrophs, the real physiological significance of the iron and manganese deposition remains unknown".

### 2.3.2 GALLIONELLA

Gallionella spp are characterized by a short curved rod, or bean shaped cell, which produces a twisted stalk consisting of filaments containing ferric hydroxides. (Ehrlich, 1981; Fenchel and Blackburn, 1979).

Gallionella ferruginea was discovered by Ehrenberg in 1836. According to Aristowskaya and Zavarzin (1971) the terminal cells are often absent. The

stalk is usually anchored to a solid surface. The stalks are composed of electron opaque strands approximately 0.05 to 0.1 microns in diameter (Vatter and Wolfe, 1955). As many as eight strands have been observed in a stalk. The strands are closely united in newly-secreted stalks but as the stalk ages the strands separate. In natural environments the stalk is composed primarily of silicon, aluminium and iron (Ridgway et al, 1981).

The cells, which may form one or two polar flagella, can detach from the stalk, swim in swimmers, and seek a new site for attachment and a stalked growth habit.

Gallionella spp grow best at low oxygen tensions and have a pH optimum between 6 and 7. It is believed that Gallionella spp can catalyse ferrous ion oxidation and derive energy by this means. Evidence for this assumption was given by Ehrlich (1981). Firstly, the organism will grow in a mineral salts medium in the absence of organic carbon (Kucera and Wolfe, 1957). It will assimilate significant quantities of labelled carbon dioxide from  $\text{NaH}^{14}\text{CO}_3$  added to an iron sulphide containing medium. Ehrlich (1981) noted however that a quantitative demonstration of  $\text{CO}_2$  uptake coupled to Fe(II) oxidation remains to be shown.

### 2.3.3 OTHER MICROORGANISMS

In addition to filamentous bacteria and Gallionella spp other organisms are commonly found in filamentous ochre samples. These include fungi, yeasts and common soil heterotrophic bacteria. For example, Ivarson and Sojak (1978) isolated a yeast - Rhodotorula spp and small numbers of Penicillium frequentans and Cladosporium in filamentous ochre. Martin et al (1977) isolated a variety of fungi from iron deposits in field drains. These were similar to species found in adjacent top soils and included Aspergillus niger, Fusarium spp, Verticillium spp and Cladosporium spp. The hydrogen sulphide oxidizing Toxothrix and Thiothrix are also common in

ochre. These are found in the anaerobic areas of the biotype (Kuntze, 1982). The occurrence of these organisms may be incidental or they may have a role in its formation. In a model simulating filamentous ochre formation Ford (1978, 1979A) used Pseudomonas and Enterobacter in addition to the normal filamentous bacteria. It was found that ochre production was enhanced by the "clear jelly slimes" of these bacteria. Similarly, Fischer and Ottow (1972) found that Pseudomonas fluorescens was responsible (with Acinetobacter) for the decomposition of ammonium iron citrate in a well aerated solution. The decomposition yielded a yellow brown precipitate. This type of complex degradation may be a mechanism of ochre formation (see section 2.5.2.3).

## 2.4 THE MICROBIOLOGY OF PYRITIC OCHRE

### 2.4.1 THE THIOBACILLI

Pyritic ochre is normally associated with bacteria of the genus Thiobacilli (Bloomfield, 1967; FDEU, 1975). The Thiobacilli are a closely related group of rod shaped, Gram negative bacteria (Thorburn and Trafford, 1976). In general these organisms derive their energy from the oxidation of one or more reduced, or partially reduced, sulphur compounds. Sulphates are the usual oxidation product but the accumulation of sulphur and polythionate is seen under certain conditions. The genus Thiobacilli includes strictly autotrophic organisms, facultative autotrophs and at least one species requiring organic matter for growth. They are all obligate aerobes except T.denitrificans (Buchanan and Gibbons, 1974).

In ochre formation two distinguishable species of Thiobacilli are involved namely T.ferrooxidans and T.thiooxidans (Thorburn and Trafford, 1976).

The former normally derives its energy from the oxidation of ferrous iron and reduced sulphur compounds whilst the latter only from the oxidation of reduced forms of sulphur. Because they obtain metabolically useful energy from the oxidation of inorganic compounds and fix atmospheric CO<sub>2</sub> they are classed as chemolithotrophic bacteria (Rittenberg, 1969).

T. ferrooxidans has been isolated throughout the world and found to be responsible for acidification of pyritic mine wastes and acid sulphate soils (Temple and Colmer, 1951; Trafford et al, 1973). The commercial leaching of ores by processes depending on T.ferrooxidans is a well established practice. Bacterial leaching can be used for uranium and copper extraction, manganese recovery, and the removal of sulphur from coal, shale or slag (Le Roux, 1969, 1972; Tuovinen, 1972; Dugan and Apel, 1978; Beck, 1967).

T.ferrooxidans and T.thiooxidans grow at pH values below 4-5, with an optimum of pH 2.0 - 2.5 (Johnson et al, 1979). The optimum pH of T.ferrooxidans depends on the substrate being used, as illustrated in table 2.2 (Tuovinen and Kelly, 1974(v); McGoran et al, 1969).

TABLE 2.2 : OPTIMUM pH OF T.ferrooxidans GROWN ON VARIOUS SUBSTRATES (from McGoran et al, 1969)

<u>SUBSTRATE</u>	<u>OPTIMUM pH</u>
ferrous ions	2.0
sulphur	1.75 - 5.0
chalcopyrite	1.8 - 3.5

The optimum temperature of the Thiobacilli is about 30°C with a range from 10°C to 45°C. The metabolic activity of T.ferrooxidans declines above 40°C and little activity is seen above 50°C (Thorburn and Trafford, 1976; Tuovinen and Kelly, 1972).

T.ferrooxidans and T.thiooxidans obtain oxygen from dissolved atmospheric oxygen and have an ability to live at very low oxygen concentrations. These organisms are autotrophic, utilizing atmospheric carbon dioxide as their sole carbon source (Thorburn and Trafford, 1976). Tuovinen and Kelly (1972) showed that CO<sub>2</sub> fixation by T.ferrooxidans depends on energy derived from ferrous ion oxidation. The number of viable cells is thus correlated with CO<sub>2</sub> fixation and Fe<sup>++</sup> oxidation (Tuovinen and Kelly, 1973).

The principal nitrogen source of T.ferrooxidans is  $\text{NH}_4^+$  but this can be replaced by urea, with little loss in activity, or by amino acids with a subsequent decrease in activity (Moss and Anderson, 1968). Mackintosh (1976) showed that T.ferrooxidans could fix atmospheric nitrogen and that the nitrogenase enzyme system was present in the microorganism.

Bloomfield (1972A) demonstrated the effect of T.ferrooxidans on the oxidation of pyrite. 900 mg/g of pyrite were oxidized in the presence of T.ferrooxidans as compared to 20-25 mg/g in a sterile control during the same time period. Lacey and Lawson (1969) found that T.ferrooxidans was able to oxidize  $\text{Fe}^{++}$  at a rate some 500,000 times as fast as that occurring by chemical oxidation. No growth of T.ferrooxidans was seen where initial  $\text{Fe}^{++}$  concentration was less than 125 ppm (Temple and Colmer, 1951). Silverman et al (1959) could find no signs of toxicity due to high ferrous iron concentration in their studies of Ferrobacillus ferrooxidans, an organism now considered identical to T.ferrooxidans.

The Thiobacilli can thrive as free floating or self motile organisms but prefer to adhere to solid surfaces, forming a thin film (Thorburn and Trafford, 1976). Mehta et al (1974) found that this adherence was strong and quick. T.ferrooxidans can attach to insoluble substrates and to the solid end product of ferrous ion oxidation (McGoran et al, 1969).

MacDonald et al (1970) explained that film formation is initiated by the attachment of T.ferrooxidans cells to a surface. The cells multiply until the surface is completely covered and then build up layers. Iron precipitates help to bind or cement the structure.

T.ferrooxidans is tolerant of wide variations in the environment and is tolerant of most toxic metals. T.ferrooxidans is able to endure over 10 g/l of zinc, manganese, copper, cobalt and nickel (Tuovinen et al, 1971; Tuovinen and Kelly, 1974 (III)). It is more sensitive to other metals but can still tolerate 50 - 100 mg/l of silver, tellurium, arsenic and selenium and up to 5 mg/l of molybdenum (Tuovinen et al, 1971). The

adaption of T.ferrooxidans to tolerate increasing levels of Zn, Ni, Cu, U and Cl have been reported (Tuovinen et al, 1971; Tuovinen and Kelly, 1974(III); Thorburn and Trafford, 1976). It has been shown that the toxic effects of metals can be affected by external factors. For example, Tuovinen and Kelly (1974(IV) ) reported that the inhibition of T.ferrooxidans by 2mM uranyl sulphate could be partially relieved by 200mM  $K^+$ ,  $Na^+$ ,  $Li^+$  or  $NH_4^+$  added to the growth medium as sulphates.

T.ferrooxidans is not tolerant of mercury and 100  $\mu\text{g/ml}$  mercuric chloride will stop its activity almost instantly (Le Roux et al, 1973).

T.ferrooxidans is tolerant of organic biocides but Le Roux et al (1973) found that panacide (200  $\mu\text{g/ml}$ ) was an effective inhibitor. Such levels would be difficult to maintain in the presence of clays and silts. Apel et al (1980) discussed the inhibitory effects of pentachlorophenol and nigericin on T.ferrooxidans. Similarly, 2:4 dinitrophenol has a marked uncoupling effect on  $\text{CO}_2$  fixation (Tuovinen and Kelly, 1974(III); Beck and Shafia, 1964).

#### 2.4.2 ACIDOPHILIC HETEROTROPHS

Several authors have isolated heterotrophic bacteria growing in acid media with Thiobacillus ferrooxidans (Zavarzin, 1972; Dugan and Apel, 1978; Harrison et al, 1980). Some of the isolates have a pH optimum near neutral while others are acidophilic. Johnson and Kelso (1980) isolated similar bacteria in the form of gelatinous acid streamers from a mine drainage stream of great acidity. The extracellular material of the streamer organisms contained polysaccharides and RNA. It has been shown that these bacteria are also associated with pyritic ochre deposits (Johnson, 1979; Johnson and Kelso, 1980). The role of these organisms in the formation of pyritic ochre is unclear but the presence of bacterial polysaccharides would accentuate the seasonal swelling and shrinking capacity of the deposits.

The adhesive properties of extracellular polysaccharides could also initiate ochre precipitation and hinder cleaning of drains.

#### 2.4.3 THE COEXISTENCE OF MICROORGANISMS ASSOCIATED WITH PYRITIC AND FILAMENTOUS OCHRE

The conventional view of ochre as being either filamentous or pyritic may be an over-simplification since there is evidence to suggest that the bacteria responsible for the formation of the two types can coexist (FDEU, 1973, 1974, 1975; Thorburn and Trafford, 1976). This is possible either because both groups of organisms are capable of adapting to a wider range of pH values in the field, than is apparent in work with defined laboratory media, or that there are other bacteria which bridge the gap in the pH range. The two types of bacteria may also exist in microenvironments of varying pH within the soil and drainage system.

Cameron et al (1981) isolated an iron oxidizing bacterium from an ochre polluted stream of pH 5.5. This organism grew autotrophically in artificial media at pH 3.5. The organism, with regard to its autotrophy and pH optimum, could not be classed as one of the filamentous bacteria described in section 2.3.1. It was also distinct from the Thiobacilli since it grew as long unbranched filaments encrusted with iron.

One bacterium that could be a "missing link" between filamentous and pyritic ochre is Metallogenium (Aristovskaya and Zavarzin, 1971; Buchanan and Gibbons, 1974). According to Walsh and Mitchel (1972) it catalysed iron oxidation in the pH range 3.5 - 5.0 with an optimum of pH 4.1.

Metallogenium spp have been isolated from soils (Yefremova et al, 1978), mining waste (Langworthy, 1978), concretions on rice roots (Sidorenko et al, 1979), and on solid laboratory media (Aristovskaya, 1961). Ivarson and Sojak (1978) observed filamentous structures resembling Metallogenium in filamentous ochre samples but experimental evidence indicated that they were aggregates of colloidal  $\text{Fe}(\text{OH})_3$ .



It appears, however, that in some areas it is possible to find purely filamentous or pyritic ochre. For example, whereas Ford (1969) isolated acidophilic Thiobacilli in filamentous ochre Ivarson and Sojak (1978) could not find these bacteria in their studies of the same ochre type. This suggests that although coexistence of the organisms associated with pyritic and filamentous ochre does occur, it should not be taken as an universal rule governing all ochre sites.

## 2.5 THE FORMATION OF IRON OCHRE

Before discussing the possible mechanisms by which ochre is formed the factors influencing its deposition will be considered. The role of some factors, such as soil type and microbiology, has been mentioned earlier.

### 2.5.1 Factors influencing ochre formation

These factors can be divided into three main groups, namely environmental factors, factors associated with the drainage system, and management factors.

Alcock (1973) noted that high rainfall increased ochre deposition. This could be explained by the longer duration of anaerobic conditions within the soil resulting in a greater extent of iron reduction and thus increased input of ferrous iron to the drains.

The effect of geology is not clear. Alcock's (1973) survey of ochre<sup>e</sup> sites in England and Wales revealed a high incidence of ochre on beds with a high iron content. However, the evidence also suggested that most of the really serious problems were associated with "bogs" where no correlation with geology would be expected.

Factors associated with the drainage system can also influence the formation of iron ochre. According to some authors the deposition of ochre is not related to the material of the drainage pipe (Grass, 1969; Spencer et al, 1963). In a detailed comparative study, using laboratory models, Talman (1978) found that clay pipes blocked more readily than plastic pipes. However, clay pipes showed less accumulation of ochre where the gap between the tiles was larger than 5 mm. Talman also found that increasing slot width decreased ochre deposition at the slot whilst increasing deposition above it (i.e, in the permeable backfill).

Alcock (1973) found a definite trend of ochre occurrence towards the East of England. Here high value cash crops are grown and thus there is more incentive and money available for drainage, "bringing to light" ochre problems.

The influence of soil mixing on ochre formation was revealed by work at Arthur Rickwood Experimental Husbandry Farm. Dennis and Wickens (1977) suggested that soil mixing could influence ochre deposition but no direct evidence was available. The purpose of soil mixing is to incorporate mineral matter into organic top soil to reduce peat wastage. In theory soil mixing should increase the rate of aeration of newly-drained profiles, hence increasing the oxidation of pyrite and ochre production (FDEU, 1976).

Scheffer and Kuntze (1979) noted that the application of pig slurry to drained land increased the amount of ochre and its iron content by 40% in relation to mineral fertilizers. This is worthy of consideration especially as mineral fertilizers become more expensive and farmers find it necessary to use more organic manures such as slurry.

#### 2.5.2 The mechanisms of ochre formation

Many ideas have been advanced concerning the origin of iron deposits in field drains but it is generally accepted that they are formed by the oxidation of ferrous iron to ferric oxides and hydroxides, (Petersen, 1966; Mackenzie, 1962; Puustjarvi and Juusela, 1952; Ford, 1969; Linder, 1977; Ivarson and Sojak, 1978; Haijjer and Wolf, 1965; Kuntze, 1978). The main sphere of dispute concerns the means by which oxidation occurs.

Some authors have explained the formation of ochre by purely chemical mechanisms (Haijjer and Wolf, 1965; Puustjarvi and Juusela, 1952; Childs et al, 1982). Davison and Seed (1983) found that the rate of ferrous ion oxidation in natural waters did not change with respect to

depth or time while the microbial population did. This suggests that the oxidation reaction is not mediated by bacteria. Other authors have concentrated on the role of microorganisms in the precipitation of iron. It has been claimed that chemical oxidation cannot account for the composition of all ochre samples (Petersen, 1966; Glathe and Ottow, 1972). It has also been claimed that non-catalysed chemical oxidation proceeds slowly at pH values below neutral. Petersen (1966) included bacteria amongst the soil water components that could catalyse the reaction. Aristovskaya (1961) regarded the accumulation of ferric and manganic hydroxides in soils, if not entirely, then to a considerable degree as a biological phenomenon. Green (1950) demonstrated the microbial oxidation of iron by reducing ferrous ion oxidation with bacterial inhibitors. This reduction was accompanied by a reduction in oxygen uptake. With regard to ochre formation it has been demonstrated (Ford, 1978, 1979A) that bacteria are the primary cause of ochre formation and clogging from ochre. Chemically precipitated iron is a poor clogging agent lacking ability to "adhere" (Ford, 1978).

In reality a combination of chemical and biological mechanisms probably operate (Mackenzie, 1962; Meek et al, 1968; Quispel et al, 1952; Odilien et al, 1975; Halvorson, 1931; Ivarson and Sojak, 1978; Petersen, 1966; Linder, 1977). There are four possible agencies by which iron could precipitate under natural conditions (Glathe and Ottow, 1972; Starkey and Halvorson, 1927; Kuntze, 1982).

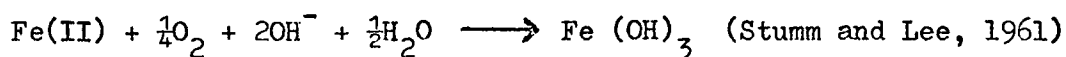
1. Chemical mechanisms involving atmospheric oxygen.
2. The action of autotrophic iron bacteria.
3. Decomposition of the organic component of soluble complexes by heterotrophic organisms.
4. The action of microorganisms causing environmental changes.

### 2.5.2.1 Chemical oxidation

Chemical oxidation can play an important part in the formation of filamentous ochre since ferrous iron oxidizes rapidly at the near neutral pH values associated with these sites. Childs et al (1982) found that ferrous ions in a spring water oxidized, on reaching the surface, to form an ochreous deposit. The rate of oxidation could be accounted for by chemical mechanisms.

The chemistry of ferrous iron oxidation is well documented and the aim in this section is to summarise this information concentrating especially on those factors relevant to the formation of ochre deposits.

In solutions of pH above 6.0 the oxidation of ferrous ions can be described by the equation:-



The rate of oxidation depends on several factors including pH, the concentration of dissolved oxygen, temperature, organic matter levels and the presence of catalysts.

In solutions of pH above 5.0 the rate of Fe(II) oxidation is first order with respect of oxygen concentration (Stumm and Morgan, 1981). However, at pH 4.0 ferrous iron is likely to remain in solution at all redox potentials (Patrick and Henderson, 1981). Oxidation is very slow at pH values less than 6.0 and in acidic media only a small dependence of the oxidation rate on hydrogen ion concentration is observed. In less acidic solutions a 100 fold increase in reaction rate occurs for each unit increase in pH (Stumm and Lee, 1961; Morgan and Stumm, 1964). There is an interaction between Eh and pH with regard to Fe(II) oxidation. Collins and Buol (1970) demonstrated that at low (negative) Eh the pH necessary to precipitate iron is higher than at more positive values. Similarly, the Eh level necessary to convert iron from Fe(II) to Fe(III) depends largely on the pH.

Temperature also affects the rate of Fe(II) oxidation. For a given pH the rate of oxidation increases tenfold for a 15°C temperature rise (Stumm and Lee, 1961; Sung, 1981).

Catalysts can increase the oxidation rate significantly, especially copper (Lamb and Elder, 1931; Morgan and Stumm, 1964), and anions that form complexes with Fe<sup>+++</sup>, for example, HPO<sub>4</sub><sup>2-</sup> (Huffman and Davidson, 1956; Stumm and Morgan, 1981). The rate of oxidation by molecular oxygen in acidic solutions is very dependent upon the nature of the anions present. The rate is increased as the complex affinity of the anion for Fe<sup>+++</sup> increases.

In neutral solutions the oxidation of Fe(II) is accelerated by the reaction product ferric hydroxide, and by the addition of ferric hydroxide (Tamura et al, 1976; Sung and Morgan, 1980). Sung (1981) showed that the product of Fe(II) oxidation was γ-FeOOH (lepidocrocite). Thus the oxidation of Fe(II) in neutral solutions can proceed via two mechanisms occurring simultaneously. There is a homogenous reaction in solution but also a heterogeneous reaction on the ferric hydroxide surface. The second mechanism involves the preliminary adsorption of Fe(II) by ferric hydroxide (Tamura et al, 1976; Sung, 1981).

According to Tamura et al (1976) a linear plot for ferrous iron concentration with time is obtained with 3 mg/l Fe(II) initially present in solution, but with 25 mg/l Fe(II) initially present a concave plot is obtained demonstrating autocatalysis. The autocatalytic effect is negligible with less than 3 mg/l Fe(II) since the amount of ferric hydroxide produced is small. Where ferric hydroxide is added to a ferrous solution a greatly increased rate of oxidation is observed. The rate constant increases linearly with the amount of ferric hydroxide. Davison and Seed (1983) found that adding ferric iron at concentrations of 10 mg/l or less had no

measurable effect on the rate of reaction at pH 7.0. However the addition of 50 mg/l (Fe(III)) at pH 7.13 - 7.18 produced significantly higher rates by a factor of two to five.

It has also been demonstrated that the ferric hydroxide surface produced by Fe(II) oxidation can catalyse the oxidation of Mn(II). In the presence of hydrous iron precipitates Mn(II) is removed from solution at Eh and pH values which are not sufficiently high to precipitate manganese in the absence of iron (Sung, 1981; Collins and Buol 1970).

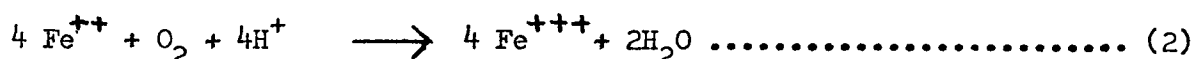
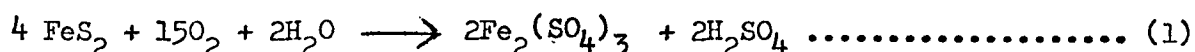
Organic compounds can affect the rate of Fe(II) oxidation, either decreasing or increasing it. Organic compounds can reduce the oxidation rate by forming complexes. Coulson et al (1960) showed that polyphenols were capable of reducing iron by forming complexes. Similarly, Bloomfield (1953) demonstrated that aqueous extracts of pine needles could mobilize ferric oxides by reducing Fe(III) to Fe(II). The rate of oxidation of these complexes is low but increases with increasing pH. However, oxidation of complexed iron does not necessarily result in its precipitation. Fe(II) can be oxidized to Fe(III) while the total amount of complexed iron in solution remains constant.

Some organic substances that can reduce Fe(III) can also catalyse the oxidation of Fe(II). Essentially ferrous ions, oxygen and an organic compound react to form a ferric - organic complex. This complex dissociates yielding ferrous iron and an oxidized organic compound. The ferrous iron is reoxidized by combination with the organic compound and oxygen. This cycle has been observed with phenols, tannic acid and cysteine (Stumm and Morgan, 1981). In these cases the ferrous - ferric system acts as a catalyst for the oxidation of organic material by oxygen.

The majority of the work described above was carried out in synthetic solutions. Davison and Seed (1983) found that the oxidation of Fe(II) by molecular oxygen was described by the same rate expressions in natural systems as in analogous synthetic solutions. The catalytic or inhibitory effects of other substances appeared to be no greater in natural waters and in some cases the effect may be less pronounced. Davison and Seed (1983) concluded that a "universal" rate constant of  $2 \times 10^{-3} \text{ M}^{-2} \text{ atm}^{-1} \text{ min}^{-1}$  (range  $1.5$  to  $3 \times 10^{-3}$ ) operates in natural waters of pH 6.5 - 7.5. Consequently, in air saturated freshwaters of pH 6.5, 7.0, 7.5 and 8.0 the half lives for Fe(II) will be 361, 36, 3.6 and 0.36 minutes at 20°C.

2.5.2.2 The action of autotrophic iron bacteria (pyritic ochre formation)

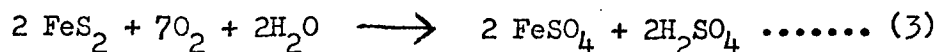
The iron of pyritic ochre deposits is derived from iron sulphides in soil and parent material. If pyrite is taken as a representative sulphide then the oxidation of iron sulphides can be described by equation 1 (Le Roux et al, 1973; Talman, 1978) or alternatively by equation 2 (Lundgren and Vestal, 1972).



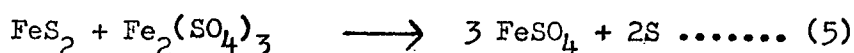
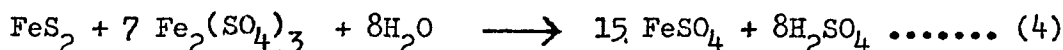
This reaction proceeds via several intermediates giving a reaction sequence for pyrite oxidation. This sequence involves chemical and microbial activity, the bacteria are the acidophilic Thiobacilli described in section 2.4.1.



The initial oxidation of pyrite can be described by reaction 3 (Le Roux, 1972; Thorburn and Trafford, 1976)



Le Roux et al (1973) suggested that two reactions were involved in the initial oxidation of pyrite in sterile ferric sulphate solutions (equations 4 and 5), and found that the dissolution occurred mainly by reaction 4.



The oxidation of pyrite to ferrous sulphate proceeds readily by chemical oxidation, a reaction which was recognised in 1875 when Case reported in Nature that:-

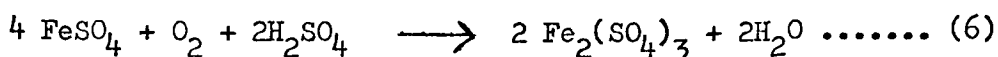
"Some iron pyrites exhibited in a particular case in the Maidstone Museum have crumbled into a coarse, finely divided mass"

The phenomenon was discussed by several authors in subsequent issues and it was realised that pyrite was oxidized in the presence of air and moisture producing acidic ferrous sulphate (Williams, 1875; Wire, 1875).

According to Lingwood (1875):-

"this salt appeared in abundant crystals, and was sufficiently strong to partially obliterate and destroy a contiguous manuscript."

Under acid conditions ferrous sulphate is oxidized to ferric sulphate (equation 6). Johnson (1979) noted that this is the rate limiting step in the oxidation of pyrite by chemical reactions and in the pH range 1.5 - 4.5 the reaction is catalysed by T.ferrooxidans

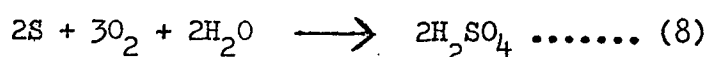


The ferric sulphate produced reacts with more pyrite to yield ferrous sulphate and sulphur (equation 7)

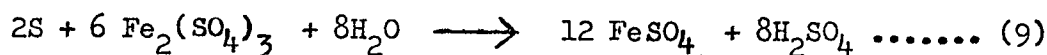


This reaction takes place readily without bacterial action. Le Roux et al (1973) found that it could proceed under sterile conditions.

Sulphur produced, as in equation 7 can be oxidized to sulphuric acid (equation 8). This reaction is extremely slow by atmospheric oxidation but is catalysed by T.ferrooxidans or T.thiooxidans (Temple and Delchamps, 1953).

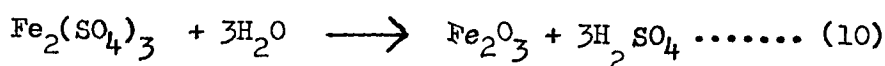


In theory sulphur could be oxidized by ferric sulphate by a chemical reaction (Temple and Delchamps, 1953; Le Roux et al, 1973) (equation 9).

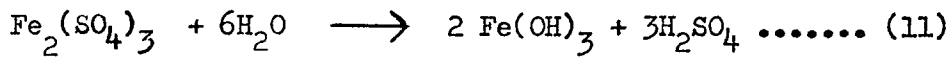


Reactions 6, 7 and 8 form a reaction cycle that is self accelerating with increasing acidity, up to a maximum at about pH 2.0. Le Roux (1969) found that the overall effect of these reactions (3 to 9) is to produce an "oxidizing acidic liquor". The role of T.ferrooxidans is to provide a continuous supply of ferric iron, whereas T.thiooxidans oxidizes elemental sulphur.

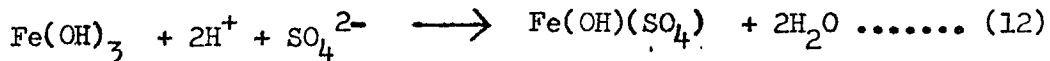
The formation of ferric compounds in acidic drainage water can take place as in reaction 6. According to Thorburn and Trafford (1976) ferric sulphate then hydrolyses yielding ferric oxide and sulphuric acid (equation 10).



The formation of anhydrous ferric oxide in drainage water is unlikely and it has been suggested (Johnson, 1979; Dugan and Apel, 1978) that ferric sulphate hydrolyses to ferric hydroxide and sulphuric acid (equation 11)

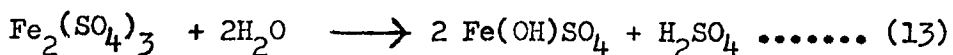


The ferric hydroxide can react with sulphuric acid to form hydroxy-sulphate complexes (equation 12)



Petersen (1966) believed that basic ferric sulphates would precipitate in solutions of low pH and high sulphate concentration. This would be characteristic of pyritic ochre sites. It is possible that combinations of such precipitates are responsible for the typical yellowish-brown or reddish-brown deposits associated with pyritic ochre sites (Johnson, 1979).

Alternatively, Leathen et al (1953) suggested that hydrolysis of ferric sulphate to produce ferric hydroxide could proceed via the formation of basic ferric sulphates (equation 13). This can be compared with equations 10 and 11 where basic ferric sulphates are formed via ferric hydroxide.



The ratio of iron, hydroxide, and sulphate depends upon dilution and acidity during hydrolysis. Complete hydrolysis results in the formation of ferric hydroxide.

Thus, the oxidation of pyrite, via several chemical reactions, results in the formation of ferric hydroxides and basic ferric sulphates. The rate of pyrite oxidation is directly related to the activity of the autotrophic Thiobacilli.

### 2.5.2.3 Decomposition of the organic component of soluble complexes by heterotrophic organisms

According to Glathe and Ottow (1972) the decomposition of organic compounds has been overlooked as an important mechanism in ochre formation. Biodegradation can be achieved by a large number of soil microorganisms that use the organic component of iron-organic complexes as an energy, carbon, or nitrogen source regardless of the metal component. Most of these microorganisms have a wide tolerance of pH (3.0 - 8.0) and are nutritionally inexacting.

Several authors have demonstrated the effect of microorganisms on iron-organic complexes. Fischer and Ottow (1972) showed that the soil bacteria Pseudomonas and Acinetobacter could decompose an iron (III) citrate complex yielding an amorphous ferric precipitate. Similarly Macrae et al (1973) studied the degradation of soluble iron-organic complexes by bacteria and the subsequent precipitation of iron from these complexes.

Aristovskaya (1961) suggested that the accumulation of ferric hydroxide by microorganisms results from their breakdown of organo-mineral complexes.

The stabilization of iron colloids is a well known property of certain organic substances (Sholkovitz, 1980). Kauritshev et al (1964) noted that organic chelates are important in iron transformation and migration processes. Normally the solubility of ferrous iron declines with rising pH. Most iron in groundwater is completely oxidized at pH values about 7.0. Chelated iron, on the other hand, will remain in solution at much higher pH values than the inorganic form (Brady, 1974; Linder, 1977). Therefore, complex formation is an important mechanism for maintaining the flow of iron to drains even where pH is high in filamentous ochre sites.

Iron can be held in solution by organic substances such as tannins, phenolics and humic acids (Theis and Singer, 1974; Aristorskaya, 1974).

Ford and Tucker (1974) noted that these compounds are present in drainage water where ochre is found.

#### 2.5.2.4 The action of microorganisms causing environmental changes

It is known that microorganisms can alter their immediate environment (Starkey and Halvorson, 1927; Brock, 1966). Such effects include changes in pH, redox potential, temperature, and changes in the concentration of gases such as oxygen and carbon dioxide. Many of these changes could cause the precipitation of iron from solution. Since iron is precipitated indirectly by these organisms they do not apparently benefit from its oxidation.

## 2.6 POSSIBLE SOLUTIONS TO THE IRON OCHRE PROBLEM

### 2.6.1 Prediction

Techniques for predicting the deposition of iron ochre would be valuable since the drainage of high risk sites could be avoided. The major problem in the development of a reliable method is that many factors, especially biological factors, can only be assumed and not evaluated before drainage (Ford, 1978).

Visual examination of a site and adjacent areas before drainage can be useful. Areas susceptible to ochre are likely to have an orange colouration, staining or deposits in old field drains and oily sheens on water in ditches and exposed soil (Alcock, 1973; Ford, 1969; Ivarson and Sojak, 1978; Thorburn and Trafford, 1976). Some of the features indicative of acid sulphate soils (Sulphaqpts), that produce pyritic ochre when drained, have been discussed by Bloomfield and Coulter (1973).

Several workers have attempted to use an estimation of total soil iron content as a method of prediction but this technique has limitations. Firstly, iron is not uniformly distributed in soil (Thorburn and Trafford, 1976; FDEU, 1977). Kuntze (1982) found that the mean soilwater Fe(II) content in a 3 ha site varied from 0.3 µg/ml to 23 µg/ml over 9 years. Secondly, factors other than soil iron content can affect ochre deposition, not least of which is the flow into drains of iron containing water from a source external to the drainage site. For this reason soil iron content can only predict the initial development of ochre. At sites where iron flows from an external source the duration and severity of ochre cannot be estimated (Spencer et al, 1963; Kuntze, 1982).

Linder (1977) reviewed the use of this technique in the East European countries and found differences of opinion concerning the concentration of iron at which ochre formation is likely. Linder found that the threshold

values (the maximum ferrous iron concentration at which it is safe to drain land) given by different authors varied widely. This is illustrated in table 2.3.

TABLE 2.3 VALUES OF Fe(II) CONCENTRATION FOR ESTIMATING THE RISK OF OCHRE FORMATION (From Linder, 1977)

<u>Author</u>	<u>Risk of ochre formation</u>		
	<u>Little</u>	<u>Moderate</u>	<u>Great</u>
		Fe (II) mg/l	
Chruckaja (1966)	10	10 - 20	20
Autorenkoll (1971)	1.6 - 3.1	3.2 - 6.3	6.3

Linder (1977) also found differences of opinion concerning when to take soil samples for iron determination.

Meek et al (1968) measured redox potential (Eh) to predict where ochre might develop. They concluded that such measurements could estimate the dissolution of iron and manganese in the soil solution and hence the possible levels of these elements entering drain lines.

Another method of prediction was advocated by Ivarson and Sojak (1980) for areas with a high risk of ochre formation. They recommended the installation of a few trial drains, and waiting for up to two years to gauge the extent and severity of the problem.

Finally, Ford and Tucker (1974) described simple field and laboratory tests for estimating ochre potential. These tests were based on the determination of several factors including iron, tannins, and pH. The value of this approach is that it conforms with the concept of ochre formation being related to many factors.

## 2.6.2 Prevention

### 2.6.2.1 Self-cleaning grades

It has been suggested that ochre deposition can be reduced by self-cleaning grades and large water entry holes in drainage pipes (Kuntze, 1982; Ford, 1969; Ford, 1973). In general ochre will form in pipes with grades up to 0.35%. At 0.4%, and above, clogging will decrease. However self cleaning grades are unlikely to reduce ochre formation substantially.

### 2.6.2.2 Special drainage systems

In the U.K. ochre has been dealt with in practical drainage work by adopting special drainage designs for less severe cases, but mainly by avoiding high risk sites. A simple layout, each drain having an independent outfall to the ditch possibly below the normal water table, would be typical (Trafford et al, 1973; Trafford, 1978). These systems do not prevent ochre but simply aid its detection and removal. In other countries drainage systems have been used in an attempt to prevent ochre.

One method that has been advocated is pre-drainage (Glathe and Ottow, 1972; Puustjarvi and Juusela, 1952). In this system land is drained by open ditches for about twelve months. At the end of this time iron deposits are removed from the ditches before installing subsurface drains. The period of twelve months is likely to be too short in soils containing pyrite which can take many years to oxidize completely (Trafford et al, 1973). Such a system would also be very costly in terms of labour, machinery, and reduced production from the temporarily drained land.



### 2.6.2.3 Aerobic and anaerobic conditions

Several authors have tried to control ochre by maintaining aerobic or anaerobic conditions in the soil or drainage system.

In theory the deposition of iron in the soil, before it can enter the drainage system, can be achieved by keeping the soil aerobic (Ford and Beville, 1970; Grass et al, 1973 (II)). The major problem associated with this method is controlling the soil water table effectively. Prolonged anaerobic conditions in the soil would reduce the deposited iron releasing it into the soil solution (Thorburn and Trafford, 1976). In soils containing pyritic material aeration achieved, for example, by subsoiling may result in extreme acidity. The low soil pH would prevent normal agricultural activity for many years.

As an alternative to maintaining soil aeration some authors have considered the prevention of ochre by keeping drains waterlogged and anaerobic (Bloomfield, 1972A); Wesseling, 1964). The use of "air traps" to prevent ochre was suggested by Mangon in 1856. In theory, when the entry of air is inhibited oxygen concentrations in the drain fall and ferrous iron oxidation is prevented. Thorburn and Trafford (1976) argued that this idea ignored the fact that the oxygen concentration in flowing water is high and anaerobic conditions would seldom be achieved except under static conditions during the summer months. Practical results on the use of this method are conflicting. Petersen (1966) in a survey of the ochre problem, concluded that drain submergence was the only effective control method. Seppala (1958) also presented results of experiments in Finland where drain traps had been used to prevent ochre. On the other hand, Ford (1969) found that drains submerged for seven years contained no ochre but iron sulphide deposits around the drains drastically reduced their efficiency. Similarly Kaptein and Zwan (from Petersen, 1966) could not prevent ochre formation

by keeping drain outlets covered with water.

It is difficult in practice to employ this system except where the land is flat and the water table easily controlled (Petersen, 1966; Puustjarvi and Juusela, 1952).

#### 2.6.2.4 Lime

At filamentous ochre sites lime could be used to encourage the precipitation of iron in the soil. However, Kuntze (1982) noted that if pH was allowed to fall then iron could migrate into the drainage system. Additionally the large quantities of lime required could cause trace element deficiencies. Limestone chips around drains have been used to raise the local pH encouraging ochre formation outside the drain (Thorburn and Trafford, 1976; Puustjarvi and Juusela, 1952; Ford, 1969). Generally this has been unsuccessful and could cause clogging of pores outside the drain (Ford, 1973; Kuntze, 1982).

It has been suggested that regular applications of lime can reduce the rate of pyrite oxidation and hence extend the life of a drainage system affected by pyritic ochre (FDEU, 1972; Bloomfield and Coulter, 1973). Trafford et al (1973) found that liming did decrease the rate at which pyrite was oxidized, thus reducing the amount of iron entering the drainage system. With a total drainage of about 1,000 mm over twelve months treating the soil with 42,000 kg of  $\text{CaCO}_3$ /ha decreased the amount of iron leached from the soil to about 20% of the amount leached from an unlimed control.

#### 2.6.2.5 Filters

Filters normally used in drainage schemes for preventing the deposition of silt or to intercept and transport soil water rarely prevent ochre formation. They can, in fact, aggravate the situation by causing ochre to form outside the pipe where it is inaccessible to cleaning operations (Petersen, 1966; Thorburn and Trafford, 1976; Talman, 1978).

The filters discussed in this section are designed to prevent ochre formation within or outside the pipe. Alkaline filters, used to cause ochre deposition outside the pipe, were discussed in section 2.6.2.4.

Organic filters such as sawdust or tannin rich filters have some bacteriocidal effect or are capable of complexing ferrous iron preventing oxidation and deposition in the drainline. Regamey and Jatou (1976) prevented ochre formation by using a woodchip filter system. Similarly, Ford (1969) found that tile lines laid in sawdust contained less ochre than tiles with fiberglass filters. Other authors have not recorded a reduction of ochre when sawdust was used as a drain filter (Spencer et al, 1963; Scheffer and Kuntze, 1979). Scheffer and Kuntze (1979) were able to control ochre deposition for two to three years using tannin rich filters. Care must be taken to avoid the toxic effects of high tannin extract concentrations in watercourses (Kuntze, 1982).

Inorganic filters include silica gravel, river gravels, sulphur based compounds and copper slag. Thorburn and Trafford (1976) described the use of sulphur based filters arguing that if the drain was surrounded by sulphur or pyrites the local pH would fall so low that ferric compounds would remain soluble preventing ochre formation. The bulk of soil could be maintained at pH values suitable for normal agriculture. The formation of iron compounds such as Jarosite at very low pH values and pollution difficulties arising from the formation of sulphuric acid would limit the use of this method.

Petersen (1966) reported work in which gas purifying material was used to cover drains. Gas purifying material is a waste product from gas works, the major constituents of which are iron and sulphur compounds. This was used on account of its assumed bacteriocidal properties. In the initial experiments some positive action was recorded but when the tests were repeated the results were not confirmed.

Since it is easier to remove ochre from inside drains than from the surrounding soil and backfill the use of filters to encourage precipitation within the drain has been advocated (Ford and Beville, 1970; Ford, 1973). It was found that silica gravel and river gravel could reduce ochre deposition around the pipe but these materials could not be recommended since they gradually became cemented with iron deposits and accumulated iron sulphide.

#### 2.6.2.6 Bacteriocides

Bacteriocides have been included in this section on "prevention" but it should be noted that they could also be used to control ochre in systems already affected.

The use of bacteriocides to control iron and manganese oxidising bacteria is well known. For example, Gleen (1950) used sodium azide, iodoacetic acid and mercuric chloride to reduce iron oxidation in a soil column through which ferrous iron was passed. Similarly, Douka (1977) found that the activity of cell-free extracts of two manganese oxidizing bacteria was inhibited by mercury chloride ( $\text{HgCl}_2$ ).

The inhibitory effect of several compounds on the organisms associated with filamentous ochre has also been demonstrated. Lackey and Wattie (1940) used various inhibitors to control the growth of Sphaerotilus natans in activated sludge. Chlorine was the cheapest bacteriocide available when the article was written but silver nitrate, chloroform,

phenol, acetic acid, and several dyes were also effective. Chang et al (1979) studied the effects of iron compounds on S.natans. Inhibition by iron citrate (20 mg/l as Fe), iron cysteine (5 mg/l as Fe) and ferrous sulphate (10 mg/l as Fe) was observed at pH 6.0. Takiguchi et al (1980) isolated compounds that specifically inhibited Sphaerotilus at low concentrations. These included peptide antibiotics, Anslimins A and B, that inhibited Sphaerotilus at 0.78 and 0.39 µg/ml respectively (Takiguchi et al, 1978; Yoshikawa et al, 1979). However, very little work has been carried out on the use of bacteriocides in ochre control. Ford (1978, 1979) was able to inhibit the formation of ochre in experimental models by using Acrolein and Sodium hypochlorite. Other reports suggest that the latter is not practical since the concentrations required would pose a pollution hazard (Thorburn and Trafford, 1976).

Although the Thiobacilli, responsible for the formation of pyritic ochre, are susceptible to bacteriocides the concentration required for their control in field drains would not be acceptable. Le Roux et al (1973) found that 100 mg/l mercuric chloride stopped pyrite oxidation by T.ferrooxidans. This concentration can be compared to the 4.1 mg/l of mercuric chloride required to kill 50% of the population of a freshwater fish in 24 hours (Hanumante and Kulkarni, 1979). Similarly, McIntyre (1978) showed that 0.1 to 10.0 µg/l of mercury (as aqueous mercuric chloride) affected all levels of an experimental marine food chain from photoplankton to fish.

### Copper

Copper is a bacteriocide that has been investigated widely in relation to ochre and thus deserves particular attention.

The effects of copper on living systems is well known. Copper sulphate was one of the first selective weedkillers used in European agriculture, early this century. Similarly, the "Bordeaux mixture" was a copper based

fungicide used to prevent potato blight. Copper has also been advocated for controlling foot rot in sheep and liver fluke in grazing stock. Iron bacteria are not exempt from the effects of copper. Lueschow & Mackenthun (1962) recorded the use of copper sulphate for controlling iron bacteria in drainage ditches and Waitz and Lackey (1959) found that S. natans was completely inhibited by 1 µg/ml of copper sulphate.

Because of copper's inhibitory effects it has been used as an ochre preventative. However, the practical results are conflicting. Some authors have found that copper prevents ochre formation. In a laboratory experiment with filamentous ochre Bloomfield (1972A) found that a piece of copper wire delayed the onset of iron precipitation in an aqueous extract of dried lucerne by about a week. Similarly, Langish (from Puustjarvi and Juusela, 1952) described an experiment where an 80 mm copper lining was placed in the joints of drain pipes. Water leaving the pipes contained copper but no Leptothrix bacteria. In the absence of copper, L. ochracea was found in the drainage water. Ford (1969) was able to minimize ochre formation for about six weeks using copper; however, the treatment did not improve the flow of water out of the drain lines.

Other authors have not found copper to be an effective control measure. Petersen (1966) noted field experiments by Jensen and Jackobsen where copper was applied in several treatments. All tests showed negative results despite promising laboratory experiments. Puustjarvi and Juusela (1952) conducted laboratory and field experiments on the use of copper and found no positive benefits.

This inconsistency in the reported effects of copper on ochre development may have arisen, at least in part, from a failure to distinguish between pyritic and filamentous ochre (Bloomfield, 1972 Bloomfield and Coulter, 1973). For example, Bloomfield (1967) was unable to prevent the formation

of pyritic ochre with copper whilst the deposition of filamentous ochre was inhibited for several days by a piece of copper wire. This was explained by the well known tolerance of T.ferrooxidans to copper and the susceptibility of filamentous bacteria such as S.natans (Thorburn and Trafford, 1976; Bloomfield and Coulter, 1973; Waitz and Lackey, 1959). Kuntz (1982) noted that copper might have only a limited effect on the whole range of organisms involved in ochre formation and believed that resistant forms and strains could develop in ecological niches.

### 2.6.3 Cure

#### 2.6.3.1 Mechanical methods

Mechanical methods for removing ochre include rodding and flushing.

##### Rodding

One of the "miscellaneous implements" exhibited at Derby in 1906 by Messrs Jordan Brothers was a "ferret drain cleaning apparatus". This consisted of a helix of steel wire, stiff enough to push through obstructions but flexible enough to pass around bends in the drainage pipe (Hippisley, 1906). This is probably one of the forerunners of today's drain rodding equipment. A variety of probes, scrappers and brushes can be used to break up and clean out all but the hardest deposits. Normally only straight, unbranched, drains with individual accessible outfalls can be treated.

Rodding was advocated as a cure for ochre by Mitchell in 1894. It is best carried out when there is sufficient drainflow to remove the loosened deposit (Thorburn and Trafford, 1976). This method is of use when internal blocking of drains by ochre is encountered. Ochre blocking drain slots, adjacent soil and backfill cannot be removed by rodding (Streutker, 1977; Mackenzie, 1962; Trafford et al, 1973). Streutker (1977) found that rodding of drains containing iron and manganese deposits, with roots associated, was only possible when the roots were young and the deposits fresh.

##### Flushing

Flushing was known as a remedy against ochre in the last century (Denton, 1883; Denison, 1850). The drains were flushed with fresh water applied from a shaft built at the head of each drain. Today, the same effect can be achieved by flushing the drains with water applied through purpose built hoses working at medium (20-30 atm pressure) or high water pressures



(80-120 atm pressure) (Raadsma, 1974). The loosened deposits washed back to the drain access opening are removed by a dewatering type pump and discharged onto the ground surface.

High pressure jetting can clean deposits inside and immediately outside the drainage pipe but can also damage the gravel envelope or soil structure around the drain (Raadsma, 1974; Ford, 1974; Thorburn and Trafford, 1976; Grass and Willardson, 1974; Grass et al, 1976).

The benefits of jetting have been demonstrated but it seems that drains over twelve months old with extensive deposits outside the pipe show only a temporary improvement in water flow as a result of jetting (Ford, 1974; Haiijer and Wolf, 1965).

#### 2.6.3.2 Chemical treatment

The most commonly used chemical for removing ochre is sulphur dioxide (Mackenzie, 1962; Haiijer and Wolf, 1965; Grass and Mackenzie, 1972; Ford and Tucker, 1975; Grass, 1969). The action of this chemical is probably threefold (Thorburn and Trafford, 1976; Dennis and Wickens, 1977; FDEU, 1975):

1. Chemical reduction of iron (III) oxide resulting in the solution of ferrous salts
2. Stabilisation of ferrous salts by a lowered pH, and if the pH is low enough, solution of iron oxides as ferric salts.
3. Bacteriocidal effects on filamentous bacteria.

Most workers have used a 2% solution of SO<sub>2</sub> gas and water introduced into the drain line via an inlet pipe (Haiijer and Wolf, 1965; Ford, 1977; Grass et al, 1976; Grass and Mackenzie, 1972). The outlet and inlet to the drainline are kept closed for over 24 hours after introducing the gas. When the stops are taken out the dissolved compounds are removed by

flushing. Provided the effluents are diluted sufficiently pollution of ditches and water courses should not occur (Aldrich, 1977). Despite the apparent success of sulphur dioxide the FDEU have not obtained comparable results. Treatment with  $SO_2$  had a marginal effect but did not restore drainage efficiency significantly. The FDEU (1975) did not see the use of  $SO_2$  to be of wide applicability on cost or safety grounds.

### 3. SAMPLING AND CHEMICAL ANALYSIS OF OCHRE SAMPLES

#### 3.1 SAMPLING

Ochre samples were collected in plastic bottles. These were washed by soaking in dilute hydrochloric acid, rinsing several times with tap water and finally distilled water. These containers were semi-sterilized by the inclusion of methylated spirits for at least 12 hours.

On site the bottles were rinsed several times in drainage water to remove traces of alcohol before collecting the sample.

In other sections the sites are referred to by the names in uppercase letters.

##### 3.1.1 Bryn Gwyn Farm, PENYGROES, Dyfed

O.S. REF. SN585146.

This farm was located at a reclaimed open cast coal site. Ochre had been reported in springs and land drains at the site (Stewart, V.I; U.C.W. Aberystwyth, personal communication). Upon examination of the area, ochre was found in three drain lines but the deposits were posing a serious problem in only one of these. Other drains in the same fields as those containing ochre showed no evidence of ochre deposition at the outlet.

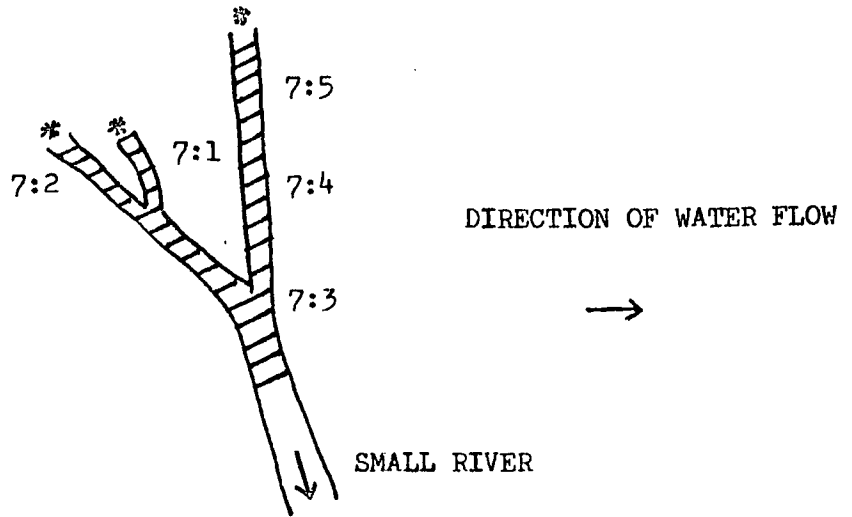
Some springs were identified by the presence of small amounts of ochre and red stains on the soil surface but there was insufficient material for sampling.


Ochre was also found in abundance in several surface water streams.

Sites 1 and 3 : Ochre was collected from drain outlets. The ochre formed a soft but tenacious mass (Plates 3.1 and 3.2).

Site 5 : Deposits were found at the mouth of a stream that ran into a small river from a "hole" in the riverbank (Plate 3.3).

FIGURE 3.1 : DIAGRAM SHOWING THE SUBDIVISION OF PENYGROES SITE 7



 AREA OF OCHRE DEPOSITION

\* WATER FLOWED INTO THESE STREAMS FROM OPEN DITCHES  
FED BY PIPE DRAINAGE SYSTEMS.

Site 6 : A small ditch below a drain outfall. Ochre was not found in the outfall itself.

Site 7 : At this site ochre was collected from a number of small streams. Site 7 is subdivided as shown in figure 3.1.

### 3.1.2 CAE COCH MINE, Trefriw, Gwynedd

O.S. REF. SH774654

In this disused pyrite mine acidic streams have resulted from the oxidation of pyrite by Thiobacillus ferrooxidans. Large "acid streamers" have been formed by heterotrophic bacteria in these streams. The chemistry and microbiology of the mine has been described in detail by Johnson (1979), Johnson et al (1979), and Johnson and Kelso (1980).

### 3.1.3 Newtowncunningham, Co.DONEGAL, Republic of Ireland

Ochre was collected from the outlets of plastic drains installed four years previously. This grassland site was 10m above sea level. The soil was a peat layer of variable depth (1m to 4m) over boulder clay derived from Silurian Shale. Where the shale was exposed ochreous mottling was evident.

### 3.1.4 LLYN COEDTY, Nr Dolgarrog, Gwynedd

O.S. REF. SH 756667

Llyn Coedty is a reservoir at 300m O.D. Ground water below the dam wall flows into a concrete ditch (Site 1, plate 3.4) which conveys the water to a small river. About 4m from the upper end of the ditch a 'V' notch flow meter has been installed. This allows ochre to accumulate in a shallow pool above the 'V' notch (Site 2, plate 3.5). Ochre deposits form at such a fast rate that the ditch has<sup>to</sup>/be cleaned with brushes at least once a week.

The concrete ditch is linked to the river by a culvert where a large amount of ochre has been deposited. Pollution of the river by ferric deposits was evident on several occasions (plates 3.6 and 3.7).

Ochre was also seen in small rock pools on the river bank especially where the water from the culvert flowed into the river (Site 3). In these pools large, readily disrupted, flocs of ochre were seen (plate 3.8).

### 3.1.5 LLYN COWLYD, Nr Trefriw, Gwynedd

O.S. REF. SH737634

This reservoir is a natural lake at 380m O.D. Ochre was found in open ditches carrying surface and ground water to a small river. The open ditches were found below the dam wall. At the upper end of the ditch water bubbled to the surface at a constant rate. An odour characteristic of hydrogen sulphide was often evident at this site indicating that the emerging water had passed through an anaerobic zone, probably at depth. Site 1, plate 3.9). The ditch contained a large amount of ochre, especially in the first 9m where the deposits were composed of dark-orange, gelatinous flocs (plate 3.10). Large fragments would stay intact and float downstream when the ochre was disturbed. Site 2 was at a 'V' notch some 9m from the head of the ditch.

The open ditch ended in a culvert which conveyed water to a small pool (Site 4, plate 3.11) at the head of the river. Water also flowed into this pool from a subsurface drain. The drain which contained much ochre, was essentially a stone ditch interspersed with concrete pipes. This channel carried water from a point at the base of the dam wall, where ochre was always evident, and from the neighbouring areas.

### 3.1.6 Nr. MACHYNLLETH, Powys

O.S. REF. SH735004

This site was found below a recently-built road. Water drained from under the road, through gravel channels, and was conveyed to a stream via open ditches. Both the ditches and gravel were subject to ochre deposition. At the lower end of the ditches the ochre was gradually replaced by gelatinous deposits of algae and aquatic plants which were abundant in the stream (plate 3.12).

Site 1 : Pieces of gravel were collected. These were coated with ferric deposits but insufficient material was available to examine the deposits in detail. (plate 3.13).

Site 2 : Thick ochre deposits immediately below the gravel. (plate 3.14).

Site 3 : Some 5m from the upper end of the ditch. (plate 3.15).

Site 4 : This was a site near the stream where the ochre was yellow and very gelatinous.

### 3.1.7 Norfolk

Several sites were visited in Norfolk in the autumn of 1980. These sites were predominantly of fen peat in arable production.

#### BECCLES MARSH, Buxton, S.Aylsham

O.S. REF.

Site 1 : White gelatinous material was collected from a drain outfall. The pH of this deposit as measured in the laboratory was 6.5. Chemical analysis was not carried out since insufficient material was available after drying the sample. (plate 3.16).

Site 2 : Ochre from a drain outfall.

Site 3 : "Crusty" deposits of ochre in an open ditch.

BURNLEY HALL, West Somerton

O.S. REF TG478199

Samples were collected from drain outfalls and odour of hydrogen sulphide was evident in places. In addition to ochre, black stains of presumably iron sulphide were seen in many outfalls.

A white deposit, as described for Beccles Marsh site 1, was seen in several drains. Some of this deposit was collected.

FIRBECK, Meeting Hill, North Walsham

O.S. REF. TG305283

Site 1 : This large open ditch (plate 3.17) has been in operation for about 25 years and had been cleaned in 1979. At the time of sampling a deep deposit of ochre had formed in the ditch and this contained many leaves and debris of aquatic plants. Deposits of black iron sulphide were found beneath the ochre.

Site 2 : Ochre was collected from a drain outfall in the bank of the open ditch described above.

HALL FARM, Upton

O.S. REF. TG402119

Sites 1 to 3 were all drain outfalls in the same field. At site 2 algae were evident in the ochre deposit.

Sites 4 to 6 were also drain outfalls. This site had been drained in 1978 and ochre had blocked the system almost immediately. The area was redrained about 12 months later.

PARK FARM, Wormegay

O.S. REF. TF673125

Ochre samples were taken from 8 sites all of which were drain outfalls from several areas of the farm.



PERSEHALL Manor Farm, Bunwell

O.S. REF. TM137915

All ochre collected at this farm came from drain outlets.

3.1.8 OCHR CEFN ISAF Farm, Nr. Ysbyty Ifan, Gwynedd

O.S. REF. SH848502

This is a marginal farm at about 900m O.D. No drainage system on the farm had suffered from ochre at the time of sampling. Ochre deposits were found near an unsurfaced road below a rush-infested, waterlogged area. Ochre was evident at several points along the road and an oily sheen could be seen on water running from the area. (plate 3.18).

3.1.9 PENTRE MAWR, Pontyberem, Dyfed

O.S. REF. SN492104

Pentre Mawr is a disused coal mine where extraction ceased about 1965. Access to the coal seams was achieved by drifts. Ochre deposits were found on the walls of one of the drifts and these were associated with a slow, but continuous, flow of water percolating from the ground above.

In some parts of the drift small, vertical, red rods were seen on the roof. A continuous flow of water was also associated with these formations.

The surface of the ochre deposits had a crusty texture and was bright orange (plate 3.19). Sometimes a very hard deposit formed, having a blood red colouration (plate 3.20). Below these surface layers the ochre was darker in colour.

3.1.10 PONTHENRI, Dyfed

O.S. REF. SN491097

Ochre was found on the soil surface in a field used for rough grazing. The field was wet with many areas dominated by rushes. Ochre had deposited in part of the field as a red coating about 1cm deep overlying

black material. An odour of hydrogen sulphide was evident when the deposit was disturbed.

#### 3.1.11 Nr. Porthmadog, Gwynedd

Two sites were sampled; both were at sea level and were used for grazing. The brown earth soils were derived from marine or river alluvium.

#### GALLT-YR-HULLDREM

O.S. REF. SH612432

Samples were taken from the outfalls of a drainage system that had been operating for over ten years. One drain was completely blocked by ochre at the outfall. When the deposit was removed water ran freely from the drain.

#### MORFA GLAS

O.S. REF. SH575403

Ochre was collected from inside a clay tile and a pit was dug to gain access to the drain line. Ochre had formed in the joints between the tiles and in the gravel backfill.

#### 3.1.12 SPANKER, Nether Heage, Derbyshire

O.S. REF. SK363505

This reclaimed open cast site had been investigated by Johnson (1979), who found that ochre deposits in the area were pyritic.

Site 1 : Ochre from an open ditch

Site 2 : Ochre deposits forming on the soil surface over an extensive area (plate 3.21).

Site 3 : An open ditch close to site 2. Dead earthworms were found in this ditch suggesting an acidic environment (plate 3.22).

Site 4 : Above the open ditch (site 3) a wet, raised mound of ochreous material was discovered. The mound was partially covered with grasses.

### 3.1.13 SYCHNANT PASS, Nr. Conwy, Gwynedd

O.S. REF. SH764772

Ochre deposits formed in a roadside ditch. The ditch ran between the road and a wet, peaty area. The ditch was blocked for several metres by aquatic weeds, mainly rushes. This allowed the ochre deposit to form and accumulate within the blocked area. Following heavy rainfall water overflowed onto the road and thin layers of ochre developed on the road surface. On drying these left a red stain (plate 3.23 and 3.24).

In the early summer ochre usually spread down the ditch for about 10 metres below the accumulation of rushes. In these parts of the ditch, where the flow of water was strong, the ochre was a bright orange/yellow colour and very gelatinous. This can be compared to the small red flocs associated with the main accumulation of ochre in the blocked area.

### 3.1.14 TAL Y BONT Isaf Farm, Tal y Bont, Nr. Bangor, Gwynedd

O.S. REF. SM603704

Ochre had developed on the soil surface over a 4 - 6 square metre area. This site lay across a watercourse where inadequate drainage caused extensive waterlogging.

### 3.1.15 TUMBLE Coal Mine, Tumble Dyfed

O.S. Ref. SN516123

At this disused coal mine water accumulating in the old workings is pumped to the surface to prevent flooding in adjacent worked coal seams.

Ochre accumulated as a red sludge about 300 - 400 metres below ground level. Despite a very fast flow rate (approximately 1,500 l/minute) ochre severely reduced the effective diameter of the 150 mm diameter steel pipes used for pumping water.

## 3.2 CHEMICAL ANALYSIS

### 3.2.1 METHODS

Before chemical analysis ochre samples were dried in an oven at 105°C. Due to the high water content of the samples it was found that air drying was very slow and thus the higher temperatures were adopted. Dried samples were ground with a pestle and mortar and stored in plastic bags. Grinding reduced the ochre to a fine powder and sieving was not necessary.

#### 3.2.1.1 pH

pH was determined using a 7020 pH meter (Electronic Instruments Limited) buffered at pH 4.0 and 9.2. The pH of undried samples was measured after thorough shaking of the sample in its collecting vessel. This was carried out as soon as possible after sampling.

To determine the pH of dried samples, ochre and distilled water were mixed in the ratio 1g ochre to 2.5 ml water. The mixture was left to stand, with intermittent stirring, for one hour.

#### 3.2.1.2 Loss on ignition

A known weight of ochre (approximately 2g) was heated in a muffle furnace at 200°C for 2 hours. The temperature was then raised to 500°C for  $3\frac{1}{2}$  hours. The percentage loss on ignition was calculated for the oven dried ochre.

#### 3.2.1.3 Total iron and manganese

Total iron and manganese contents were determined for ignited samples of ochre. Ignited ochre was used since insufficient material was available to use oven dried ochre for all the chemical analyses. Large volumes of some samples were reduced to a few grammes after oven drying.

The methods outlined below were investigated.

a. Low temperature extraction with hydrochloric acid

0.2 g ignited ochre was extracted in 3 ml concentrated hydrochloric acid for 18 hours (overnight). 0.5 ml of extract was diluted to 50 ml with distilled water and 2 ml of this solution diluted further to 100 ml.

b. Extraction with hydrochloric acid at 95°C

0.1 g ignited ochre and 3 ml concentrated hydrochloric acid were heated for 3 hours at 95°C in a dry block heater. A marble was placed on top of each extraction tube. After extraction the volume was made up to about 10 ml with distilled water, thoroughly shaken, transferred into a 25 ml volumetric flask and made up to volume. After all the ochre fragments had settled to the bottom of the flask 1 ml was withdrawn and made up to 250 ml with distilled water.

c. High temperature extraction with perchloric acid

0.2g ignited ochre was extracted in 2 ml perchloric acid at 200°C for 4 hours in a dry block heater. A small marble was placed on top of each extraction tube to prevent loss of extractant by evaporation. 0.5 ml of extract was diluted to 50 ml and 2 ml of this solution diluted further to 100 ml with distilled water.

In all the methods total iron in the final dilutions was determined using a Pye Unicam SP2900 atomic absorption spectrophotometer. The results were compared to those obtained for standard ferric nitrate solutions in the range 0 to 10 µg/ml.

The efficiency of the three methods was investigated by determining total iron content of ochre from three sites. 3 replicates per site were used. The mean results are given in Table 3.1.

Table 3.1 : TOTAL IRON CONTENT OF IGNITED OCHRE SAMPLES USING

3 EXTRACTION METHODS

% Fe in ignited ochre (mean of 3 replicates)

<u>METHOD</u>	<u>SPANKER 1</u>	<u>BECCLLES MARSH 3</u>	<u>PERSEHALL 4</u>	<u>METHOD MEAN</u>
HYDROCHLORIC ACID (OVERNIGHT)	34.26 (18.90)	24.24 (11.77)	40.55 (10.14)	33.02
HYDROCHLORIC ACID (95°C)	48.81 (0.49)	32.48 (4.28)	47.01 (0.28)	42.77
PERCHLORIC ACID	49.15 (2.97)	22.05 (1.71)	52.82 (0.98)	41.34

(THE STANDARD DEVIATION FOR EACH TREATMENT IS GIVEN IN PARENTHESES)

The mean iron content determined <sup>in</sup> by the perchloric digest, the overnight extraction, and hydrochloric acid at 95°C were 41.34%, 33.02% and 42.77% respectively. Statistical analysis, using analysis of variance and Tukey's test for the comparison of means (Honestly Significant Difference at  $p = 0.05 = 23.96$ ) showed that there was no significant difference between the extraction methods for ochre samples from any of the three sites. Comparison of the means shows that the overnight extraction with hydrochloric acid did produce values indicating an iron content about 9% lower than those obtained by the other methods. Additionally, standard deviations obtained for the overnight extraction results were much greater showing that with this method it was more difficult to obtain consistent results. The standard deviations for the perchloric and hydrochloric at 95°C extractions were less than 3 and 5 respectively. The standard deviations for the overnight extraction exceeded 10 for all the sites.

The method adopted for analysing further ochre samples was extraction with concentrated hydrochloric acid at 95°C. This gave more consistent results than the overnight extraction and was more convenient to use than the high temperature perchloric digestions.

An experiment was conducted to determine whether or not the results obtained by extracting iron from ignited ochre differed substantially from those using oven dried ochre. 3 replicate samples of oven dried ochre from Spanker 1, Beccles Marsh 3 and Persehall 4 were digested in hydrochloric acid at 95°C as described earlier. The mean results were compared to those obtained for the ignited samples from the same sites. The results for percentage iron in ignited ochre were adjusted to the percentage in oven dried ochre by taking into account the loss on ignition result for the sample (Table 3.3). The results are given in Table 3.2.

Overall there was no significant difference between the two methods. However for the sample from Beccles Marsh 3 the result obtained using ignited ochre (27.32%) was significantly higher than that obtained using oven dried ochre (21.04%). It is possible, therefore, that a longer extraction period is required to remove all the iron from samples of oven dried ochre.

Table 3.2 : TOTAL IRON CONTENT OF OCHRE SAMPLES USING IGNITED AND OVEN DRIED SAMPLES

	<u>SPANKER 1</u>	<u>BECCLLES MARSH 3</u>	<u>PERSEHALL 4</u>	<u>MEAN</u>
OVEN DRIED	44.75 (0.38)	21.04 (0.97)	35.50 (1.38)	33.76
IGNITED SAMPLE*	41.19 (0.42)	27.32 (3.60)	35.12 (0.21)	34.54

(Standard deviation for each treatment is given in parentheses)

\* % Fe in oven dried ochre calculated by taking into account % LOI

HONESTLY SIGNIFICANT DIFFERENCE AT  $p = 0.05 = 4.50$  and AT  $p = 0.01 = 5.78$

#### 3.2.1.4 Total organic carbon

Total organic carbon was determined by Tinsley's method (Bremner and Jenkinson, 1960). To replicate samples of ochre (0.1 g to 0.25 g oven dried sample) were added 25 ml 0.4N potassium dichromate, 25 ml concentrated

sulphuric acid and 12.5 ml orthophosphoric acid (S.G. 1.75). This mixture was heated under reflux for 2 hours. After cooling 100 ml distilled water was added and 5 ml indicator solution (N-phenyl anthranilic acid, 0.2 g in 100 ml of 0.2%  $\text{Na}_2\text{CO}_3$ ).

Residual dichromate was determined by titrating against 0.2N ammonium ferrous sulphate (78.44 g/L including 20 ml conc  $\text{H}_2\text{SO}_4$ ) until the colour changed from dark purple to dark green. A blank containing all reagents but no ochre was included for each batch of samples.

Total organic carbon was calculated on the basis that 1 ml of 0.4N dichromate is consumed by 1.2 mg carbon.

#### 3.2.1.5 Colour

The colour of oven dried and ignited samples was described by reference to standard Munsell colour charts.

#### 3.2.1.6 Absolute sugar levels (from Johnson, 1979, except for total sugars)

3.0 g of ochre and 4 ml 72% (w/v) sulphuric acid were mixed to a slurry in a 250 ml round-bottom flask. After two hours at room temperature 100 ml of distilled water was added. The flasks were heated under reflux for 16 hours. The final hydrolysate volume was estimated and 20 ml withdrawn for determining absolute sugar levels.

The 20 ml of hydrolysate was diluted to about 100 ml, neutralised with NaOH and filtered to remove iron hydroxide precipitates. The volume was reduced by evaporation under vacuum to 20 ml. Colorimetric methods were used to determine different classes of monosaccharides.

#### (a) Total sugars (Mongomery, 1961)

To 2 ml of solution was added 0.1 ml of 80% phenol. 5 ml of concentrated sulphuric acid was added slowly. The acid was directed onto the surface of the solution to aid mixing. The reagents were mixed thoroughly. Absorbance was read at 489 nm after 30 minutes at room temperature.



(b) Hexoses

A known volume of solution (containing 10-60  $\mu\text{g}$  hexose, usually 1 ml) was thoroughly mixed with anthrone reagent (0.2% anthrone in a 5:2 mixture of concentrated sulphuric acid and water) in an ice bath. The mixture was heated at  $100^{\circ}\text{C}$  for 10 minutes and then rechilled. Absorbance was read at 620 nm after 5 minutes. Glucose was used as a standard.

(c) Pentoses

Aniline reagent was prepared by mixing together 100 ml of glacial acetic acid, 10 ml 5% aqueous solution of oxalic acid dihydrate, 24 ml distilled water and 16 ml of freshly redistilled aniline. 6 ml of this reagent was added to 2 ml of sugar solution (containing 10-70  $\mu\text{g}$  pentose). The mixture was incubated at room temperature, in the dark, for 24 hours. Absorbance was measured at 480 nm using ribose as a standard.

(d) Methyl pentoses

1 ml of solution, containing 10-50  $\mu\text{g}$  methyl pentose, was mixed in an ice bath with 4.5 ml of a 6:1 solution of concentrated sulphuric acid and water. After heating at  $100^{\circ}\text{C}$  for 10 minutes the mixture was cooled for 5 minutes in an ice bath. 0.1 ml of thioglycollic acid solution (1.0 ml thioglycollic acid in 29 ml  $\text{H}_2\text{O}$ ) was added, and the solution incubated in the dark at room temperature for 3 hours. Absorbance was read at 400 nm and 430 nm. The difference between the two absorbances corresponded to the optical density attributable to methyl pentose. Rhamnose was used as a standard.

(e) Uronic acids

0.4 ml of solution, containing 2-20  $\mu\text{g}$  uronic acid, was mixed with 3.0 ml of sodium tetraborate solution (0.952 g in 100 ml concentrated sulphuric acid). The mixture was chilled in ice, heated at  $100^{\circ}\text{C}$  for 5 minutes, and then rechilled. 50  $\mu\text{L}$  of *m*-hydroxydiphenyl solution (0.15% in 0.5% aqueous sodium hydroxide) was added. After five minutes absorbance was read at 520 nm. Glucuronic acid was used as a standard.

#### (f) Hexosamines

1.0 ml of solution, containing 15-150  $\mu\text{g}$  hexosamine, was mixed with 1.0 ml of acetylacetone reagent (4% acetylacetone in 1.5N sodium carbonate). 1.0 ml of distilled water was added. The solution was then heated for 20 minutes at 100°C and cooled. The volume was made up to 10.0 ml with 95% ethanol and then 1.0 ml of Ehrlich reagent was added (2.66% p-dimethylaminobenzaldehyde in a 1:1 mixture of concentrated hydrochloric acid and 95% ethanol). Absorbance was read at 520 nm after 45 minutes. Glucosamine hydrochloride was used as a standard.

#### 3.2.1.7 Relative sugar levels (Johnson, 1979)

Ochre samples were hydrolysed in sulphuric acid as described in section 3.2.1.6.

50 ml of hydrolysate was neutralised with solid barium carbonate and aqueous barium hydroxide and reduced to dryness by rotoevaporation under vacuum. The samples were taken up in 0.1 ml distilled water and reduced to sugar alcohols by adding 0.1 ml 0.8M sodium borohydride. After two hours two drops of acetic acid were added to stop the reaction. The solution was evaporated to dryness under vacuum. Approximately 5 ml of methanol/acetic acid solution (200:1) was added and the mixture again evaporated to dryness under vacuum. The addition of methanol/acetic acid solution followed by drying was repeated three times. After drying overnight in a vacuum desiccator over phosphorus pentoxide the sugar alcohols were acetylated by heating with 0.3 ml acetic anhydride for two hours at 100°C in an oven. The sugar derivatives were washed three times by adding 2 ml of toluene and evaporating to dryness under vacuum. The alditol acetates were taken up in 2 ml chloroform. 2 ml of distilled water was added and the chloroform phase separated by centrifugation. This phase was evaporated and thoroughly dried in clean, dry roundbottom flasks.

Reference alditol acetates were prepared by the procedure outlined above for glucose, galactose, mannose, rhamnose, ribose, fucose, arabinose and xylose.

The sugar derivatives were separated by Gas Liquid Chromatography using a Pye Unicam series 104 gas chromatograph, using a flame ionisation detector.

The column used was composed of:-

Polyethylene glycol succinate	0.2%
Polyethylene glycol adipate	0.2%
GE <u>XE</u> 60	0.4%

The flow rates of nitrogen and hydrogen were both 40 cm/min. and a constant temperature of 170°C was employed.

The derivatives of reference sugars were taken up in a small volume of acetone, injected, and their retention times recorded. The hydrolysed ochre samples were treated in the same way and the monosaccharide components identified by reference to the retention times of the standards. The relative amounts of each sugar in the ochre hydrolysates was determined by comparing the areas of peaks.

### 3.2.2 RESULTS

#### 3.2.2.1 pH

Table 3.3, Figure 3.2

The pH of the undried ochre samples ranged from 3.4 (Spanker 4) to 7.8 (Persehall 2) with a mean of 6.0. Over 75% of the samples had a pH between 5.0 and 7.0 with 50% of the observations being between pH 6.0 and pH 7.0. 15% of the samples had a pH below 5.0 and the remaining 9% had a pH above 7.0.

The minimum pH of the oven dried samples was 2.2 (Spanker 4) and several samples had the maximum pH of 7.9. The mean pH for oven dried ochre was 6.2. The results recorded for oven dried samples showed a definite shift of values away from the pH 5-7 range. 39% of the samples had a pH between 5.0 and 7.0. This was almost half the percentage recorded for the undried samples. 41% of the oven dried ochre samples had a pH above 7.0 and 20% of the observations were below pH 5.0.

#### 3.2.2.2 Loss on ignition

Table 3.3, Figure 3.3

The mean loss on ignition percentage for the samples was 25% with values ranging from 10.6% (Persehall 3) to 52.6% (Hall Farm 46). The histogram in figure 3.3 shows that there was no definite trend in the frequency distribution of the values. In 59% of the samples the loss on ignition percentage was less than 25. 37% of samples had a loss on ignition between 25% and 50% and only two samples had a loss on ignition greater than 50%. No samples had less than 10% loss on ignition.

#### 3.2.2.3 Total iron and manganese

Table 3, Figure 3.4

The total iron content of oven dried samples ranged from 2% (Penygroes 5) to 50% (Spanker 4) with a mean of 27%. Half of the samples analysed had a

total iron content between 20% and 40%. 30% of the samples had less than 20% iron and 20% of the samples had a total iron content greater than 40%.

In over 75% of the samples no manganese was detected. In 20% of the observations some manganese was found but in concentrations less than 1%. Manganese contents greater than 1% were only found in 2 samples, namely Penygroes 3 (4% total manganese) and Pentre Mawr (10% total manganese).

#### 3.2.2.4 Total organic carbon

Table 3, Figure 3.5

The results showed an even distribution of organic carbon values within the range 0.8% (Tumble) to 13.2% (Donegal). The mean organic carbon content was 5.9%. Almost half the samples had an organic carbon content of between 2% and 6%. 13% of the samples had less than 2% organic carbon. The remaining values (39%) were above 6% organic carbon.

THE CHEMICAL COMPOSITION AND COLOUR OF OCHRE SAMPLES (ALL PERCENTAGES IN OVEN DRIED OCHRE)

TABLE 3.3	SITE	pH		OVEN DRY	%LOI	%Fe (2)	%Mn(2) (3)	% ORGANIC CARBON	MUNSELL COLOUR	
		WET(1)	OVEN DRY						OVEN DRY	IGNITED SAMPLE
	PENYGROES 1	5.5-5.8	6.9	18.0	17	-	0.8	REDDISH YELLOW 7.5YR 6/8	DARK RED 2.5YR 3/6	
	PENYGROES 3	5.4	6.8	21.7	6	4	3.2	YELLOWISH RED 5YR 5/8	DARK RED 2.5YR 3/6	
	PENYGROES 5	5.7-6.2	7.1	12.3	2	-	1.9	YELLOWISH RED 5YR 5/8	DARK REDDISH BROWN 5YR 3/3	
	PENYGROES 6	5.7	6.8	19.6	9	-	6.9	BROWN 7.5YR 5/4	DARK REDDISH BROWN 5YR 3/4	
	PENYGROES 7:1	5.5-6.2	7.1	16.5	7	-	3.4	YELLOWISH RED 5YR 5/8	REDDISH BROWN 2.5YR 4/4	
	LILYN COEDDY 2	5.8	4.9	31.5	31	-	5.9	RED 2.5YR 4/8	RED 2.5YR 4/8	
	LILYN COMILD 1	6.1	4.9	25.3	28	-	4.9	RED 2.5YR 4/8	RED 2.5YR 4/8	
	MACHYNLETH 2	6.7	5.1	11.2	12	-	3.0	STRONG BROWN 7.5YR 5/6	RED 2.5YR 4/6	
	MACHYNLETH 3	6.9	5.2	13.2	10	-	3.3	STRONG BROWN 7.5YR 5/6	RED 2.5YR 4/6	
	MACHYNLETH 4	7.2	5.1	13.3	12	<1	3.8	STRONG BROWN 7.5YR 5/6	RED 2.5YR 4/8	
	BECCLES MARSH 2	6.6	6.8	32.5	24	-	10.8	STRONG BROWN 7.5YR 5/6	RED 2.5YR 4/6	
	BECCLES MARSH 3	6.4	7.3	15.5	22	-	4.8	REDDISH YELLOW 7.5YR 6/8	RED 2.5YR 4/6	
	FIRBECK 1	7.0	7.2	19.3	34	<1	4.1	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6	
	FIRBECK 2	6.2	6.5	21.0	47	-	5.8	YELLOWISH RED 5YR 5/8	DARK RED 2.5YR 3/6	
	HALL FARM 1	6.5	7.6	21.2	41	-	6.0	REDDISH YELLOW 7.5YR 6/8	RED 2.5 YR 4/6	
	HALL FARM 2	6.6	7.5	50.3	13	<1	5.4	STRONG BROWN 7.5YR 5/6	RED 2.5 YR 4/6	
	HALL FARM 3	6.6	7.7	21.5	42	<1	6.2	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6	

TABLE 3.3 CONTINUED

SITE	pH		%LOI	%Fe (2)	%Mn(2) (3)	% ORGANIC CARBON(4)	MUNSELL COLOUR	
	WET(1)	OVEN DRY					OVEN DRY	IGNITED SAMPLE
HALL FARM 4a	6.6	7.9	20.6	40	<1	5.8	YELLOWISH RED 5YR 5/8	DARK RED 2.5YR 3/6
HALL FARM 4b	6.4	7.5	52.6	18	-	6.1	STRONG BROWN 7.5YR 5/8	DARK RED 2.5YR 3/6
HALL FARM 5	6.5	7.8	17.0	29	<1	5.1	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6
HALL FARM 6	6.6	7.7	26.6	38	-	8.3	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6
PARK FARM 1	5.8	6.3	28.2	29	-	8.6	REDDISH YELLOW 7.5YR 6/6	DARK RED 2.5YR 3/6
PARK FARM 2	5.6	5.6	38.4	29	-	*	YELLOWISH RED 5YR 5/8	DARK REDDISH BROWN 2.5YR 3/4
PARK FARM 3	5.4	6.3	37.2	30	-	12.4	YELLOWISH RED 5YR 5/6	DARK REDDISH BROWN 2.5YR 3/4
PARK FARM 4	6.2	6.6	38.2	28	-	*	YELLOWISH RED 5YR 5/6	DARK RED 2.5YR 3/6
PARK FARM 5	6.1	7.8	34.0	30	-	9.4	REDDISH YELLOW 7.5YR 6/6	RED 2.5YR 4/6
PARK FARM 6	6.2	6.4	36.9	27	-	12.7	STRONG BROWN 7.5YR 5/6	DARK REDDISH BROWN 2.5YR 3/4
PARK FARM 7	5.7	5.8	39.6	29	-	11.2	YELLOWISH RED 5YR 5/6	DARK REDDISH BROWN 2.5YR 3/4
PARK FARM 8	6.0	6.3	39.6	22	-	10.7	REDDISH YELLOW 7.5YR 6/6	RED 2.5YR 4/6
PERSEHALL 1	6.7	7.9	20.5	44	-	3.8	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6
PERSEHALL 2	7.6	7.9	19.0	22	-	4.7	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6
PERSEHALL 3	6.5	7.8	10.6	18	-	2.5	REDDISH YELLOW 7.5YR 6/8	DARK RED 2.5YR 3/6
PERSEHALL 4	6.5	7.9	24.0	37	-	6.2	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6
PERSEHALL 5	6.6	7.2	14.8	40	<1	3.6	STRONG BROWN 7.5YR 5/6	DARK RED 2.5YR 3/6

TABLE 3.3 CONTINUED

SITE	pH		%LOI	%Fe (2)	%Mn(2) (3)	% ORGANIC CARBON	MUNSELL COLOUR	
	WET(1)	OVEN DRY					OVEN DRY	IGNITED SAMPLE
PERSEHALL 6	6.5	7.5	27.7	36	-	3.9	RED 2.5YR 4/6	DARK REDDISH BROWN 2.5YR 2.5/4
PENYRE MAWR	7.0	7.8	12.2	30	1.0	4.7	YELLOWISH RED 5YR 4/6	RED 2.5YR 4/6
PONPHENRI	6.0	5.8	32.9	16	< 1	9.8	DARK REDDISH BROWN 5YR 3/4	DARK REDDISH BROWN 2.5YR 3/4
GALLT YR HULLDREM	4.6	4.5	33.6	9	-	11.4	BROWN/DARK BROWN 7.5YR 4/4	DARK REDDISH BROWN 2.5YR 3/4
MORFA GLAS	4.8	4.2	36.7	20	-	11.8	BROWN/DARK BROWN 7.5YR 4/4	RED 2.5YR 4/6
SPANKER 1	4.2	2.5	15.9	41	-	1.0	REDDISH YELLOW 7.5YR 6/6	DARK RED 2.5YR 3/6
SPANKER 2	3.5	2.4	21.2	33	-	3.1	STRONG BROWN 7.5YR 5/8	DARK RED 2.5YR 3/6
SPANKER 3	3.9	2.6	14.4	35	-	1.3	STRONG BROWN 7.5YR 5/8	DARK RED 2.5YR 3/6
SPANKER 4	3.4	2.2	16.9	50	-	1.1	REDDISH YELLOW 7.5YR 6/6	DARK RED 2.5YR 3/6
TAL Y BONT FARM	6.5	6.4	16.4	9	< 1	4.9	STRONG BROWN 7.5YR 5/6	DARK REDDISH BROWN 2.5YR 3/4
TUMBLE	4.5	3.6	13.1	48	-	0.8	STRONG BROWN 7.5YR 5/8	RED 10 YR 4/8
DONEGAL	5.9	5.6	44.3	27	-	13.2	RED 2.5YR 4/6	DUSKY RED 10R 3/4

(1) RANGES REPRESENT DIFFERENT SAMPLING DATES

(2) TOTAL Fe and Mn WERE DETERMINED FOR IGNITED SAMPLES AND ADJUSTED TO PERCENTAGE IN OVEN DRIED OCHRE USING LOI%

(3) - NO MANGANESE DETECTED

(4) \* INSUFFICIENT MATERIAL FOR DETERMINING % ORGANIC CARBON



FIGURE 3.2 : HISTOGRAMS SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED  
pH VALUES FOR UNDRIED AND OVEN DRIED SAMPLES (46 SAMPLES)

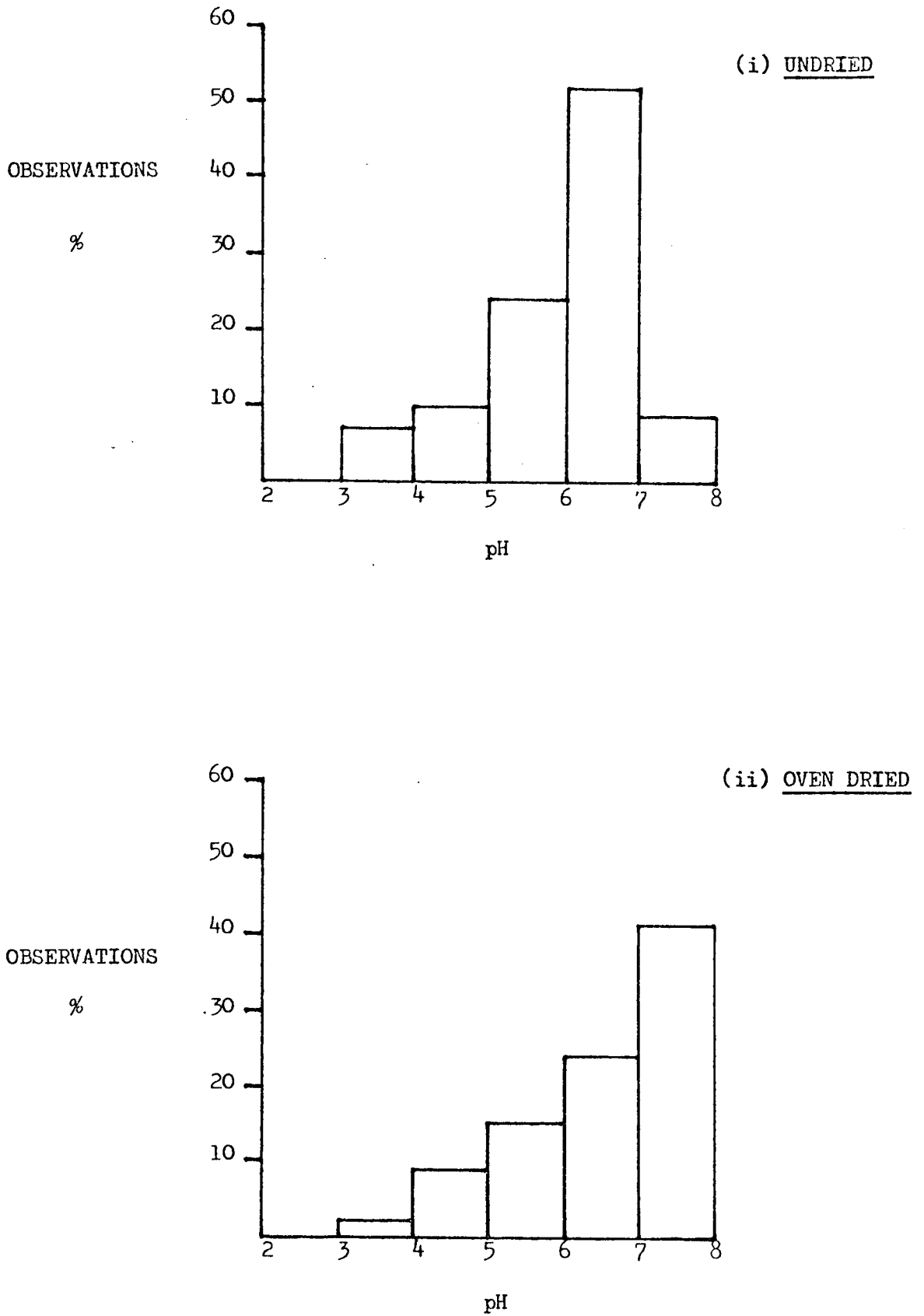


FIGURE 3.3 HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED LOSS ON IGNITION PERCENTAGES FOR OVEN DRIED OCHRE SAMPLES

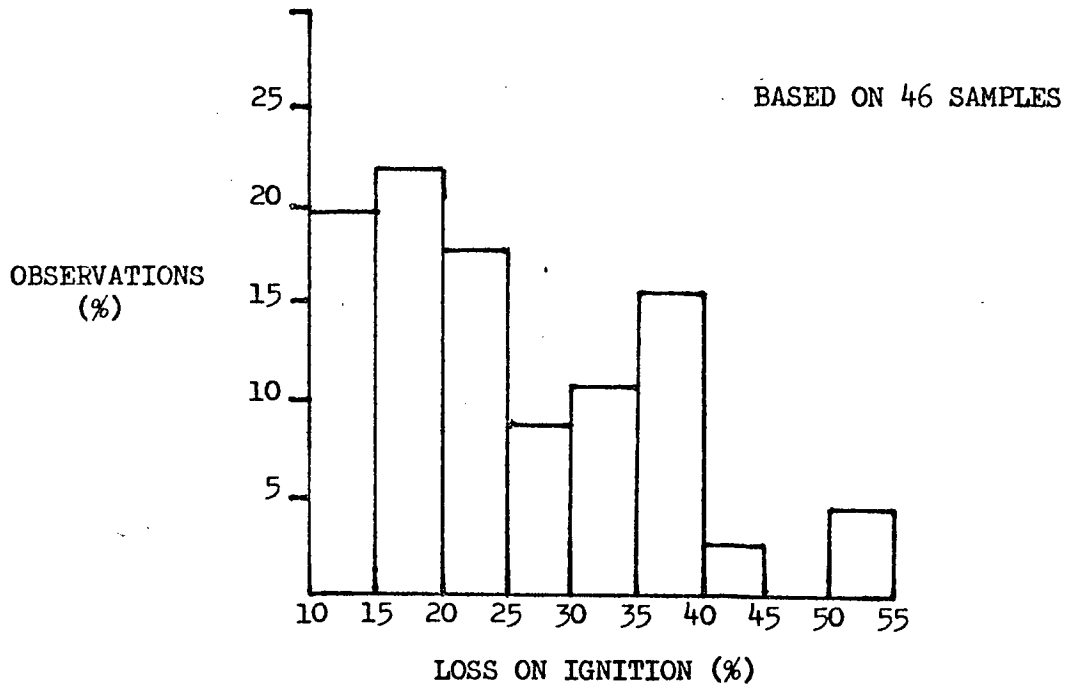


FIGURE 3.4 HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED TOTAL IRON PERCENTAGES OF OVEN DRIED OCHRE

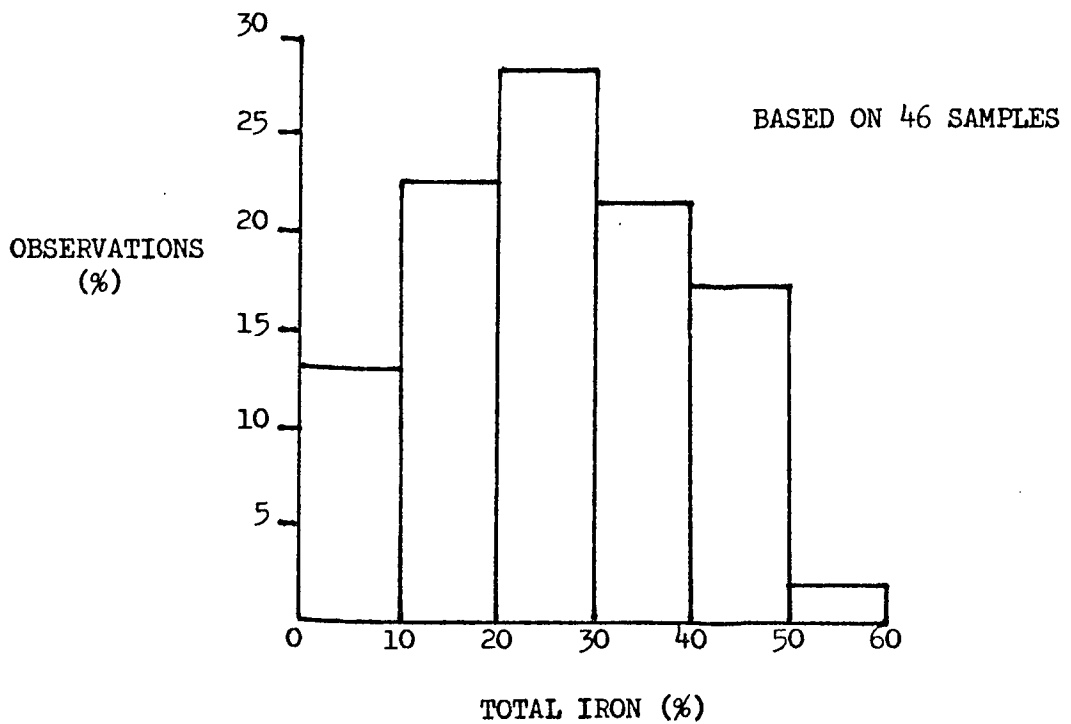


FIGURE 3.5 HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED  
ORGANIC CARBON PERCENTAGES FOR OVEN DRIED OCHRE SAMPLES

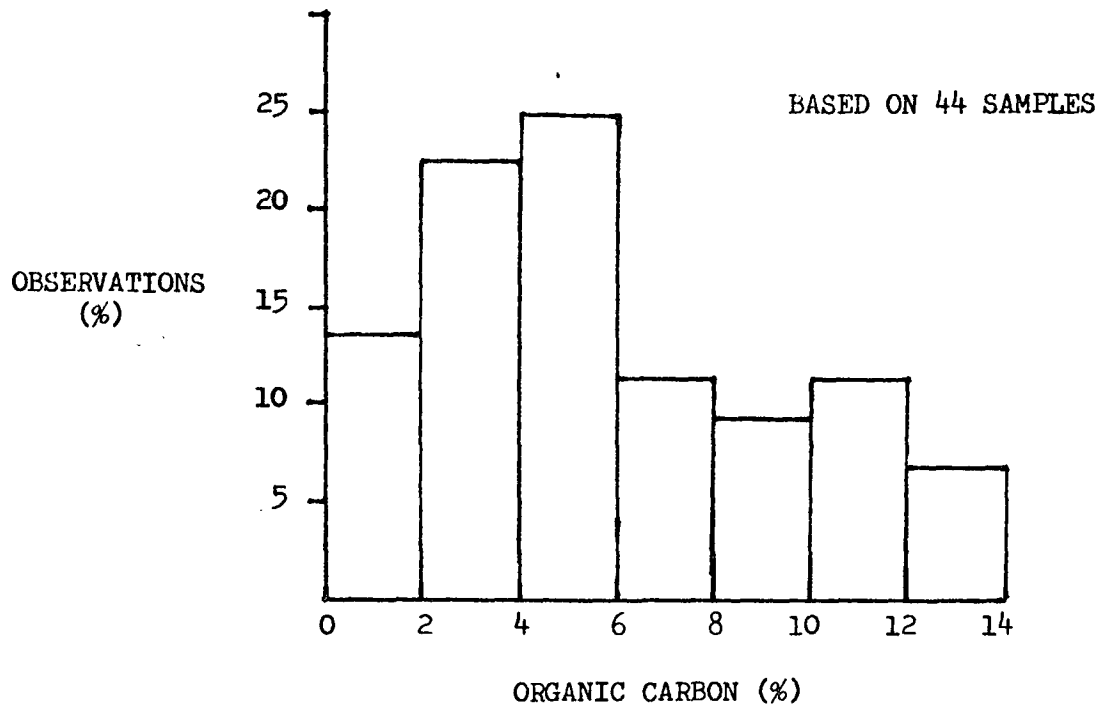
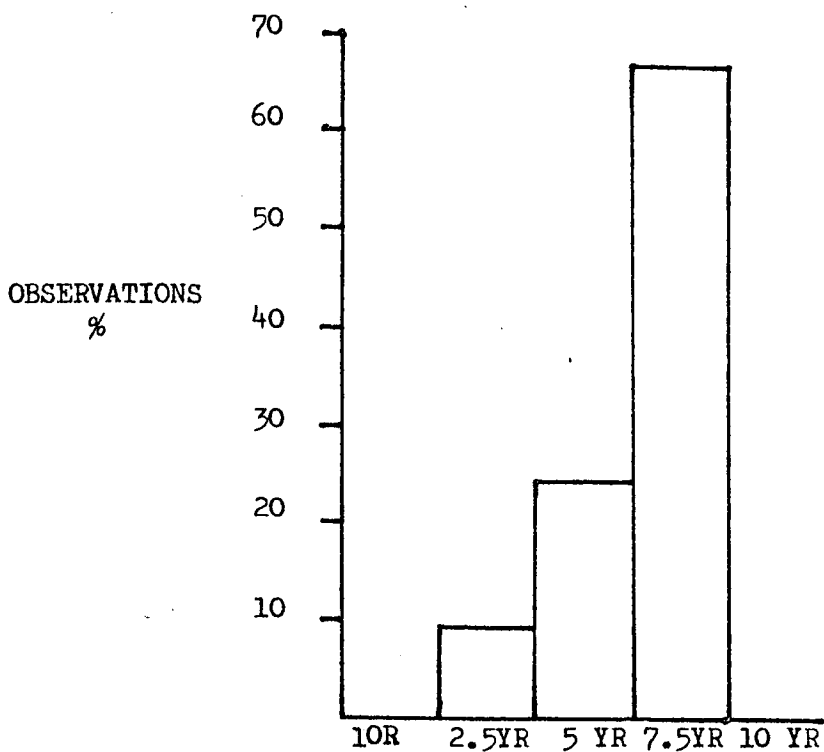
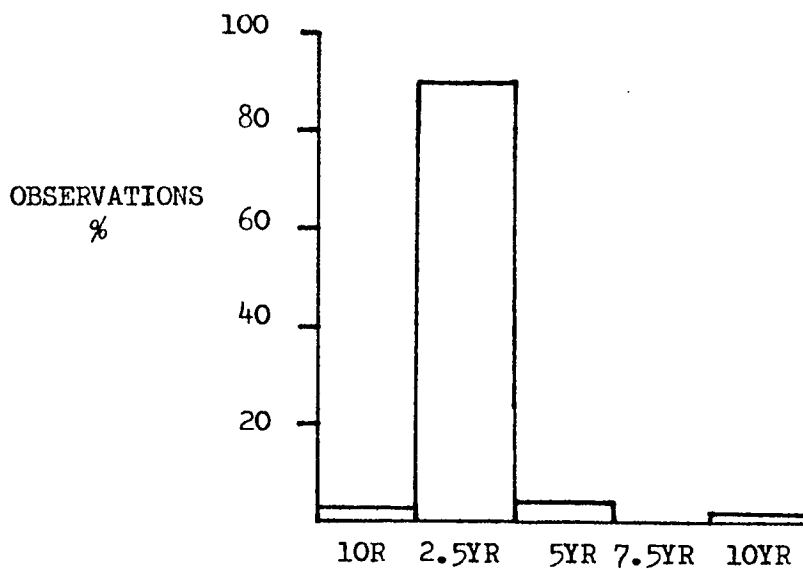


FIGURE 3.6 HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED MUNSELL COLOURS OF OVEN DRIED OCHRE. (46 SAMPLES)



HISTOGRAM SHOWING THE FREQUENCY DISTRIBUTION OF RECORDED MUNSELL COLOURS OF IGNITED OCHRE



### 3.2.2.5 Colours

Table 3.3, Figure 3.6

Most oven dried samples (67%) had a colour represented by Hue 7.5YR on the Munsell scale. This reflects a reddish yellow to strong brown colouration. 24% of the samples fell into the 5YR Hue (yellowish-red, reddish brown) and the remaining 9% had a distinct red colour, Hue 2.5YR.

After ignition the colours were dominated by red ferric compounds. 90% of the samples had a red to dark red or dark reddish brown colour classified as Hue 2.5YR. One sample (Donegal) had a dusky red colour after ignition, 10R 3/4 on the Munsell scale.

### 3.2.2.6 Absolute Sugar levels

Table 3.4

The carbohydrate fraction of the 18 samples analysed for absolute sugar levels was dominated by hexoses. Hexoses accounted for over 50% of the total sugar content for all samples except two. On average the total carbohydrate (sum of each absolute sugar level) was composed of hexoses 61%, pentoses 6%, methyl pentoses 6%, uronic acids 25%, and hexosamines 2%.

Uronic acids were found in all samples but at approximately half the concentration of hexoses. Pentoses were also identified in all samples except two. Methyl Pentoses and hexosamines were not common in the analysed samples. Over half of the samples contained neither of these sugar types.

The results cast doubt on the determination of total sugars by the method used. In all cases the total sugar value was substantially less than the sum of the absolute sugar concentrations. In 12 out of the 18 samples the total sugar content was less than the value calculated for the hexoses.

### 3.2.2.7 Relative Sugar Levels

The relative sugar levels for ten ochre samples are given in table 3.5.

The GLC traces for each sample are shown in figure 3.7.

TABLE 3.4 : ABSOLUTE SUGAR LEVELS IN OVEN DRIED OCHRE SAMPLES

	$\mu\text{g/g}$ (to nearest 10 $\mu\text{g}$ )			<u>METHYL PENTOSE</u>	<u>URONIC ACIDS</u>	<u>HEXOSAMINES</u>
	<u>TOTAL</u>	<u>HEXOSES</u>	<u>PENTOSES</u>			
SPANKER 1	700	980	50	*	300	*
SPANKER 4	300	470	*	*	300	*
HALL FARM 1	1000	680	150	*	470	*
HALL FARM 2	1300	1280	160	500	500	*
HALL FARM 3	2100	2390	490	300	430	30
HALL FARM 4a	1700	2270	110	800	840	30
HALL FARM 5	1200	1390	240	100	1190	30
HALL FARM 6	1900	2530	140	400	570	120
PERSEHALL 2	1900	1670	90	*	360	240
PERSEHALL 3	900	600	110	*	220	*
PERSEHALL 4	400	480	< 10	*	440	120
PERSEHALL 5	700	900	280	*	480	*
PARK FARM 1	1000	1170	70	*	440	30
PARK FARM 5	800	1080	20	*	360	*
BECCLES MARSH 2	1600	2530	430	900	1030	*
BECCLES MARSH 3	1600	1280	40	400	540	*
FIRBECK 1	900	1080	*	*	210	30
FIRBECK 2	1200	960	190	100	460	*

\* NONE DETECTED

TABLE 3.5 : RELATIVE SUGAR LEVELS IN OCHRE HYDROLYSATES

<u>SAMPLE</u>	<u>GLUCOSE</u>	<u>GALACTOSE</u>	<u>MANNOSE</u>	<u>RIBOSE</u>	<u>RHAMNOSE</u>	<u>FUCOSE</u>	<u>ARABINOSE</u>	<u>XULOSE</u>
SPANKER 1	100	22	27	25	22	23	43	13
SPANKER 4	100	44	15	29	55	55	61	44
HALL FARM 2	100	44	30	26	74	123	51	16
HALL FARM 4a	100	36	14	74	104	96	209	89
HALL FARM 6	100	79	63	16	87	26	221	179
PERSEHALL 3 <sup>(1)</sup>	100	45	33	12	39	28	70	59
PERSEHALL 4 <sup>(1)</sup>	100	48	33	21	52	34	44	30
PERSEHALL 5 <sup>(1)</sup>	100	61	65	2	42	35	68	58
PARK FARM 5	100	*	*	78	65	65	100	61
BECCLES 2 <sup>(2)</sup>	100	59	*	113	113	212	174	50

(1) RATIOS BASED ON THE MEAN OF SEVERAL REPLICATES (SEE TABLE 7)

(2) DETERMINATION OF RELATIVE LEVELS DIFFICULT SINCE THE GLUCOSE PEAK WAS NOT EASILY DEFINED

\* NONE DETECTED

It proved difficult to obtain reasonable traces from the GLC separation of ochre hydrolysates. Although separation of individual monosaccharides at retention times comparable to those of the standards was clearly observed the peaks produced were often small and showing only a slight deviation from the baseline. Additionally the "peaks" of the sugars glucose, galactose and mannose had pronounced flat tops.

By calculating the mean of each column in table 3.5 it is possible to derive an overall Glucose : Galactose : Mannose : Ribose : Rhamnose : Fucose : Arabinose : Xylose ratio for the 10 ochre samples of 100 : 44 : 28 : 40 : 65 : 70 : 104 : 60. This ratio would suggest that arabinose was slightly more abundant than glucose in the hydrolysates. The least abundant monosaccharide was mannose with galactose and ribose being less than half as abundant as glucose. However, the trends suggested by the overall ratio are not applicable to each sample. For example, arabinose is more abundant than glucose in only 4 samples. As revealed in table 3.5 a great variation was observed between the samples. The ranges in the levels of each sugar relative to glucose are given in table 3.6.

More than one chromatogram were obtained for samples from Persehall by analysing several subsamples of the alditol acetate derivatives of the hydrolysates. These results are shown in table 3.7. For some sugars the results obtained for the replicates were identical or very similar. For example, the relative xylose ratios for Persehall 3(1) and Persehall 3(2) were both 0.59. Similarly the relative mannose levels for Persehall 4(1), 4(2) and 4(3) were 0.35, 0.32 and 0.31 respectively. Other sugars however showed a marked difference between the replicates. The relative levels of mannose, for example, for Persehall 3(1) and 3(2) were 0.15 and 0.51 respectively.



TABLE 3.6 : MAXIMUM AND MINIMUM RELATIVE SUGAR LEVELS  
BASED ON THE ANALYSIS OF 10 OCHRE SAMPLES

\* ALL RATIOS ARE RELATIVE TO GLUCOSE (1.0)

	<u>MINIMUM</u>	<u>MAXIMUM</u>
GALACTOSE	0.22	0.82
MANNOSE	+	0.65
RIBOSE	0.02	1.13
RHAMNOSE	0.22	1.13
FUCOSE	0.23	2.12
ARABINOSE	0.43	2.21
XYLOSE	0.13	1.79

+ NONE DETECTED

FIGURE 3.7 - INDEX

GL = GLUCOSE

GA = GALACTOSE

M = MANNOSE

X = XYLOSE

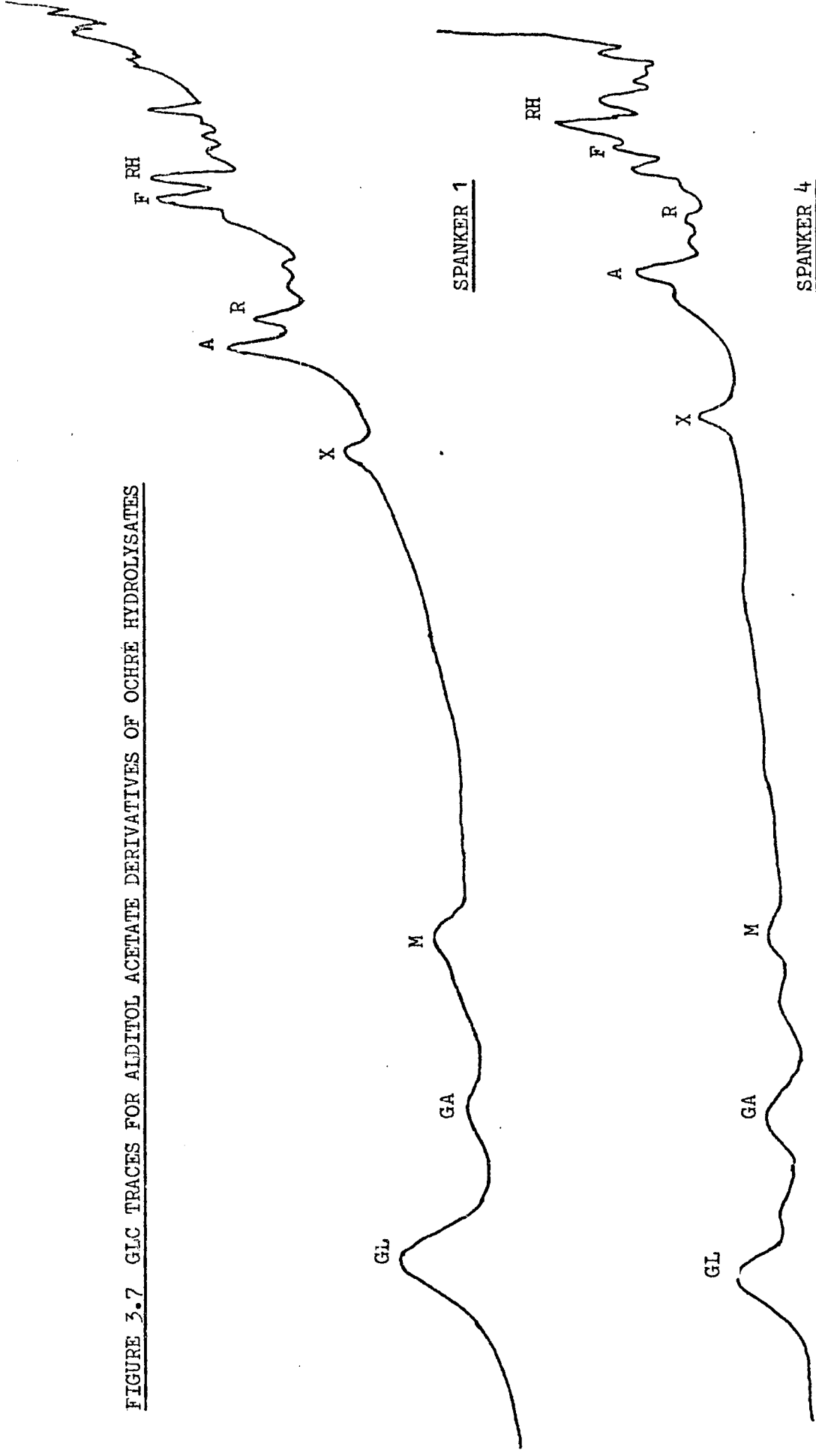
A = ARABINOSE

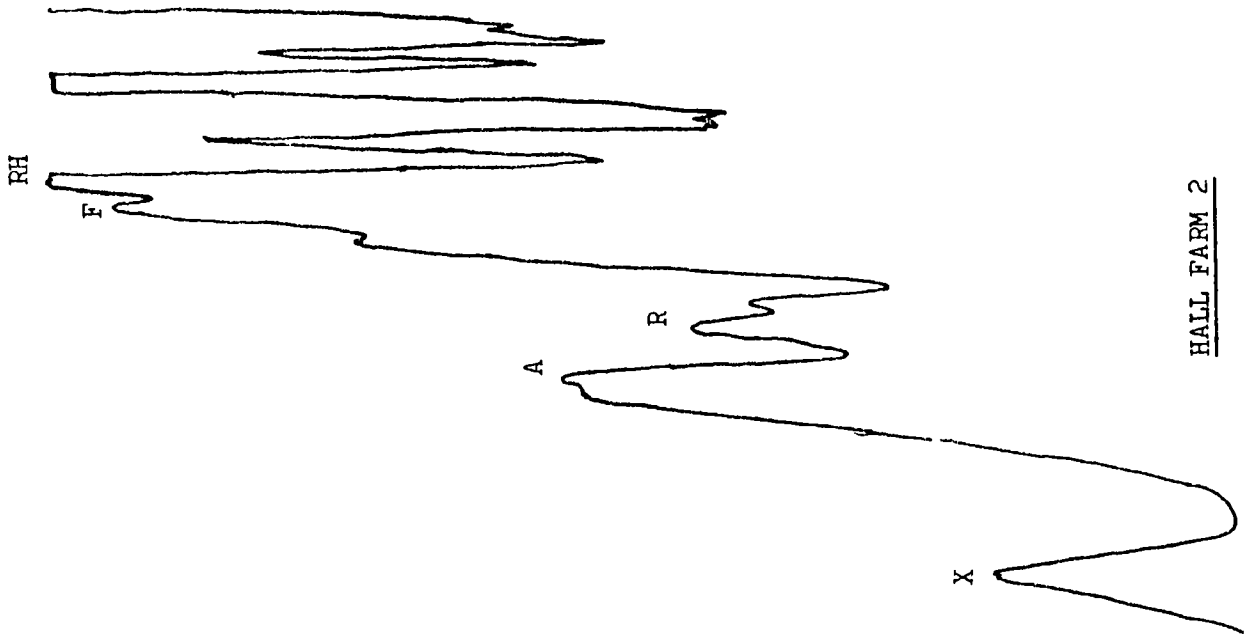
F = FUCOSE

RH = RHAMNOSE

Note : These are not standard abbreviations —  
See Biochim J.

FIGURE 3.7 GLC TRACES FOR ALDITOL ACETATE DERIVATIVES OF OCHRE HYDROLYSATES





HALL FARM 2

FIGURE 3.7 CONTINUED

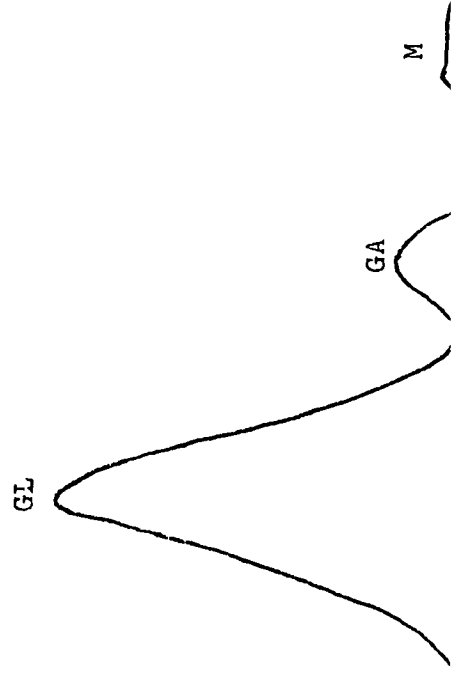


FIGURE 3.7 CONTINUED

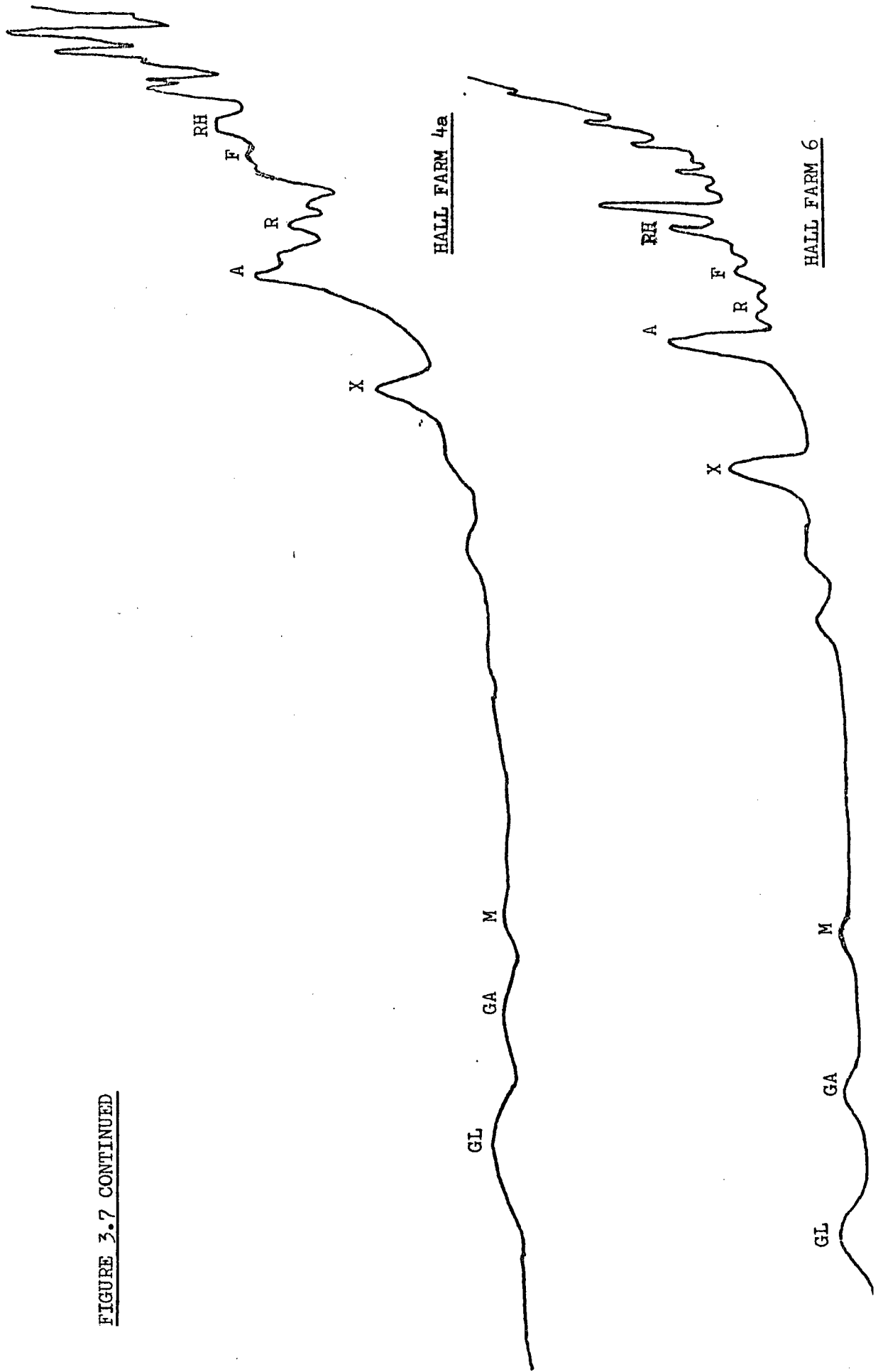


FIGURE 3.7 CONTINUED

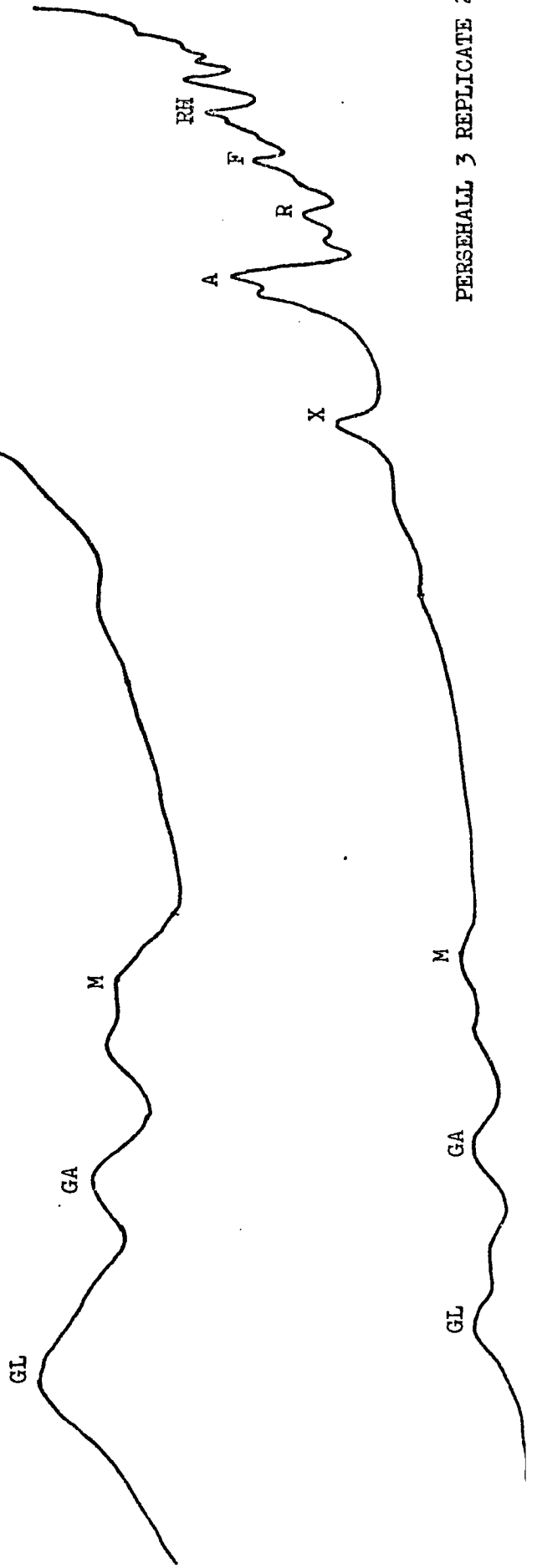
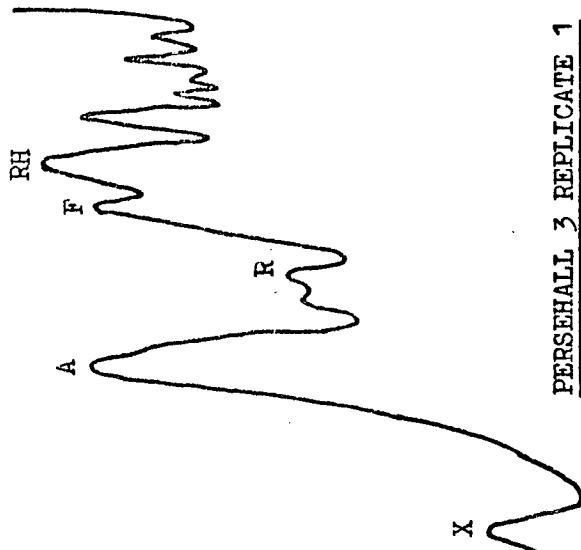


FIGURE 3.7 CONTINUED

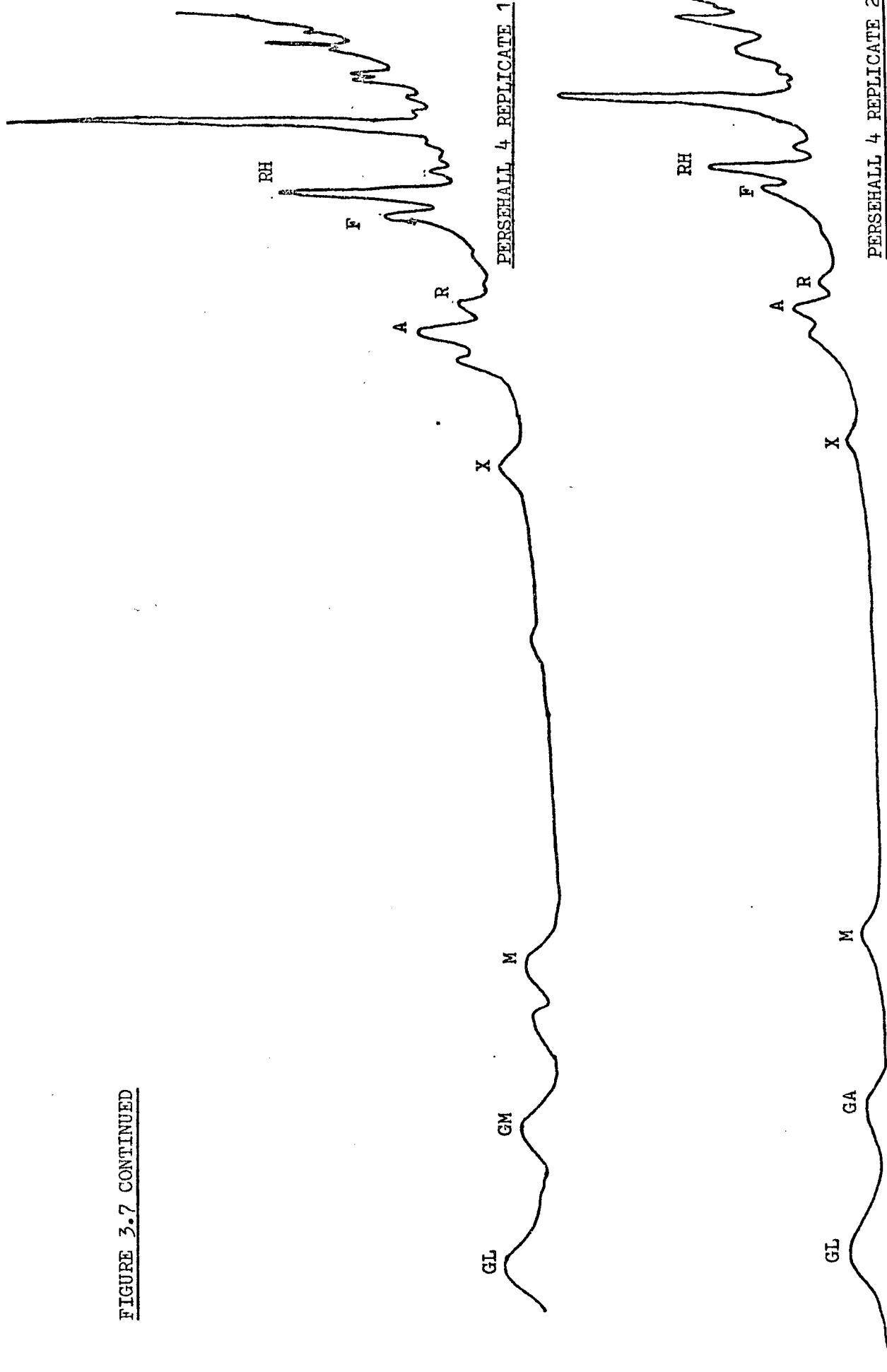


FIGURE 3.7 CONTINUED

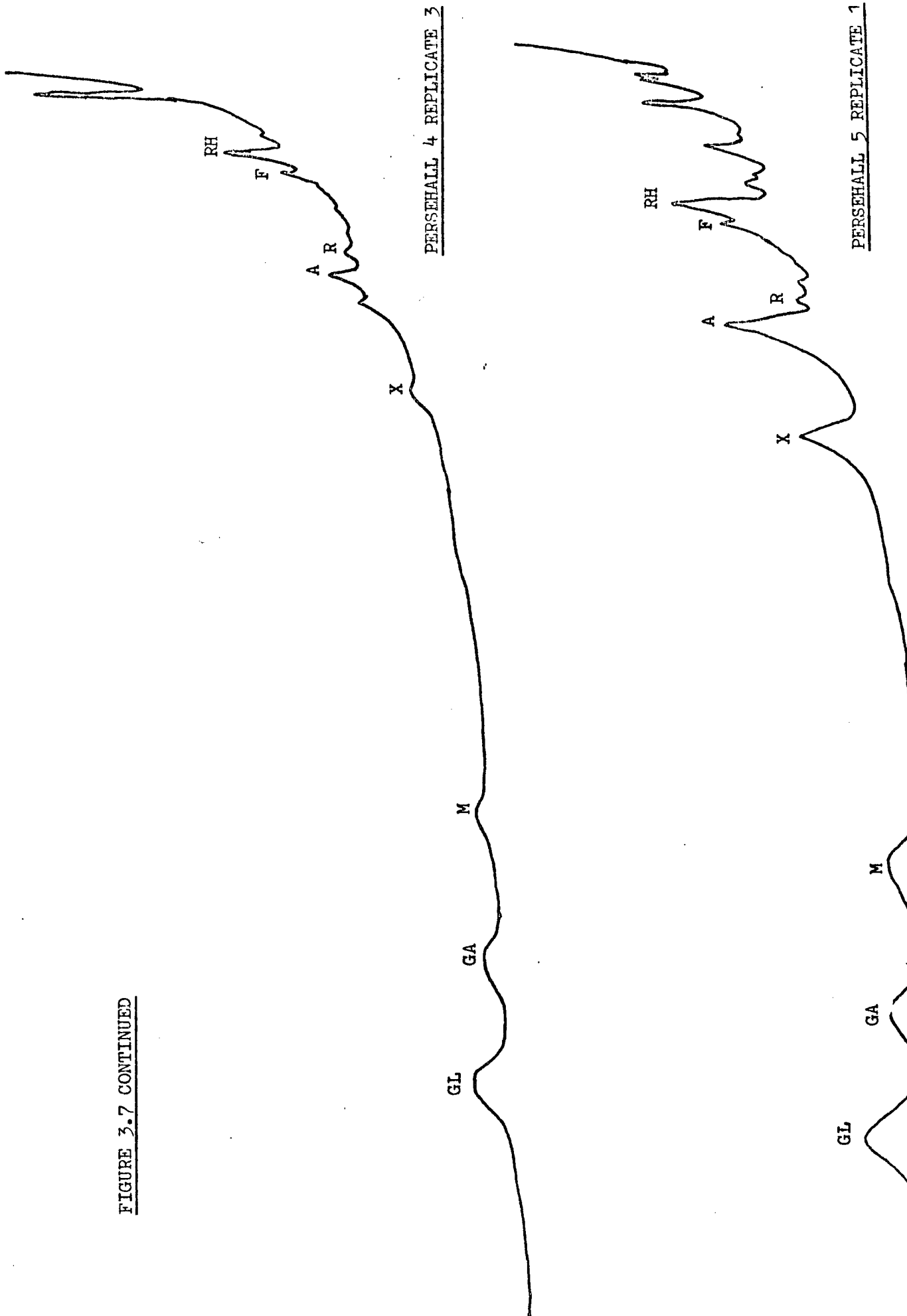




FIGURE 3.7 CONTINUED

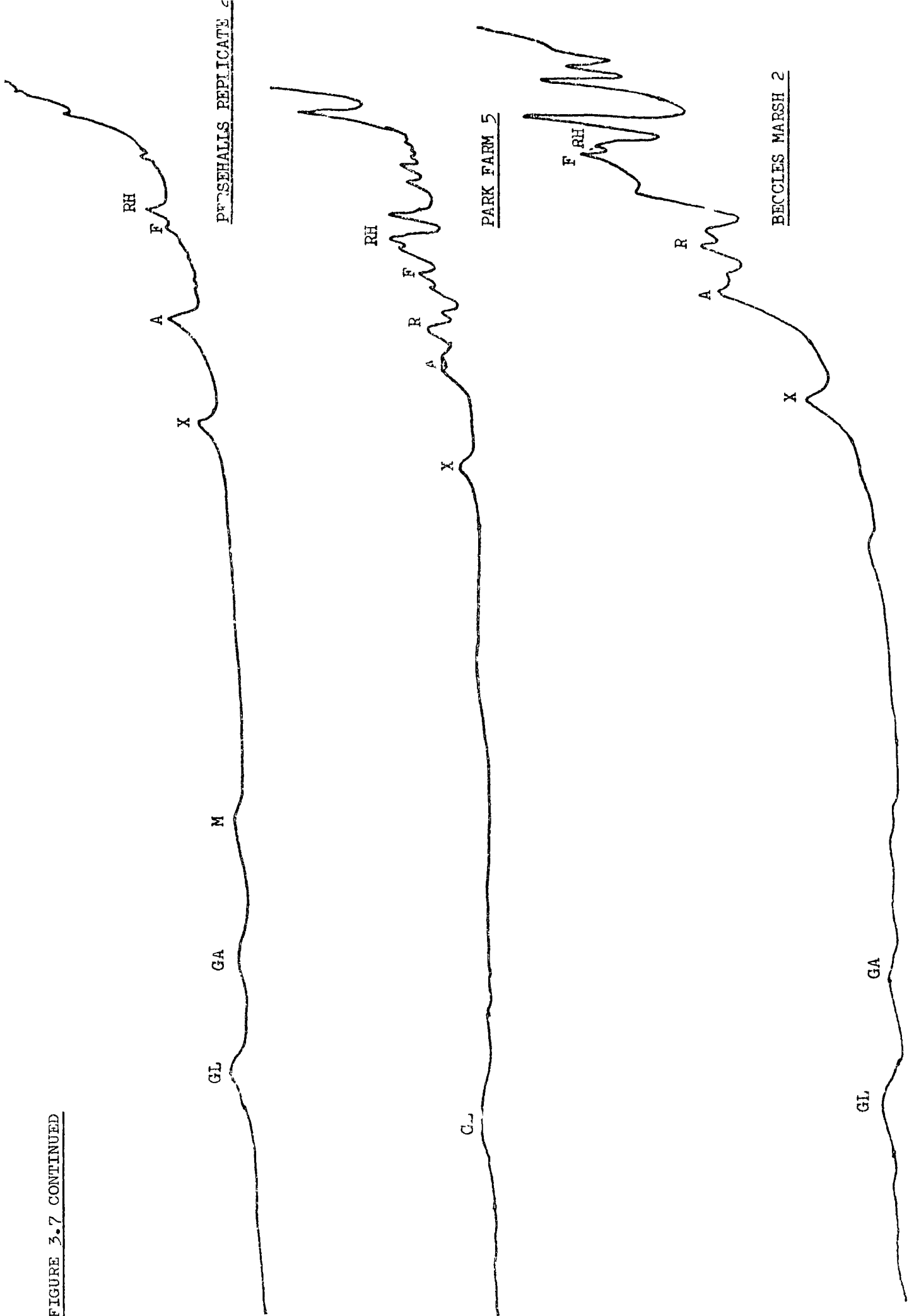


TABLE 3.7 : THE RELATIVE SUGAR LEVELS IN REPLICATE SUBSAMPLES OF OCHRE HYDROLYSATES

<u>SAMPLE</u>	<u>REPLICATE</u>	<u>GLUCOSE</u>	<u>GALACTOSE</u>	<u>MANNOSE</u>	<u>RIBOSE</u>	<u>RHAMNOSE</u>	<u>FUCOSE</u>	<u>ARABINOSE</u>	<u>XYLOSE</u>
PERSEHALL 3	1	100	44	15	9	31	22	53	59
PERSEHALL 3	2	100	46	51	14	47	33	86	59
PERSEHALL 4	1	100	53	35	23	60	33	42	55
PERSEHALL 4	2	100	47	32	12	52	41	38	12
PERSEHALL 4	3	100	45	31	27	45	27	52	24
PERSEHALL 5	1	100	40	38	4	34	28	47	37
PERSEHALL 5	2	100	82	91	*	50	41	88	78

\* NONE DETECTED

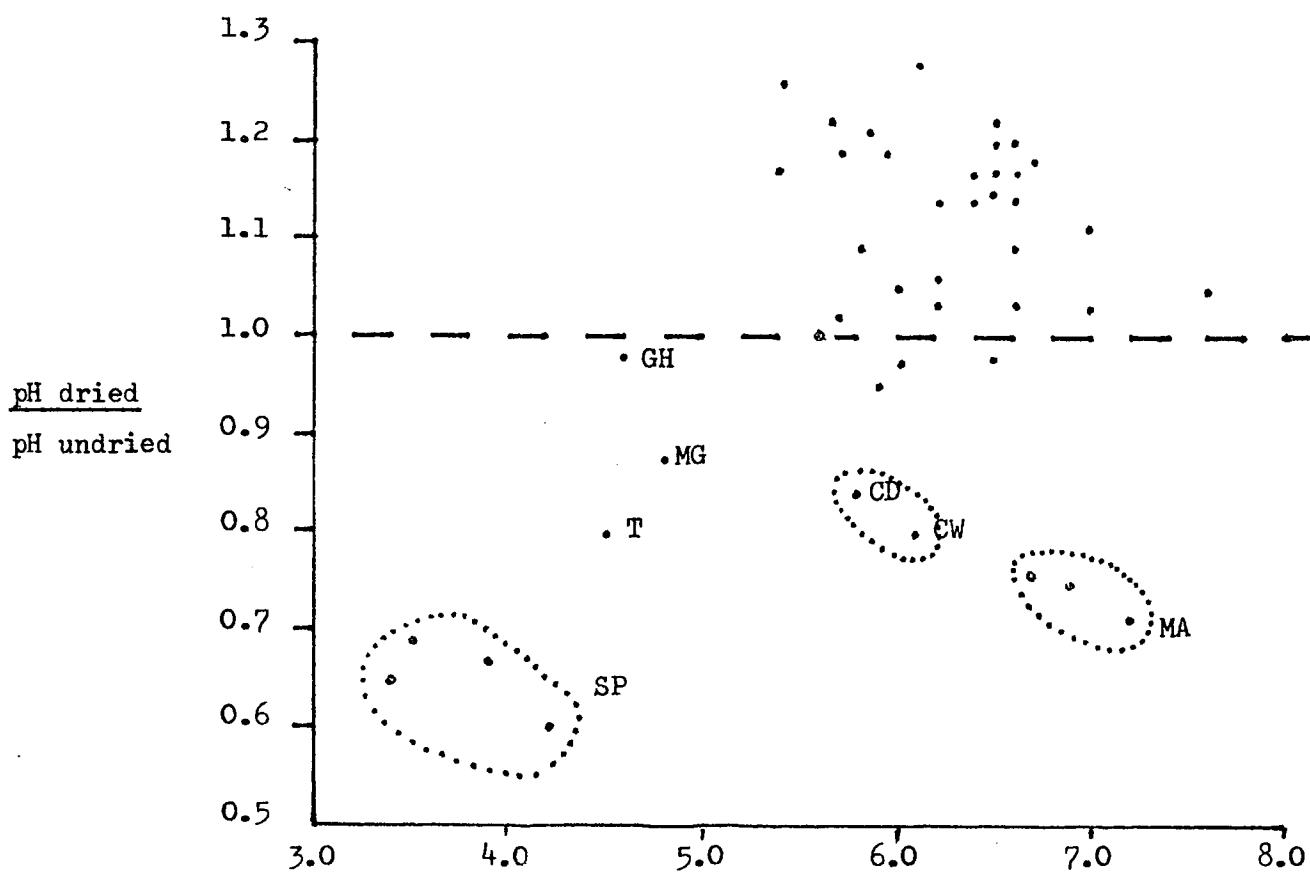
### 3.3 DISCUSSION

Ochre was collected from a variety of sites including drain outfalls, open ditches, streams, and the soil surface. The appearance of ochre in situ was highly variable. Most of the deposits in open ditches were composed of small flocs of dark orange material (plate 3.25) whereas samples from drain outlets were a bright orange colour, harder, well structured and gelatinous (plate 3.27). Where ochre had dried it formed a crusty and brittle deposit. The difference in appearance between ochre in ditches and in drain outlets was also observed in open ditches at Llyn Cowlyd, Llyn Coedty and Sychnant Pass. Where the flow of water increased (for example, at the 'V' notches) ochre changed from a well flocculated dark orange colour to a very gelatinous, bright orange-yellow colour. The ochre at Spanker was also orange and compact (plate 3.26). A very distinct type of ochre was observed in the shallow pools at Llyn Coedty (plate 3.8). These pools contained very large, but fragile, flocs and filaments of ochre.

An examination of the sites where ochre was found substantiates the view that these deposits develop at the interface between aerobic and anaerobic zones (see section 2.2.1.). For example, at Sychnant Pass and Ochr Cefn ochre deposits formed below a very wet, peaty area. At Llyn Cowlyd and Llyn Coedty water bubbled into the open ditches from an anaerobic zone. This was evident by the odour of hydrogen sulphide where the water emerged and by the high ferrous ion concentration of the water (section 6.1.). Hydrogen sulphide and deposits of ferrous sulphide were found at several sites.

A marked variation was seen in the extent to which ochre developed within a site. For example, at Penygroes only two drains were affected by ochre in a field containing several drain lines. At Penygroes 1 two outlets emerged at the head of an open ditch. One of the drains contained ochre

FIGURE 3.8 PLOT OF pH (UNDRIED) AGAINST pH DRIED/pH UNDRIED



- GH - Gallt yr Huddrem
- MG - Morfa Glas
- CD - Llyn Coedty
- CW - Llyn Cowlyd
- MA - Machynlleth
- SP - Spanker
- T - Tumble

whilst the other did not. Similarly, at Llyn Cowlyd an open ditch a few metres away from the ochre containing ditch contained no iron deposits and iron could not be found in the water by atomic absorption spectrophotometry (plate 3.28).

Examination of the various deposits showed that ochre formation had occurred at different rates. Some drainage systems had been operating for several years and the ochre deposits in them were not preventing the discharge of water. Other drains had blocked completely within a very short time. The most severe case was the site at Hall Farm (Sites 4 to 6) where ochre had blocked a drainage system within 12 months. Repeated visits to some sites revealed that ochre could form very quickly. At Llyn Coedty and Llyd Cowlyd ochre had to be cleared from the open ditches at least once a week to ensure their continual operation.

Most of the samples had a pH between 5.0 and 7.0 in the undried state. This rose to pH 6.0 - pH 8.0 on oven drying. This reflects the fact that most ochre deposits form when the drainage water has a near neutral pH. Some of the sites, however, were characterized by a very low pH. When undried samples had a pH below 5.0 then oven drying reduced the pH even further. Ochre at these sites, especially those at Spanker, would normally be classified as pyritic deposits (Section 2.2.1) on the basis of their acidity. The degree to which this distinction is appropriate to the ochre problem will be discussed in chapter 4.

It should be noted that the effect of oven drying on pH was not simple. In general oven drying increased pH if the undried sample had a pH greater than 5.0. If the undried sample had a pH lower than 5.0 then drying caused the pH to fall. The samples collected at Llyn Coedty, Llyn Cowlyd and Machynlleth were, however, anomalous in that their pH fell after drying although the undried samples had a pH above 5.0. This is illustrated in Figure 3.8 where undried pH is plotted against oven dry pH/undried pH.

The complex nature of this effect is illustrated also by the fact that some samples having the same pH before drying yielded different pH values when oven dried. For example, Hall Farm 2, Hall Farm 3 and Hall Farm 4a had a pH of 6.6 in the undried state. On oven drying the pH of these samples was 7.5, 7.7 and 7.9 respectively. These differences in pH between and within dried and undried samples reflects the inherent variability of ochre deposits with respect to chemical reaction and external influences such as the pH, flow rate and composition of the waters in which they are formed.

The total organic matter content of the samples is reflected in the loss on ignition and organic carbon percentages. The results show that the proportion of organic matter in the samples is highly variable. For example, the loss on ignition percentage ranged from 10 to 52. However, all the samples analysed did contain organic matter. The minimum values recorded for percentage loss on ignition and percentage organic carbon were 10.6 and 0.8 respectively. This reflects the importance of organic material in the formation of ochre deposits. An ochre deposit of purely chemical origin, with no organic component, was not found.

The nature of the carbohydrates in the organic matter was investigated by colorimetric methods and GLC. Hexoses and uronic acids were the dominant sugars in the hydrolysed samples. The abundance of hexoses is in agreement with the fact that hexoses are the most common monosaccharides in biological systems (Lehninger, 1975). Uronic acids are also an important component of many polysaccharides (Lehninger, 1975; Russell, 1961). The levels of the other sugar classes namely, pentoses, methyl pentoses and hexosamines were relatively low and were not detected at all in some samples.

The fractionation of organic matter by GLC yielded some anomalous results. Johnson (1979) quoted the following ratio as being typical of relative sugar levels in mineral soils.

GLUCOSE	100
GALACTOSE	46
MANNOSE	50
XYLOSE	42
ARABINOSE	53
RHAMNOSE AND FUCOSE	23

The overall ratio obtained from the results of table 3.5 is substantially different to this ratio:-

GLUCOSE	100
GALACTOSE	44
MANNOSE	28
XYLOSE	60
ARABINOSE	104
RHAMNOSE AND FUCOSE	135

It can be seen that mannose levels in the ochre hydrolysates are lower than in soils, whereas the amounts of xylose, rhamnose, fucose and arabinose are much higher. The high levels (relative to glucose) recorded for the pentose sugar arabinose are especially anomalous since the absolute level of pentoses in the samples was low in relation to hexoses.

Johnson (1979) found that the level of ribose in hydrolysates of bacterial slimes from acid streamers was high giving ribose : xylose ratios greater than 10. Xylose however is a major component of soil carbohydrates and the ribose to xylose ratio of most soils is usually less than 0.1.

Johnson suggested that the ribose : xylose ratio of ochre hydrolysates could indicate the importance of the acid streamer bacteria in the formation of ochre deposits. The ribose : xylose ratios of the ochre hydrolysates analysed in the present study are given in table 3.8. A ratio greater than 10 was not found in any of the samples. The highest ratio recorded was 2.3 (Beccles 2). However, all the samples, except two (Persehall 5 and Hall Farm 6), had a ribose to xylose ratio greater than that normally found in soils. These results imply that bacteria similar to those found by Johnson (1979) are also present in the ochre samples analysed. This conclusion will be discussed further in chapter 4.

TABLE 3.8 THE RIBOSE : XYLOSE RATIOS OF OCHRE HYDROLYSATES

	<u>RIBOSE : XYLOSE RATIO</u>
SPANKER 1	1.92
SPANKER 4	0.66
HALL FARM 2	1.64
HALL FARM 4a	0.83
HALL FARM 6	0.09
PERSEHALL 3	0.20
PERSEHALL 4	0.70
PERSEHALL 5	0.03
PARK FARM 5	1.28
BECCLLES 2	2.27

As expected all the ochre samples contained significant quantities of iron. Half the samples had a total iron content when oven dried of between 20% and 40%. 50% of the dry weight of the sample from Spanker 4 was composed of iron. At the pH values associated with most of the sites, all easily oxidized iron would precipitate out and accumulate. At some sites, however, the pH values were below 5.0. At Spanker all the sites had a pH below 4.2. At these pH values the chemical oxidation and precipitation of iron would be slow. Therefore the accumulation of iron



at these sites of low pH might suggest the catalysis of ferrous iron oxidation by acidophilic microorganisms.

The high iron content of the ochre samples demonstrated that the total amount of iron deposited at each site must be very large. Additionally the rapidity and duration of deposition would suggest a large reserve or source of iron. At these sites ochre is likely to be a long term, recurrent problem and could be described as allochthonous or permanent according to Kuntze's (1982) classification (See Section 2.2).

Manganese was not found in significant quantities in the samples except for Pentre Mawr and Penygroes 3 where the percentage of manganese in dried ochre was 10 and 4 respectively. This may reflect low levels of manganese in the drainage water at the other sites. Additionally manganese does not oxidize rapidly at pH values below 8.0 although the rate of oxidation is increased at lower pH values in the presence of ferric oxides and hydroxides. Therefore even if manganese was present in the drainage water its deposition in large quantities is not to be expected at the pH values associated with the ochre sites.

The colour of the samples reflects the importance of iron in their composition. The colours recorded are characteristic of the naturally occurring minerals of iron as shown below.

Lepidocrocite ( $\delta$ -FeOOH)	5YR - 7.5YR
Goethite ( $\alpha$ -FeOOH)	7.5YR - 10YR
Ferrihydrite	5YR - 7.5YR
Haematite ( $\alpha$ Fe <sub>2</sub> O <sub>3</sub> )	5YR - 2.5YR

Schwertmann + Taylor (1977)

The results of the chemical analysis of ochre were analysed statistically to determine whether any of the variables were correlated. The matrices obtained are shown in Table 3.9.

Table 3.9 : CORRELATION MATRICES FOR VARIABLES DETERMINED BY  
CHEMICAL ANALYSIS OF OCHRE SAMPLES

(a) ALL VARIABLES (44\* SAMPLES)

\* THE SAMPLES WITH MISSING ORGANIC CARBON DATA WERE IGNORED.

pH (WET)	(1)	1.000				
pH (OVEN DRY)	(2)	0.799	1.000			
LOI %	(3)	-0.005	0.090	1.000		
TOTAL Fe %	(4)	-0.158	-0.127	-0.073	1.000	
ORGANIC CARBON %	(5)	<u>0.121</u>	<u>0.161</u>	<u>0.740</u>	<u>0.084</u>	<u>1.000</u>
	(1)	(2)	(3)	(4)	(5)	

(b) ALL VARIABLES EXCEPT ORGANIC CARBON % (46 SAMPLES)

pH (WET)	(1)	1.000		
pH (OVEN DRY)	(2)	0.800	1.000	
LOI %	(3)	-0.009	0.083	1.000
TOTAL Fe %	(4)	<u>-0.158</u>	<u>-0.127</u>	<u>-0.061</u>
	(1)	(2)	(3)	(4)

The only significant correlations found were between the undried and dried pH values (0.80) and between LOI% and organic carbon % (0.74). The association between undried and dried pH has been discussed earlier. The exceptions or anomalies to this relationship have also been considered and are probably responsible for lowering the correlation coefficient.

The positive correlation between the percentage loss on ignition and organic carbon content was also to be expected. Loss on ignition is often used as an estimate of soil organic matter content. However, the loss of

structural water from clay minerals and dehydroxylation of ferric hydroxides can also contribute to the loss on ignition especially at temperatures above 375°C (Ball 1964; Jackson 1958). Such losses would have reduced the correlation between loss on ignition and organic carbon percentage.



Plate 3:1 Ochre in a field drain outlet at Penygroes site 1.



Plate 3:2 Ochre in a field drain outlet at Penygroes site 3.



Plate 3:3 Ochre at the mouth of a stream running into a small river from a "hole" in the riverbank, Penygroes site 5.



Plate 3:4 Llyn Coedty site 2.



Plate 3:5 Llyn Coedty site 2 : Water entered the ditch via a culvert. The culvert is not evident in the photograph but runs diagonally from the bottom right to the centre.



Plate 3:6 Llyn Coedty : Large amounts of ochre accumulated where water from the ditch shown in plate 3:5 flowed through the culvert in the bottom right of the photograph.



Plate 3:7 Llyn Coedty : Pollution by iron deposits entering a small river.



Plate 3:8 Llyn Coedty : Ochre in a shallow pool on a riverbank.



Plate 3:9 Llyn Cowlyd site 1 : Water bubbling to the surface at the head of an open ditch.



Plate 3:10 Llyn Cowlyd : A portion of the open ditch conveying water from Llyn Cowlyd site 1.





Plate 3:11 Llyn Cowlyd site 4 : This culvert conveyed water from the ditch shown in Plate 3:10 to a small river.



Plate 3:12 Nr. Machynlleth : Where drainage ditches entered a small river ochre was replaced by algae and aquatic plants.



Plate 3:13 Machynlleth site 1 : gravel coated with ferric iron deposits.



Plate 3:14 Machynlleth site 2 : Ochre in an open ditch.

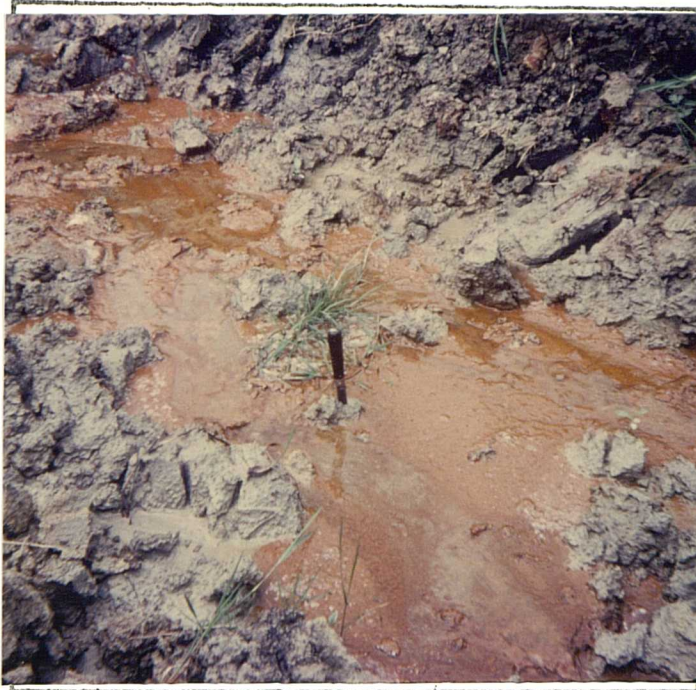


Plate 3:15 Machynlleth site 3 : Ochre in an open ditch.



Plate 3:16 Beccles Marsh site 1 : a white gelatinous material in a field drain outfall.



Plate 3:17 Firbeck site 1 : Ochre in a large drainage ditch.



Plate 3:18 Ochr Cefn Isaf : ochre deposits below a rush-infested, waterlogged area.



Plate 3:19 Pentre Mawr, ochre deposits  
in a disused coal mine.



Plate 3:20 Pentre Mawr, ochre deposits in  
a disused coal mine.

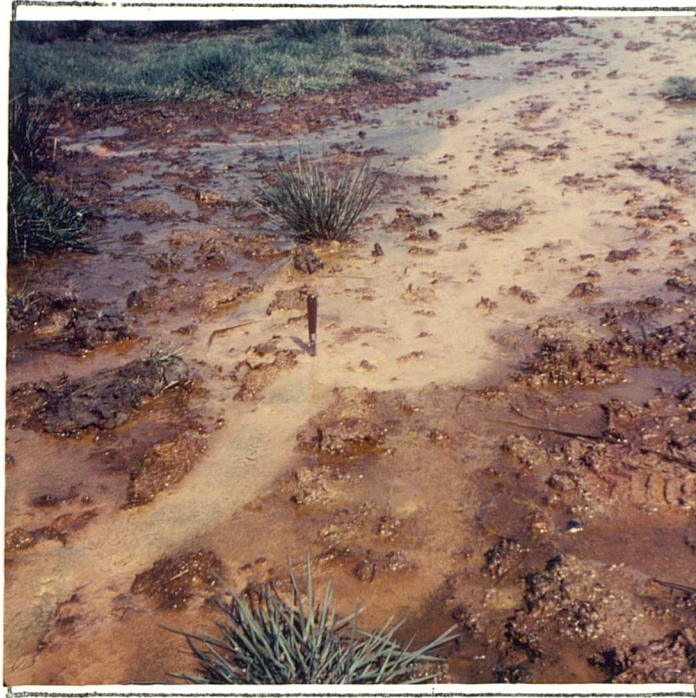


Plate 3:21 Spanker site 2 : Ochre forming on the soil surface over an extensive area.



Plate 3:22 Spanker site 3 : ochre deposits in an open ditch.



Plate 3:23 Sychnant Pass : Ochre in a roadside ditch.



Plate 3:24 Sychnant Pass : following heavy rainfall water overflowed onto the road and thin layers of ochre developed on the road surface. On drying these left a red stain.



Plate 3:25 Petri dish containing well flocculated ochre from Sychnant Pass.



Plate 3:26 Petri dish containing compacted, orange, ochre from Spanker.





Plate 3:27 Petri dish containing ochre from a field drain outfall from Park Farm.



Plate 3:28 Llyn Cowlyd : this open ditch did not contain ochre and iron could not be detected in the water. The confluence of this ditch and the ditch containing ochre is shown in Plate 3:10.

#### 4. THE MICROBIOLOGY OF OCHRE SAMPLES

##### 4.1 LIGHT AND SCANNING ELECTRON MICROSCOPY

###### 4.1.1 LIGHT MICROSCOPY

The general microbiology of ochre samples was investigated by observing a few drops of sample on a microscope slide using a Leitz ortholux microscope, with phase contrast facilities (Ernst Leitz, GMBH, Wetzal, W.Germany), at a magnification of x250.

Filamentous bacteria were observed in most samples in association with orange-red particles of ferric compounds. These bacteria were distinguished from fungi by their narrower filaments and lack of branching. The identification of different types of filamentous bacteria was made difficult by the masking effect of iron precipitates. In some samples the filaments were hardly visible except at the edge of iron masses.

The types of organism observed are described below. They were tentatively identified using the keys in Bergey's Manual (Buchanan & Gibbons, 1974), Godinho-Orlandi (1980) and by reference to the literature quoted where relevant.

###### Type 1 and 2:

Type 1 : Long filaments 2-3  $\mu\text{m}$  thick with no living cells evident within the sheath. The filaments were straight or slightly curved and ranged in length from 10  $\mu\text{m}$  to over 100  $\mu\text{m}$ . This organism was not encrusted with ferric compounds. Sheaths were found projecting from accumulations of ferric particles and also found unattached in solution.

Type 2 : Filaments 2-3  $\mu\text{m}$  thick with no living cells within the sheath. The filaments were straight or slightly curved and ranged in length from 10  $\mu\text{m}$  to 100  $\mu\text{m}$ . Iron accumulated on the sheath but not uniformly giving a "globular" effect with some portions of the sheath having no iron.

Type 1 and 2 are probably the same organism since filaments could be described as Type 1 outside an accumulation of iron particles but Type 2 inside it. Type 1 and Type 2 represent encrusted and non-encrusted growth forms of the same organism. This sheathed filamentous bacterium is therefore characterised by filaments, with no living cells within the sheath, sometimes having accumulated iron. Where iron does accumulate its distribution along the sheath is irregular.

It was found that the unencrusted filaments were most common at the edges of iron masses and free swimming. This suggests that newly-formed filaments were unencrusted. With time iron would accumulate on the sheath and thus the iron mass would get larger or, in the case of unattached filaments, a new mass would form. This phenomenon was described by Brown in 1903.

The irregular distribution of accumulated iron and the lack of living cells within the sheath suggests that this organism belongs to the genus Leptothrix. It is tentatively identified here as Leptothrix discophorus although it also bears similarities to Leptothrix cholodnii (Van Veen et al, 1978).

Plate 4.1

TYPE 3 :

Sheaths 2-3  $\mu\text{m}$  thick with their length being shorter than filaments of type 1 and 2. No cells were seen within the sheaths. The filaments were usually straight. Ferric compounds accumulated evenly on the sheath giving it an orange colour throughout its length.

The orange colour of the sheaths suggests that Type 3 could be Leptothrix ochracea. It should be noted that L.discophora is also said to have uneven golden brown sheaths (Mulder and Van Veen, 1963; Mulder, 1964; Van Veen et al, 1978).

TYPE 4 :

A "banded" or "spiral" organism 2-3  $\mu\text{m}$  thick. The length was variable but rarely exceeded 100  $\mu\text{m}$ . Type 4 was usually observed without iron accumulation but occasionally iron particles were seen adhering to the organism.

This Type was tentatively identified as a Gallionella sp. Gallionella spp. are usually described as having kidney shaped cells on the ends of long twisted stalks (Section 2.3.2). Cells were not observed in association with organisms of Type 4. However, according to Aristovskaya and Zavarzin (1971) the terminal cells of Gallionella spp are often absent. Gallionella spp are normally reported as having accumulated iron on the stalks. In this respect Type 4 differed since accumulations of iron were not usually evident.

Plate 4.2

TYPE 5 :

This organism was composed of rods in chains. No sheath was visible and iron was not accumulated. Type 5 could be Streptothrix sp, which is described as thin rods in chains within a hardly visible hyaline sheath. This organism also resembles Type 1863 isolated by Eikelboom (1975) from activated sludge. Type 1863 did not have a detectable sheath and was excluded from the Clamydobacteriaceae on this basis.

TYPE 6 :

This filamentous organism was found in association with iron masses and also unattached in solution. The filaments themselves were short, not encrusted with iron and contained no living cells. The organism was characterised primarily on the basis of its zigzag shape. The angles formed by the change in direction were always equal. An organism of similar description was not found in the literature.

The distribution of the various types of organisms according to site are given in Table 1.

#### RESULTS

Of the 49 samples examined only 5 had no filamentous bacteria, Spanker 1 and 2, Penygroes 1 and 3 and Hall Farm 4b. These did have a large population of unicellular bacteria. A further two samples, Hall Farm 4a and Park Farm 5, had predominantly unicellular organisms but with a few filamentous bacteria also being present. The lack of filamentous forms in these samples should be treated as comparative rather than absolute since it was shown that filamentous organisms were present in drainage water from Penygroes 1 (Section 5.3.1). (Continued on page 134).

TABLE 4.1 THE MICROBIOLOGY OF OCHRE SAMPLES AS REVEALED BY LIGHT

MICROSCOPY

THE POSSIBLE IDENTITY OF THE TYPES MENTIONED BELOW  
ARE DISCUSSED IN THE TEXT.

<u>SAMPLE</u>	<u>TYPES IDENTIFIED</u>
PENYGROES 1	UNICELLULAR BACTERIA ONLY. MOTILE RODS 4-8 $\mu$ m LONG (1)
PENYGROES 3	UNICELLULAR BACTERIA ONLY. MOTILE RODS 4-8 $\mu$ m LONG (1)
PENYGROES 5	TYPES 1; 2; 3; 4
PENYGROES 6	TYPES 1; 2; 3; 4
PENYGROES 7:1	TYPES 1; 2; 3; 4; 5
LLYN COEDTY	TYPES 1; 2; 3
LLYN COWLYD	TYPES 1; 2; 3; 4; 6
MACHYNLLETH 2	TYPES 1; 2; 3
MACHYNLLETH 3	TYPES 1; 2; 3
MACHYNLLETH 4	TYPES 1; 2; 3
BECCLES MARSH 1	THIN BLACK FILAMENTS ATTACHED TO BLACK MINERAL MASSES
BECCLES MARSH 2	TYPES 1; 2; 3
BECCLES MARSH 3	TYPES 1; 2; 3
BURNLEY HALL	THIN BLACK FILAMENTS ATTACHED TO BLACK MINERAL MASSES
FIRBECK 1	TYPES 1; 2; 3
FIRBECK 2	TYPES 1; 2; 3
HALL FARM 1	TYPES 1; 2; 3; 4
HALL FARM 2	TYPES 1; 2; 3
HALL FARM 3	TYPES 1; 2; 3
HALL FARM 4a	PREDOMINANTLY UNICELLULAR RODS WITH A FEW FILAMENTS OF TYPE 1; 2; 3
HALL FARM 4b	UNICELLULAR BACTERIA ONLY. MOTILE RODS 4-8 $\mu$ m LONG
HALL FARM 5	TYPES 1; 2; 3
HALL FARM 6	TYPES 1; 2; 3
PARK FARM 1	TYPES 1; 2; 3; 4
PARK FARM 2	TYPES 1; 2; 3; 4

TABLE 4.1 : CONTINUED

PARK FARM 3	TYPES 1; 2; 3
PARK FARM 4	TYPES 1; 2; 3; 4
PARK FARM 5	PREDOMINANTLY UNICELLULAR RODS WITH A FEW FILAMENTS OF TYPE 1; 2; 3
PARK FARM 6	TYPES 1; 2; 3; 4
PARK FARM 7	TYPES 1; 2; 3; 4
PARK FARM 8	TYPES 1; 2; 3; 4; 6
PERSEHALL 1	TYPES 1; 2; 3
PERSEHALL 2	TYPES 1; 2; 3
PERSEHALL 3	TYPES 1; 2; 3
PERSEHALL 4	TYPES 1; 2; 3
PERSEHALL 5	TYPES 1; 2; 3
PERSEHALL 6	TYPES 1; 2; 3
PENTRE MAWR	TYPES 1; 2; 3; 4
PONTHENRI	TYPES 1; 2; 3
GALLT YR HULLDREM	TYPES 1; 2; 3; 5; 6
MORFA GLAS	TYPES 1; 2; 3 AND PREDOMINANTLY TYPE 4
SPANKER 1	UNICELLULAR BACTERIA ONLY
SPANKER 2	UNICELLULAR BACTERIA ONLY
SPANKER 3	TYPES 1; 2; 3 BUT NOT NUMEROUS
SPANKER 4	TYPES 1; 2; 3 BUT NOT NUMEROUS
SYCHNANT PASS	TYPES 1; 2; 3; 4
TALY Y BONT FARM	TYPES 1; 2; 3; 4
TUMBLE	TYPES 1; 2; 3; 4
DONEGAL	TYPES 1; 2; 3

(1) THERE WAS SOME EVIDENCE TO SUGGEST THAT FILAMENTOUS ORGANISMS ARE PRESENT IN THE DRAINAGE WATER ALTHOUGH NONE WERE DETECTED IN OCHRE AT THE OUTFALL (SECTION 5.3.1)

At this site about 150 ml of drainage water was collected in a sterile polypropylene bottle. After a few days ochrous flocs were seen in the bottle. These were shown to contain filamentous bacteria by microscopic examination. Thus, even if filaments cannot be detected at the outlet they may be present in the drainage system.

Two samples were atypical. At Beccles Marsh 1 and Burnley Hall the white deposit contained many free living unicellular organisms and small filamentous bacteria mostly attached to black mineral masses.

The microbiology of most samples was dominated by filamentous bacteria of the genus Leptothrix. It should be noted that unicellular microorganisms were invariably present and in some cases algae and protozoa were also seen. The iron bacterium Gallionella was identified in many samples but it was not as numerous or widespread as Leptothrix spp. Several other types of filamentous organisms were also seen but only on a few occasions.

The organisms Leptothrix spp and Gallionella spp have been identified in ochre by many authors. Another genus that is commonly associated with ochre is Sphaerotilus spp. None of the organisms observed in the present study were similar to Sphaerotilus spp which is usually composed of chains of cells within a distinct sheath. According to some authors Leptothrix spp and certain species of Sphaerotilus are synonymous (See Section 2.3.1.2). This would explain to some extent the often quoted association between Sphaerotilus spp and ochre deposits.

#### 4.1.2 SCANNING ELECTRON MICROSCOPY

Samples of ochre in small terylene bags were fixed in 5% glutaraldehyde, dehydrated with acetone and dried in liquid carbon dioxide using a Polaron critical point drying apparatus. The samples were gold coated in a Polaron E3000 Sputter coater and examined using an ISI M - 7 scanning electron microscope operating at 15kV. Photographs were taken with a



Pentax 35 mm camera on a Kodak fine grain positive release film.

Negatives were developed in D19 high resolution developer.

A selection of scanning electron micrographs of ochre samples are shown in plates 4.3 to 4.29.

The structure of ochre is revealed clearly at low magnifications.

Ochre is composed of long filaments in association with iron particles (plates 4.3 to 4.5). The arrangement of the filaments and particles appears to be entirely random forming a "tangled mass". This would explain the tenacity and gelatinous nature of the deposits in situ.

The intertwined filaments are again illustrated in plates 4.5 to 4.9.

The filaments are seen here as being of uniform width and variable length.

These electron micrographs also show that the filaments are actually tubular. The rough edges at the end of many filaments, especially in plates 4.8 and 4.9, suggest that they have been broken on site, during collection, or during preparation. The tubular nature of the filaments confirms the observation made during light microscopy that the filaments are relics of bacterial growth, containing no living cells.

The nature of the ferric precipitates is shown in plate 4.10 where they appear to be accumulations of particles forming a "fluffy", almost coral-like, aggregate. These masses seem to encompass the filaments. In plate 4.11 the close association between filament and ferric iron particles is demonstrated. The ferric iron particles are dispersed along the filaments and in the centre of the photograph a ferric mass bridges the gap between two filaments. The accumulation of ferric particles on a filament is illustrated in plate 4.12 where the central filament in plate 4.11 is magnified.

Most of the filaments observed were of uniform width. One exception is shown in plates 4.13 and 4.14 which show a short filament about 35  $\mu\text{m}$  long. One end of the filament is a tube-like opening, whilst the other tapers to a point.

Plates 4.15 to 4.17 reveal a spiral structure composed of twisted bands. It is assumed that these are the stalks of Gallionella spp. Twisted bands associated with bean shaped cells were not seen.

On the whole all the samples analysed had a similar appearance being dominated by the tubes of filamentous bacteria and by iron particles. Occasionally other features were observed. For example, plate 4.18 illustrates a feature composed of a number of intertwined thin strands. The strands are no more than 0.25  $\mu\text{m}$  in diameter as compared to the filament in the top right of the photograph which has a diameter of over 1  $\mu\text{m}$ . Iron particles are loosely associated with this structure.

The feature shown in plate 4.19 is probably a fungal propagule. Plates 4.20 and 4.21 show in increasing magnifications a tangled structure of very thin strands or fibres. These are much thinner than the filamentous tubes.

A gold coated SEM sample of ochre from Llyn Cowlyd was analysed by SEM and EDAX to determine the composition of the sample. Several analyses of pin-point areas of different filaments were obtained. The traces are shown in figures 4.1 to 4.4. The trace obtained by analysing an iron accumulation is shown in figure 4.5. All the results are similar showing an abundance of iron. Silicon, sulphur and calcium were also detected in small quantities.

FIGURE 4.1 LLYN COWLYD FILAMENT 1

0 1505EC 6077INT  
VS: 25K HS: 50EV/CH

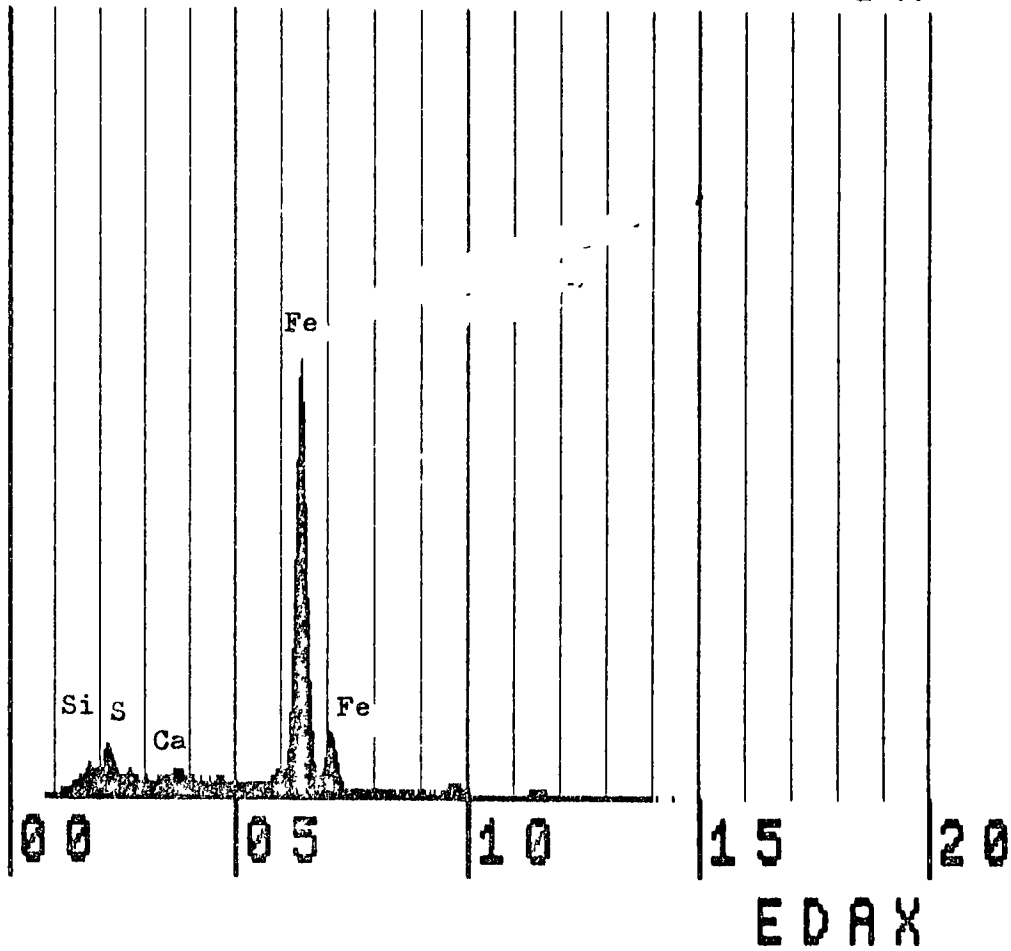


FIGURE 4.2

LLYN COWLYD

FILAMENT 2

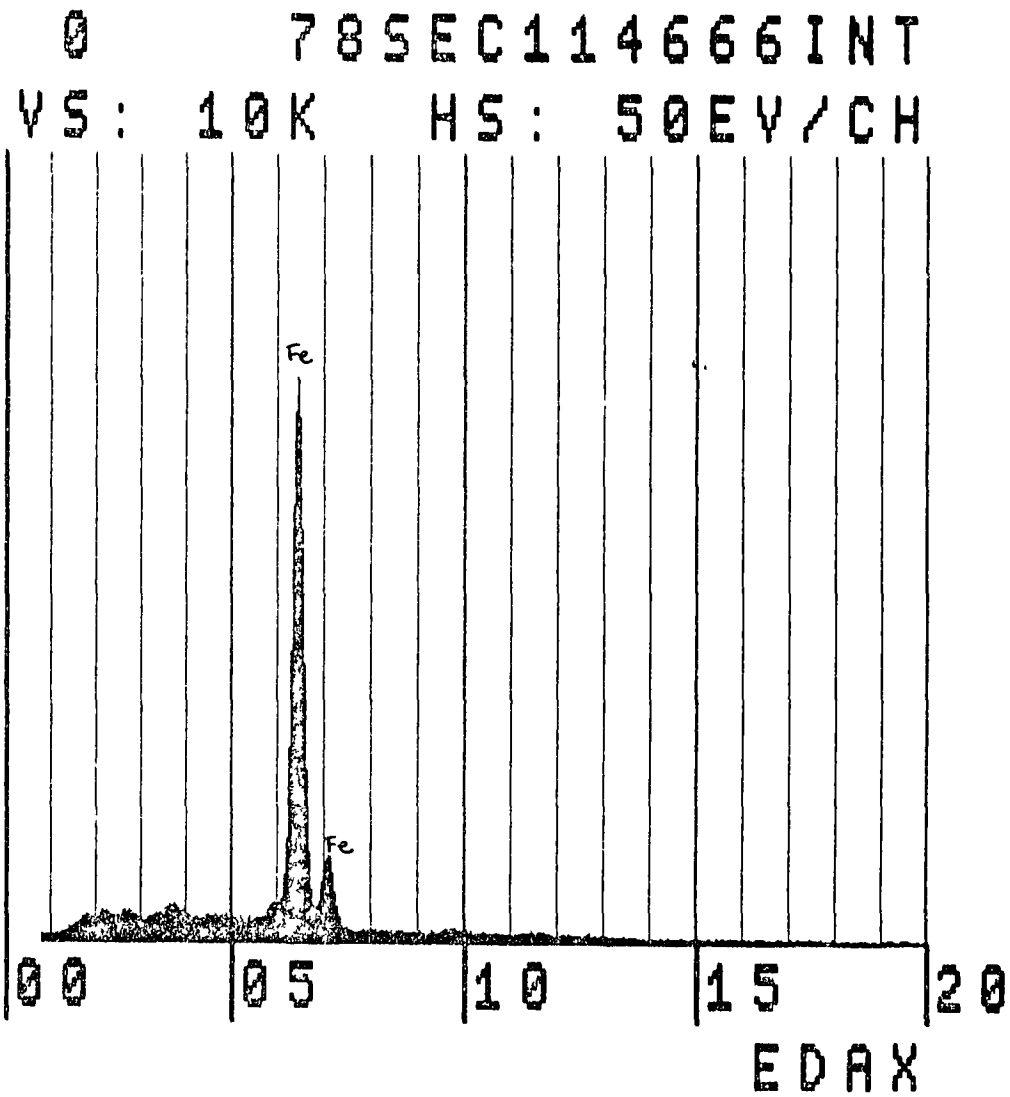


FIGURE 4.3

LLYN COWLYD

FILAMENT 3

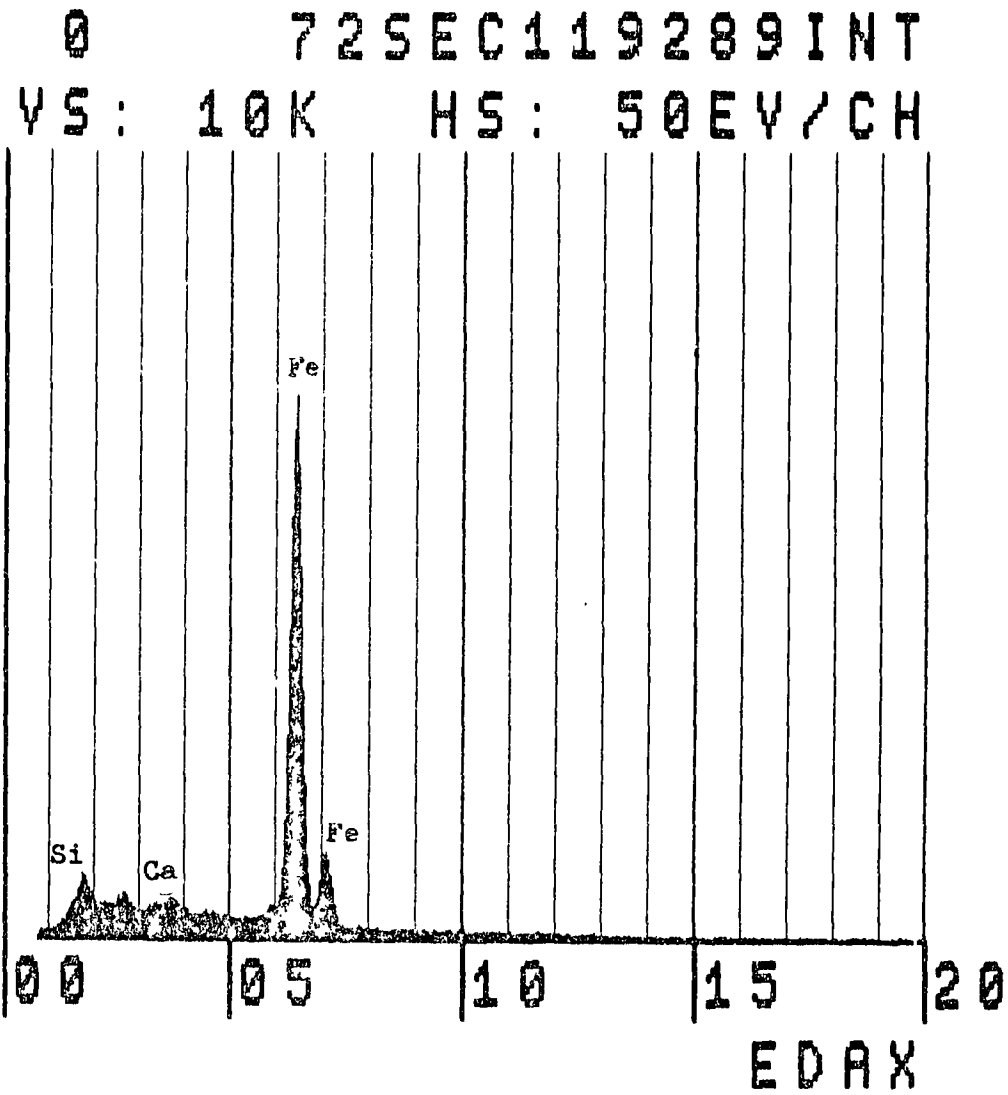


FIGURE 4.4

LLYN COWLYD

FILAMENT 4

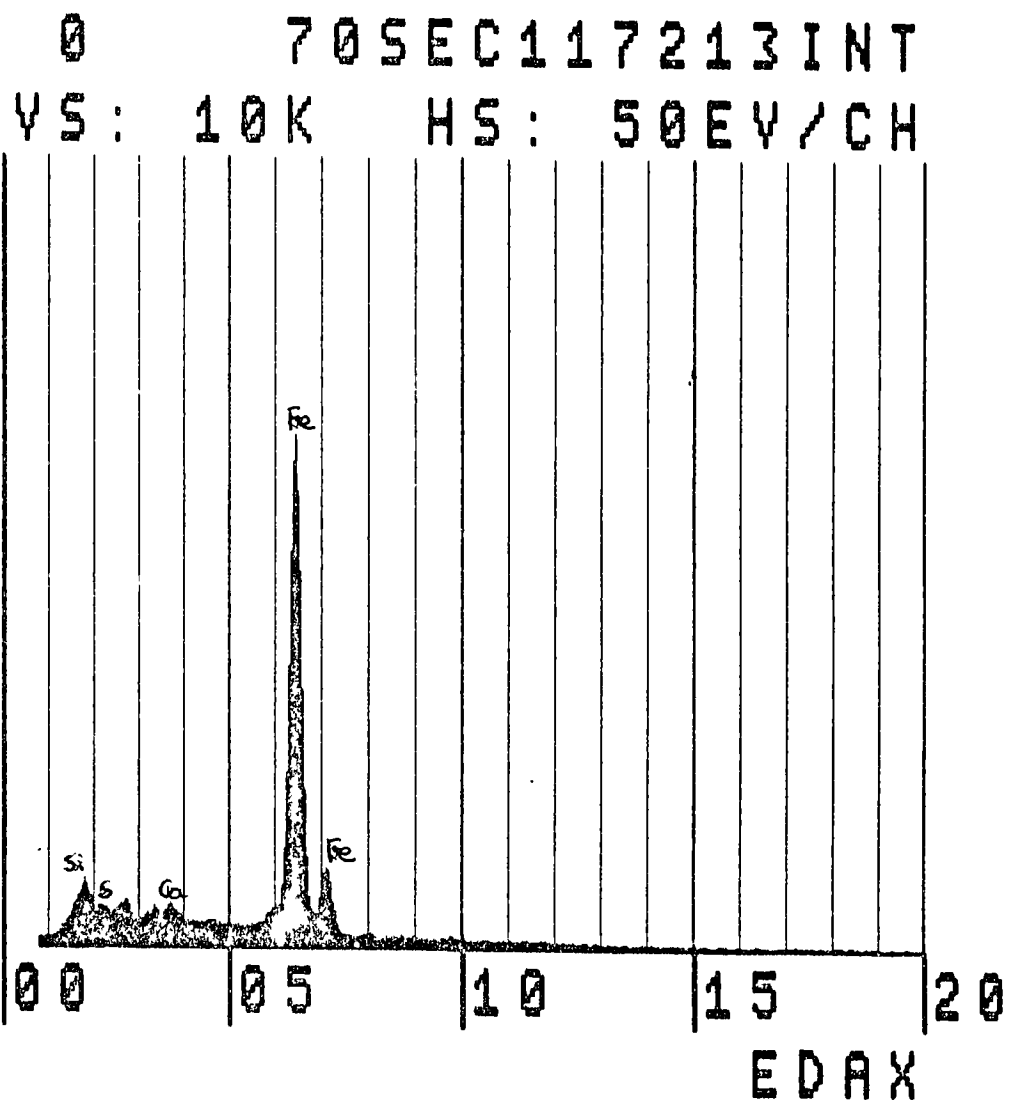
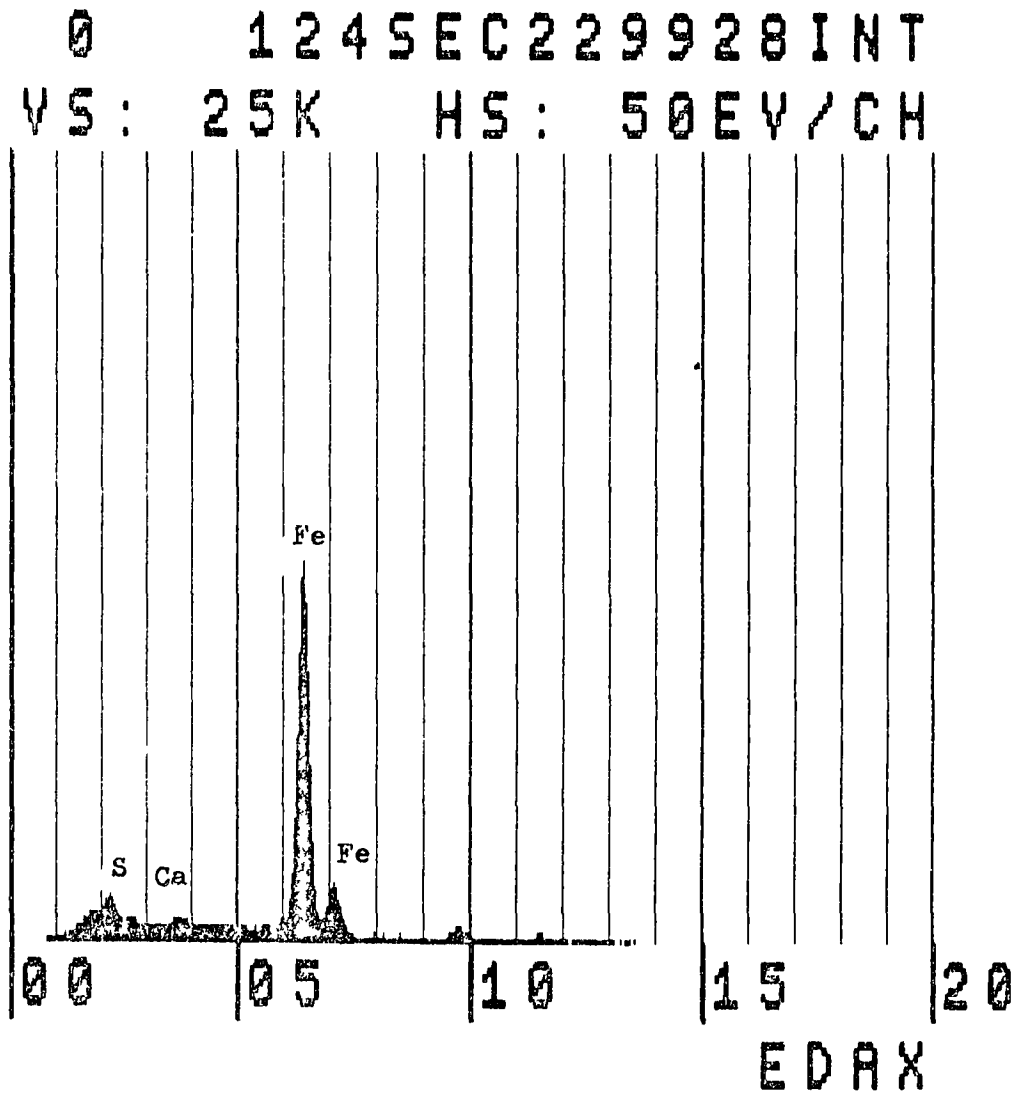


FIGURE 4.5 LLYN COWLYD MASS 1



#### 4.2 AUTOTROPHIC MICROBIOLOGY

The role of Thiobacillus ferrooxidans in the formation of ochre was discussed in section 2.4.1. T.ferrooxidans were isolated from ochre samples using the 9K medium of Silverman and Lundgren (1959). The composition of the medium is shown below:-

##### SOLUTION A

	g
$(\text{NH}_4)_2 \text{SO}_4$	3.0
KCl	0.1
$\text{K}_2\text{HPO}_4$	0.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
$\text{Ca}(\text{NO}_3)_2$	0.01
Distilled Water	700 ml

##### SOLUTION B

300 ml of a 14.74% (44.22g) solution (w/v) of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  including 1.0 ml of 10N  $\text{H}_2\text{SO}_4$ .

Solution A was sterilized by autoclaving at 15 lbs pressure for 15 minutes. Solution B was filter sterilized through a 0.22  $\mu\text{m}$  Millipore filter and the two solutions mixed. 10 ml portions of the medium were poured into sterile 25 ml or 50 ml conical flasks.

stored moist at  $-18^\circ\text{C}$   
Ochre samples<sub>A</sub> were diluted in the ratio 1 ml ochre to 9 ml sterile, acidified (pH 2.5) distilled water. The samples were homogenized for one minute using a Silverson homogenizer. It was found that this dilution gave no growth for some samples in which case the experiment was repeated using undiluted ochre.

The total number of T.ferrooxidans in each sample was determined by using a most probable number technique (Rand et al 196<sup>76</sup>~~5~~). 7 portions of 9K medium were used for each sample. 5 flasks were inoculated with 1.0 ml



of diluted ochre. The two remaining flasks were inoculated with 0.1 ml and 0.01 ml respectively. The flasks were incubated for at least 12 days at 25°C. Growth of T.ferrooxidans in the medium was clearly observed since the oxidation of ferrous iron resulted in a colour change from greenish-blue to orange.

Growth was confirmed by titrating 3 ml of medium and 3 ml of 1.0M H<sub>2</sub>SO<sub>4</sub> against 0.01M KMnO<sub>4</sub> to a permanent pink. A titre less than or equal to 1.0 ml was taken as a positive result indicating that 90% or more of the ferrous iron initially present in the medium had been oxidized.

The Most Probable Number of T.ferrooxidans per 100 ml of original diluted or undiluted sample was calculated by comparing the number of positive results obtained for the 7 tubes to results in standard tables. (Rand et al, 1976).

The numbers of T.ferrooxidans in 17 ochre samples are given in table 4.2. The number of organisms per gramme oven dried ochre ranges from none (Park Farm 2, Park Farm 3 and Penygroes 5) to 34,290 (Hall Farm 1). All samples, except Hall Farm 1, had less than 10<sup>4</sup> organisms per gramme of oven dried ochre.

The results show that the occurrence of T.ferrooxidans is widespread but also related to area or site. For example, samples from Spanker and Hall Farm had high numbers of the organism. On the other hand they could not be detected in samples from Park Farm. Similarly the samples from Penygroes, Llyn Cowlyd, and Llyn Coedty had less than 100 organisms per gramme dried ochre. One exception was Penygroes 3 where 5.2 x 10<sup>3</sup> organisms/g dried ochre were found. It is noteworthy that Penygroes sites 1 and 3 were drain outlets in the same field showing that the number of T.ferrooxidans can vary widely within small areas.

TABLE 4.2 : THE NUMBERS OF THIOBACILLUS FERROOXIDANS IN OCHRE SAMPLES

<u>SAMPLE</u>	<u>MPN* ORGANISMS/g OVEN DRIED OCHRE (1)</u>
PENYGROES 1	30
PENYGROES 3	$5.2 \times 10^3$
PENYGROES 5	NONE DETECTED
PENYGROES 6	20
PENYGROES 7:1	40
LLYN COEDTY	20
LLYN COWLYD	40
BECCLES MARSH 3	$1.09 \times 10^3$
BURNLEY HALL	190
FIRBECK 2	$1.01 \times 10^3$
HALL FARM 1	$3.43 \times 10^4$
HALL FARM 4b	$1.07 \times 10^3$
PARK FARM 2	NONE DETECTED
PARK FARM 3	NONE DETECTED
PERSEHALL 6	$4.92 \times 10^3$
SPANKER 2	150
SPANKER 4	530

\* TO THE NEAREST 10 ORGANISMS.

(1) The MPN organisms was initially determined for known volumes of ochre suspension. The value was converted to MPN/g oven dried ochre using the dry wt/ml ochre suspension calculated for each sample by drying a known volume of ochre suspension overnight at 105°C.

### 4.3 HETEROTROPHIC MICROBIOLOGY

#### 4.3.1 ACIDOPHILIC HETEROTROPHS

Johnson (1979) isolated polysaccharide-producing bacteria from streamers in acid mine drainage water. These bacteria were also isolated from ochre. The presumed effect of these bacteria on ochre deposits was discussed in section 2.4.2.

The most suitable medium found in the literature for isolating the acidophilic heterotrophs was the medium of Harrison et al (1980). The composition of the medium is shown below.

	<u>g/l</u>
$(\text{NH}_4)_2 \text{SO}_4$	2.0
KCl	0.1
$\text{Ca}(\text{NO}_3)_2$	0.01
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.50
$\text{K}_2\text{HPO}_4$	0.50
Glucose or D-Mannitol	1.0
Tryptone soya broth (oxoid)	0.10

Tryptone soya broth replaced the dehydrated trypticase soy broth (BBL) used by Harrison et al. The pH of the medium was adjusted to  $3.4 \pm 0.2$  with  $\text{H}_2\text{SO}_4$ . The medium was sterilized by autoclaving for 15 minutes at 15 lbs pressure. When glucose was used in place of mannitol it was autoclaved separately. To solidify the medium 12g/l of agar were added. The agar solution was adjusted to pH 4.5 and autoclaved separately.

Liquid and solid media incorporating glucose or mannitol were prepared. The liquid media were inoculated with acid mine drainage water (Cae Coch). 1 ml of water was diluted to 50 ml with sterile distilled water acidified to pH 2.5. 8 50 ml portions containing glucose and 8 containing mannitol were inoculated per sample according to a Most Probable Number technique.

5 portions were inoculated with 1.0ml of diluted drainage water, 1 portion with 0.1ml and 1 portion with 0.01ml. The remaining flask was kept as an uninoculated control.

Agar plates of Harrison's medium were inoculated with undiluted and diluted mine drainage water. 4 plates with glucose and 4 with mannitol were inoculated with 0.5ml undiluted water. 4 plates of each medium were also inoculated with 0.5ml of diluted drainage water. The acid mine drainage water was diluted 1ml to 50ml with acidified (pH 2.5), sterile, distilled water. Uninoculated plates were kept as controls.

The results from these inoculations were poor in terms of colony development. No growth was observed in liquid media containing mannitol. Growth was evident in 6 out of the 7 flasks containing glucose. No growth was seen in the uninoculated control flask. Examination of the flocs under a microscope revealed that they were composed of fungi. Colonies were seen on all of the inoculated plates. Some bacterial colonies were evident but fungi were dominant. Fungal colonies were also seen on the uninoculated controls.

In order to improve the performance of the medium an anti fungal agent, pimaricin, was incorporated. This was added as a 2.5% sterile solution (2.5% Pimaricin, Sigma) into sterile medium, to give a final concentration of 100 µg/ml.

7 50ml portions of Harrison's medium (with pimaricin) containing glucose and 7 containing mannitol were inoculated according to the Most Probable Number technique described above. Undiluted acid mine drainage water from Cae Coch was used as an inoculum. Uninoculated portions of media were also prepared. 9 plates of glucose medium with pimaricin were inoculated with 0.5ml undiluted acid mine water. One plate was kept as an uninoculated control. Flocculated, streamer-like growths were seen in most of the inoculated flasks. A mixed population of unicellular motile

and non-motile rod shaped bacteria were observed in the flocs. No growth was detected in the uninoculated flasks.

The streamer like growths were also seen on the agar plates. The colonies were a yellow-cream colour having an irregular shape. A mixed population of unicellular bacteria were found by examining the colonies under a microscope. The type of growth encountered is shown in plate 4.22. Plate 4.23 illustrates the effect of omitting anti fungal agent. Fungal colonies are seen to dominate the plate in the absence of pimaricin. The uninoculated plates showed no colony formation.

Harrison's glucose medium (solidified) containing pimaricin was adopted as a suitable media for isolating acidophilic heterotrophs from ochre samples.

## Isolation of heterotrophic bacteria from ochre

6 media were used to isolate heterotrophic bacteria from ochre samples.

1. Nutrient agar (Oxoid) pH 7.1. This was chosen because some of the heterotrophs isolated by Johnson (1979) from acid streamers had a pH optimum near neutral. Total counts on nutrient agar were also an estimate of the total numbers of heterotrophic organisms in the sample.

2. Malt Extract Agar (Oxoid). 980ml of agar and 20ml of sterile 10% lactic acid solution were mixed to give a medium of pH 3.5.

3. Harrison's glucose medium. (Final pH of approximately 3.5)

The composition of the medium was as described earlier. 1000ml of basal salts solution (pH  $3.2 \pm 0.2$ ), 250 ml glucose (pH  $3.2 \pm 0.2$ ) and 750ml agar solution were autoclaved separately and then mixed. The agar solution was not acidified to avoid hydrolysis.

4. Harrison's glucose medium with pimarinic. The medium was prepared as Harrison's glucose agar. Pimarinic was added before the plates were poured.

5. Citrate - Tryptone medium

	<u>g/l</u>
tryptone	0.5
tri-Sodium Citrate	2.0
$(\text{NH}_4)_2\text{SO}_4$	3.0
$\text{K}_2\text{HPO}_4$	0.5
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
KCl	0.1
$\text{CaNO}_3$	0.1

The pH of this solution was adjusted to 3.2 with 1.0 M H<sub>2</sub>SO<sub>4</sub>. The solution was mixed in the ratio 3:1 (V/V) with a suspension of separately sterilized Oxoid No 1 agar (5%). The final pH of the medium was about 3.6.

6. Citrate - Tryptone medium with pimaricin. The medium was prepared as Citrate - Tryptone agar. Pimaricin was added before pouring the plates giving a final concentration of 100 µg/ml.

A known volume (approximately 2ml) or weight (1-2g) of ochre, depending on the nature of the sample, was suspended in 200ml of sterile tap water. The suspension was homogenized for one minute using a Silverson homogenizer, cleaned and semi-sterilized by immersing in alcohol. 1.0ml of homogenized solution was pipetted into 9ml sterile tap water and mixed using a mechanical stirrer. The tap water was sterilized by autoclaving and then dispensed into graduated test tubes that had been sterilized by heating at 160°C for several hours. A further 9ml portion of tap water was inoculated with 1.0ml of this dilution. The process was repeated until four dilutions had been prepared, excluding the initial homogenized dilution.

4 plates of each medium were inoculated with 0.5ml aliquots of each dilution. The inoculum was dispersed over the agar surface using a glass spreader flamed in alcohol. The plate was allowed to dry for 1-2 hours and then inverted and incubated at 25°C. The number of colonies on each plate was monitored and recorded regularly.

TABLE 4.3 : NUMBERS OF HETEROTROPHIC BACTERIA IN OCHRE SAMPLES

	NOS PER g OVEN DRIED SAMPLE					
	NUT AGAR	MALT EXTRACT	HARR	HARR + PIM	CT	CT + PIM
SPANKER 2	$1.54 \times 10^6$ (2)	-	$4.10 \times 10^7$ (1)	$5.95 \times 10^6$ (1)	$2.87 \times 10^6$ (1)	$1.64 \times 10^6$ (1)
SPANKER 4	$5.00 \times 10^7$ (1)	-	$2.25 \times 10^7$ (2)	$2.45 \times 10^7$ (3)	$1.55 \times 10^7$ (1)	$1.08 \times 10^6$ (2)
FTRECK 2	$>1.6 \times 10^8$ (2)	-	$5.33 \times 10^7$ (1)	$2.38 \times 10^6$ (2)	$5.33 \times 10^4$ (1)	-
BURNLEY HALL	$3.00 \times 10^6$ (1)	-	-	-	-	-
HALL FARM 1	$5.97 \times 10^7$ (3)	-	$5.71 \times 10^7$ (1)	$1.46 \times 10^8$ (2)	-	-
HALL FARM 4b	$1.39 \times 10^7$ (1)	$1.39 \times 10^5$ (1)	-	$2.64 \times 10^5$ (1)	-	$2.78 \times 10^4$
BECLES MARSH 3	( * 1 )	-	$1.00 \times 10^6$ (1)	$1.82 \times 10^6$ (1)	-	-
PERSEHALL 6	$8.31 \times 10^6$ (1)	-	-	-	-	-
PARK FARM 2	( * 1 )	-	-	-	-	-
PARK FARM 3	$7.87 \times 10^6$ (2)	-	-	-	-	-
PENYGROES 1	$5.60 \times 10^7$ (2)	-	-	$4.90 \times 10^5$ (1)	-	-
PENYGROES 3	$1.30 \times 10^6$ (1)	-	-	$8.7 \times 10^5$ (1)	-	-
PENYGROES 5	$2.60 \times 10^7$ (1)	-	$1.4 \times 10^6$ (2)	-	-	-
PENYGROES 6	$3.5 \times 10^7$ (1)	-	-	-	-	-
PENYGROES 7:1	$3.7 \times 10^7$ (2)	-	-	-	-	$1.6 \times 10^5$ (1)
LLYN COWLYD	( * 2 )	-	-	-	-	-
LLYN COEDTY	( * 2 )	-	-	-	-	-



NOTES

(a) THE NUMBER IN BRACKETS FOLLOWING EACH BACTERIAL COUNT IS THE NUMBER OF DILUTIONS USED IN

CALCULATING THE VALUE GIVEN

(b) ① NUTRIENT AGAR PLATES WERE LOST WHEN TEMPERATURE OF INCUBATOR ACCIDENTALLY RAISED

② BACTERIAL NUMBERS FOR THE DILUTIONS WERE TOO NUMEROUS TO COUNT

(c) NO COLONIES OBSERVED (EXCLUDING FUNGAL COLONIES)

ABBREVIATIONS

NUT AGAR: NUTRIENT AGAR

HARR: HARRISON'S AGAR

HARR + PIM: HARRISON'S AGAR WITH PIMARICIN

CT: CITRATE - TRYPTONE

CT + PIM: CITRATE - TRYPTONE WITH PIMARICIN

Ochre dry weights were calculated by drying approximately 20 ml or a known weight of ochre at 105°C for several days.

The total number of organisms in the samples according to the counts on different media are given in Table 4.3. Bacterial colonies were observed on nutrient agar plates for all the samples examined, excluding Beccles Marsh 3 and Park Farm 2 (see note b(1) in table 3). Numbers greater than  $1 \times 10^6$ /g dried ochre were recorded in all samples and the number rose above  $1 \times 10^7$ /g in ten samples. No growth, except fungal colonies, was seen on Malt<sup>Extract</sup>/agar. The only exception was one bacterial colony isolated on malt agar from Hall Farm 4b.

Heterotrophic bacteria capable of growing on the acidic media were found in 10 out of the 17 samples examined. None were found in samples from Llyn Cowlyd and Llyn Coedty. The numbers in Penygroes samples were also very low with organisms being observed on only a few plates. At Spanker organisms were observed on all the acidic media and the numbers ranged from  $1.08 \times 10^6$ /g (Spanker 4, Citrate - Tryptone + pimaricin) to  $4.10 \times 10^7$ /g (Spanker 2, Harrison's medium). Similarly the numbers at Firbeck 2 ranged from  $5.33 \times 10^4$  (Citrate - Tryptone) to  $5.33 \times 10^7$  (Harrison's). The highest number of organisms was recorded for Hall Farm 1 using Harrison's medium with pimaricin where  $1.46 \times 10^8$  organisms per gramme were found.

It should be noted that fungi were found in all sites except at Llyn Cowlyd and Llyn Coedty. All the malt extract plates had fungal colonies except those inoculated with ochre from Hall Farm 1, Park Farm 3, Penygroes 5 and Penygroes 6. However, fungi were isolated from these sites on one or more of the other plates. No attempt was made to determine the number of fungi in the ochre samples.

The total number of bacterial colonies isolated on each media are shown in Table 4.4.

Table 4.4 : Total number of bacterial colonies isolated from ochre samples using several agar media

	<u>NUMBER OF COLONIES</u>
NUTRIENT AGAR	125
MALT EXTRACT AGAR	1
HARRISON'S	41
HARRISON + PIMARICIN	76
TRYPTONE - CITRATE	16
TRYPTONE-CITRATE + PIMARICIN	21

It can be seen from Table 4.4 that the highest number of colonies were found on nutrient agar plates. A significant number of isolates were seen on all the acidic plates except malt agar. The results also show that Harrison's medium incorporating pimaricin was the most suitable medium for isolating acidophilic heterotrophs from ochre.

The most commonly observed isolates were transferred to fresh Harrison's medium (incorporating pimaricin). They are described in detail in Table 4.5. Their distribution according to media and sites is shown in Table 4.6.

TABLE 4.5 : DETAILED DESCRIPTIONS OF THE MOST COMMONLY OBSERVED  
HETEROTROPHIC BACTERIA ISOLATED FROM OCHRE ON ACIDIC  
MEDIA AND NUTRIENT AGAR

<u>ISOLATE</u>	<u>COLONY MORPHOLOGY</u>
1	CREAM, DARK CENTRE, CRENATED
2	PALE YELLOW, WHITE RIM, ENTIRE, RAISED
3	WHITE-OPAQUE, ENTIRE, FLAT
4	ORANGE CENTRE, OPAQUE-CREAM RIM, ENTIRE-IRREGULAR, RAISED
5	CREAM-WHITE, ENTIRE, RAISED
6	LARGE YELLOW WITH CREAM-WHITE RIM, CRENATED, RAISED
7	PINK, ENTIRE, RAISED

<u>ISOLATE</u>	<u>SHAPE</u>	<u>SIZE</u>	<u>MOTILITY</u>	<u>SPORES</u>	<u>GRAM</u>	<u>CATALASE</u>
1	ROD	2-5 $\mu\text{m}$	+	+	-	-
2	ROD	2 $\mu\text{m}$	+	-	-	-
3	ROD-COCC1	2-3 $\mu\text{m}$	+	-	-	-
4	ROD	2-3 $\mu\text{m}$	+	-	+	-
5	ROD	2 $\mu\text{m}$	+	-	+	-
6	ROD	3-4 $\mu\text{m}$	+	+	+	-
7	ROD	4 $\mu\text{m}$	-	-	-	-

TABLE 4.6 : THE DISTRIBUTION OF THE MOST COMMONLY OBSERVED HETEROTROPHIC BACTERIA ISOLATED FROM OCHRE

ACCORDING TO SITES AND MEDIA

<u>ISOLATE</u>	<u>NUTRIENT AGAR</u>	<u>MALT AGAR</u>	<u>HARRISON'S</u>	<u>HARRISON'S + PIMARICIN</u>	<u>TRYPTONE-CITRATE</u>	<u>TC + PIMARICIN</u>
1	S2, PF3, Pen 6	-	HFI	HF4b, S4	-	HF4b
2	S2, F2, HF1, Pen 7	-	Pen 5	-	-	S4, Pen 7
3	S2, HF1, Pen 3, Cod	-	Pen 5	S4, S2	S2	S2
4	S2, Pen 3, S4, Pen 6, HF1	-	S2, S4	S4, Pen 3	S4	S2
5	HF1, Pen 5, Pen 6, HF4b, Cod, BH	-	S2, B3	S2, S4, HF1	-	-
6	Pen 3; S4; HF1	-	S2, S4	-	S2	S4
7	-	-	S2	S2, S4	S2	S2, S4

Abbreviations:

- S - SPANKER
- PF - PARK FARM
- Pen - PENYGROES
- F - FIRBECH
- HF - HALL FARM
- Cod - LLYN COEDTY
- BH - BURNLEY HALL
- B - BECCLES MARSH

Most of the isolates were observed on both the acidic media and on nutrient agar. The exception was isolate 7 which was only seen on the acidified media. There were some types isolated on nutrient agar that were not seen on any of the acidic plates. For example, an orange, crenated, raised colony was isolated on nutrient agar from Firbeck 2, Burnley Hall, Penygroes 1 and Penygroes 7. Similar colonies were not observed on acidic media. Similarly, a yellow, irregular, slightly raised colony was isolated on nutrient agar from all the Penygroes sites and from Hall Farm 4b. This colony type was only observed once on acidic media, isolated from Spanker 2 on Harrison's agar.

It should be noted that the distribution of the isolates was not straightforward. This is best illustrated by considering isolate 3 as an example. This type was isolated on nutrient agar and acidic media from Spanker 2. However it was only isolated on acidic media from Spanker 4 and Penygroes 5 and only on nutrient agar from Hall Farm 1, Penygroes 3 and Llyd Coedty.

The prominence of the Spanker sites in table 6 should be noted. Several isolates were only found on acidic plates from these sites although they were isolated on nutrient agar from several sites. Isolate 7 was only found at Spanker. Most of the organisms isolated using the Citrate-Tryptone medium were from Spanker.

#### 4.3.2 COMPLEX-DEGRADING HETEROTROPHS

The decomposition of organic-iron complexes has been proposed as a possible mechanism of ochre formation (Section 2.5.2.3). It is thought that a variety of soil bacteria can utilize the organic component of the complexes releasing iron, which is subsequently oxidized and precipitated. Bacteria of this type were isolated from ochre samples using the Ferric Ammonium Citrate (FAC) medium of Macrae et al (1973).

	<u>g/l</u>
$K_2HPO_4$	0.1
$(NH_4)_2 HPO_4$	0.5
$MgSO_4 \cdot 7H_2O$	0.2
$Ca(NO_3)_2 \cdot 2H_2O$	0.01
Ferric Ammonium Citrate	5.0
Yeast Extract	0.2
Agar	14.0

Agar plates and 100 ml liquid portions of the medium, with and without ferric ammonium citrate, were initially prepared.

1 ml of Donegal ochre was diluted and homogenized in 200 ml of sterile distilled water. 1 ml of this suspension was diluted to 9 ml with sterile distilled water (dilution 1) in a test tube that had been pre-sterilized by heating at 160°C for several hours. This process was repeated until 3 dilutions had been obtained.

1 flask of each medium was inoculated with 1.0 ml of each dilution. Similarly, 1 plate of each medium was inoculated with 0.5 ml of each dilution.

Colonies formed on all plates except the final dilution for the medium without FAC. Colony development was extensive with a variety of small rods, spheres and larger rods being evident by microscopic examination.

Where FAC was incorporated into the medium the plates were coloured dark red and red particles could be seen under the microscope.

Liquid cultures without FAC had a "cloudy" appearance. A variety of rod and spherical shaped organisms were observed in microscope slides of the solution. In the flasks containing FAC a distinct red precipitate had formed in the bottom of the flask with a clear solution above it. A thin orange layer was seen on the surface of the cultures. Before inoculation the medium had a uniform, clear, orange colour. Under the microscope a variety of bacteria were observed including rods and spheres. Some longer rods of about 20  $\mu\text{m}$  in length were also seen. Additionally many red-orange particles were evident on the microscope slides and these were regular or angular in shape.

Aliquots from liquid cultures containing FAC and colonies from solid media, with and without FAC, were transferred onto agar plates of FAC, Nutrient agar and malt agar to characterize the isolates further. Eight isolates were obtained by maintaining and purifying on malt agar or nutrient agar. The isolates are described below.

Isolate 1A : Originally isolated on FAC agar, dilution 1 and purified by three transfers on malt agar. It formed cream-yellow colonies that were crenated and raised. Under the microscope it appeared as motile rods about 4  $\mu\text{m}$  long.

Isolate 1B : Originally isolated on FAC agar, dilution 1 and purified by three transfers on malt agar. The isolate was a motile rod about 4  $\mu\text{m}$  long forming opaque-white colonies that were entire and domed.



Isolate 2 : This type was also isolated on FAC agar, dilution 1. Pure colonies were obtained by transferring the organism 4 times on malt agar. It formed opaque-white colonies that were entire and domed. Under the microscope isolate 2 appeared as motile rods between 4 and 8  $\mu\text{m}$  long. Some rods were up to 20  $\mu\text{m}$  long. The organism was able to form straight and irregular shaped chains.

Isolate 3 : Originally isolated on FAC agar, dilution 1 and transferred twice on malt agar. It produced diffuse white colonies and appeared under the microscope as motile rods about 4-8  $\mu\text{m}$  long.

Isolate 4 : This type was isolated on FAC agar, dilution 1 and pure cultures obtained by 2 transfers on nutrient agar. Cream-white, entire, raised colonies were observed. The isolate was a motile rod 4-8  $\mu\text{m}$  long. Chains of rods were often seen. The chains were usually about 40-60  $\mu\text{m}$  long.

Isolate 5 : Originally isolated on the solid medium without FAC, dilution 2. Pure cultures were obtained by transferring the organism five times on malt agar. Initially the colonies were cream-orange, about 1-2 mm in diameter, being entire and domed. After a few days, when colonies grew and began to merge an orange colour developed at the centre whilst the edges became white or opaque. The orange portions contained unicellular rods about 4-8  $\mu\text{m}$  long. No chains of rods were observed. The white-opaque outer edges, however, contained motile chains ranging from 20  $\mu\text{m}$  to 100  $\mu\text{m}$  long. Most chains were straight but a few showed an irregular, twisted, or zig-zag shape.

On nutrient agar isolate 5 produced yellow colonies that were entire and flat. On FAC medium an opaque film developed. Motile rods were seen (4  $\mu\text{m}$  long) as were particles of ferric compounds.

After pure cultures had been obtained isolate 5 was used to inoculate a liquid FAC medium. A distinct red precipitate, indicating complete release of iron from solution, was not seen. However, microscopic examination showed that the organism was growing in the medium. Small motile rods about 8  $\mu\text{m}$  long were seen. Some larger rods up to 20  $\mu\text{m}$  long were also observed. Red particles were clearly evident indicating release of iron from the ferric ammonium citrate complex.

Isolate 7 : Originally isolated from a liquid FAC culture, dilution 1. Further cultures were obtained by two transfers on nutrient agar. The colonies were cream-white, entire and raised. Some colonies were crenated. Isolate 7 was composed of very motile rods about 4-8  $\mu\text{m}$  long. Occasionally motile rods between 8 and 16  $\mu\text{m}$  long were observed. Long, non motile, chains of rods up to 80  $\mu\text{m}$  long were also formed.

Isolate 8 : Isolated originally from a liquid FAC culture, dilution 1 and purified by three transfers on malt agar. On agar small white colonies were formed and these were entire and raised. Under the microscope chains of 4-8  $\mu\text{m}$  long rods were seen.

The isolates above were originally separated on the basis of colony appearance. When this evidence was associated with the characteristics of the isolates under the microscope all the types appear to be similar. The bacteria isolated from ochre using FAC medium were motile rods 4-8  $\mu\text{m}$  long capable of forming chains of various shapes. The chains were mostly straight or slightly curved; some were motile whilst others were not. Some isolates, 1a, 1b and 3, showed no tendency however to form chains. Occasionally different types were observed, for example, the 8-16  $\mu\text{m}$  rods associated with Isolate 7 and the irregular shaped chains found in cultures of Isolate 5.

Because of the pronounced tendency of the isolates to form chains it was thought at one stage that these heterotrophs might have been filamentous bacteria of the genus Sphaerotilus spp or Leptothrix spp. It is thought that these organisms are capable of utilizing organic complexes of iron. Ferric ammonium citrate was used by Rogers and Anderson (1976) in a medium for isolating and purifying cultures of Sphaerotilus spp (Section 5.1.15). However, the characteristic sheath associated with organisms of the Sphaerotilus - Leptothrix group was not observed in any of the isolates.

It was also postulated that the chains formed by the isolates might serve as a template for iron accumulation resulting in the formation of the iron tubes characteristic of ochre. Such a phenomenon was never observed

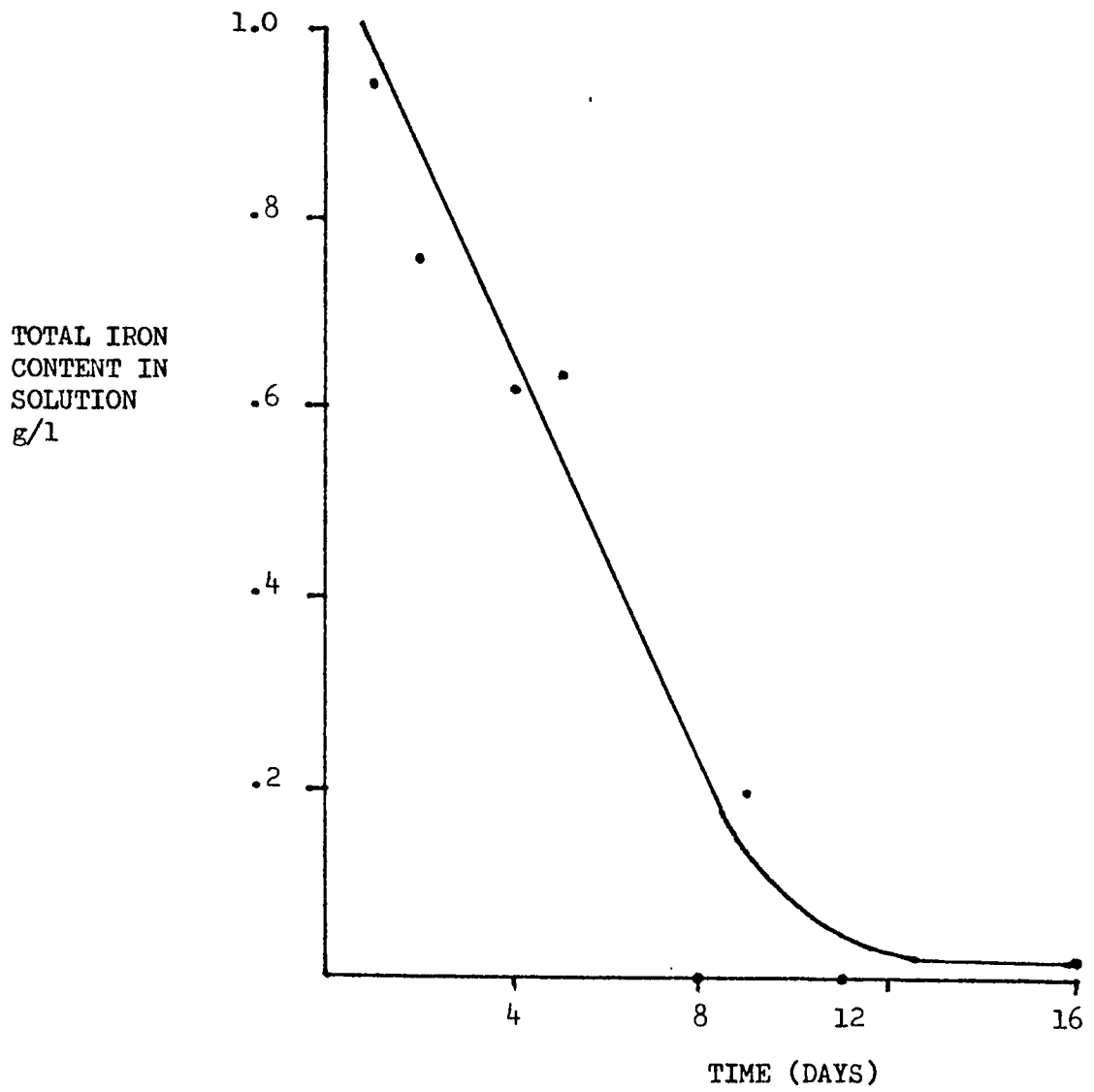
in FAC cultures. In these cultures chains of organisms were often seen as were particles of ferric compounds. The chains and particles were always independent of one another and deposition of iron onto a bacterial chain was not encountered.

#### THE RATE OF IRON RELEASE IN FERRIC AMMONIUM CITRATE MEDIUM

Two 250 ml portions of FAC were prepared and inoculated from existing FAC cultures. Total iron in solution was determined at regular intervals by withdrawing 2 ml and filtering through a 0.22  $\mu$ m Millipore filter to remove any ferric precipitate. 1 ml of filtrate was diluted to 50 ml with distilled water. 4 ml was withdrawn and acidified with 1 drop of concentrated hydrochloric acid. Total iron in solution was determined by atomic absorption spectrophotometry and compared with standard solutions of ferric nitrate in the range 0  $\mu$ g/ml to 10  $\mu$ g/ml. It was sometimes necessary to dilute the sample to bring the iron concentration within the range of standards.

The results shown in figure 4.6 reveal that most of the iron was removed from solution within about eight days. All iron was removed after 12-16 days.

FIGURE 4.6 PLOT OF TOTAL IRON CONTENT FOR INOCULATED FERRIC AMMONIUM CITRATE MEDIUM



The curve in figure 30 follows the exponential pattern characteristic of bacterial growth. The initial plateau, corresponding to a slow growth rate, was not observed, presumably because iron content was not determined at regular intervals within the first 24 hours of growth.

COMPLEX DEGRADING ORGANISMS AS A COMPONENT OF THE TOTAL MICROBIAL  
POPULATION OF OCHRE

An experiment was conducted to determine the relationship between complex-degrading organisms and the total microbial numbers determined by isolating bacteria on nutrient agar.

Plates of FAC and nutrient agar were prepared.

Initially 1.0 ml of ochre from Penygroes (collected 9.8.1982) and Llyn Coedty was diluted to 100 ml with sterile distilled water (dilution 1). The suspension was homogenized for one minute using a semi-sterilized Silverson homogenizer. Plates inoculated from dilution 1 had numbers too high to count and are ignored in the results. 1 ml of homogenized solution was diluted to 9 ml with sterile distilled water in a pre-sterilized test tube (heated at 160°C for several hours). This process was repeated until 3 tubes, 3 dilutions, were obtained. 1 plate of each medium was inoculated with 0.5 ml of each dilution.

The dry weights of ochre were determined by drying a known volume at 105°C for several days.

The colonies that formed on the plates, especially on FAC agar, tended to spread and therefore the experiment was repeated with ochre from Penygroes (collected 4.3.1983). The same procedure was used except that 2 ml of ochre was initially diluted to 100 ml with sterile tap water and 0.2 ml of each dilution was used to inoculate the agar plates.

The results are shown in table 4.7 and table 4.8.

No strong trend towards either of the media emerges from the results. In some sites the number of organisms growing on nutrient agar was greater than on FAC. For example, in Penygroes 7:2 (dilution 2) 8 colonies were observed on nutrient agar, whereas none were observed on FAC. Conversely in other sites a greater number of organisms was found on FAC medium. The number of organisms per gramme dried ochre isolated on FAC medium (dilution 2) from Llyn Coedty was  $2.9 \times 10^8$ . The number determined on nutrient agar was  $2.7 \times 10^7$  organisms per gramme. Thus at Llyn Coedty (dilution 2) the number of organisms seen on FAC was over 10 times as great as the number seen on nutrient agar.

Much variation was encountered between the two sampling dates. The number of colonies on nutrient agar for Penygroes 1, dilution 2, on 9.8.1982 was 3 whilst the number on FAC was too high to determine. On 4.3.1983 the results were reversed. The number of colonies on nutrient agar was too numerous to count, whereas only one colony was observed on the FAC plate.

The ratio of bacterial numbers on FAC to the number growing on nutrient agar could not be calculated for 10 out of the 27 results shown in Table 8.

This was because the number of organisms on both media were too numerous to count or because no growth had occurred. Of the 17 results calculated, 10 showed a ratio greater than 1.0 indicating that the number of organisms on FAC was higher than the number on nutrient agar. The remaining 7 results showed a higher number of observations on nutrient agar. This

TABLE 4.7 : PLATE COUNTS AND TOTAL NUMBERS OF BACTERIA IN OCHRE SAMPLES  
AS DETERMINED BY ISOLATING ON NUTRIENT AGAR AND FAC<sup>(1)</sup>

<u>SITE</u>	<u>MEDIUM<sup>(1)</sup></u>	NUMBERS PER GRAMME OVEN DRIED		
		OCHRE (NUMBER OF OBSERVATIONS PER PLATE)		
		<u>DILUTION<sup>(4)</sup>(5)</u>		
		<u>1</u>	<u>2</u>	<u>3</u>
LLYN COEDTY	NUT	2.1x10 <sup>7</sup> (8)	2.7x10 <sup>7</sup> (1)	NG
	FAC	>1.3x10 <sup>8</sup> (>50)	2.9x10 <sup>8</sup> (11)	NG
PENYGROES 1 <sup>(2)</sup>	NUT	7.9x10 <sup>6</sup> (20)	>1.6x10 <sup>8</sup> (>40)	7.9x10 <sup>7</sup> (2)
	FAC	>1.2x10 <sup>7</sup> (>30)	4.0x10 <sup>6</sup> (1)	8.7x10 <sup>8</sup> (22)
PENYGROES 1 <sup>(3)</sup>	NUT	TN	3.0x10 <sup>6</sup> (3)	9.9x10 <sup>6</sup> (1)
	FAC	TN	>4.0x10 <sup>7</sup> (>40)	1.98x10 <sup>7</sup> (2)
PENYGROES 5 <sup>(2)</sup>	NUT	>4.1x10 <sup>6</sup> (>50)	6.5x10 <sup>6</sup> (8)	1.6x10 <sup>7</sup> (2)
	FAC	>3.3x10 <sup>6</sup> (>40)	1.6x10 <sup>7</sup> (20)	2.4x10 <sup>7</sup> (3)
PENYGROES 5 <sup>(3)</sup>	NUT	TN	4.8x10 <sup>6</sup> (2)	4.8x10 <sup>7</sup> (2)
	FAC	TN	>9.5x10 <sup>7</sup> (>40)	NG
PENYGROES 7:1 <sup>(2)</sup>	NUT	>4.6x10 <sup>6</sup> (>50)	2.8x10 <sup>7</sup> (30)	3.7x10 <sup>7</sup> (4)
	FAC	>4.6x10 <sup>6</sup> (>50)	2.6x10 <sup>7</sup> (28)	2.8x10 <sup>7</sup> (3)
PENYGROES 7:2 <sup>(3)</sup>	NUT	TN	4.7x10 <sup>7</sup> (8)	NG
	FAC	TN	NG	NG
PENYGROES 7:3 <sup>(3)</sup>	NUT	TN	6.25x10 <sup>7</sup> (5)	>1.25x10 <sup>9</sup> (>10)
	FAC	TN	5.00x10 <sup>8</sup> (>40)	NG
PENYGROES 7:4 <sup>(3)</sup>	NUT	TN	TN	8.3x10 <sup>7</sup> (2)
	FAC	TN	TN	4.2x10 <sup>7</sup> (1)

(1) NUT = NUTRIENT AGAR; FAC = FERRIC AMMONIUM CITRATE MEDIUM

(2) SAMPLES COLLECTED ON 4.3.83

(3) SAMPLES COLLECTED ON 9.8.82

(4) FOR SAMPLES COLLECTED ON 9.8.82 AND LLYN COEDTY DILUTIONS 1 TO 3 REPRESENT DILUTIONS OF 2x10<sup>3</sup>, 2x10<sup>4</sup> AND 2x10<sup>5</sup> RESPECTIVELY

FOR SAMPLES COLLECTED ON 4.3.83 DILUTIONS 1 TO 3 REPRESENT DILUTIONS OF 2.5x10<sup>3</sup>, 2.5x10<sup>4</sup> AND 2.5x10<sup>5</sup> RESPECTIVELY

(5) NG; NO GROWTH OBSERVED TN; TOO NUMEROUS TO COUNT, MANY SPREADING COLONIES.

TABLE 4.8 A COMPARISON BETWEEN THE NUMBERS OF COMPLEX DEGRADING BACTERIA<sup>(1)</sup>  
AND THE NUMBERS DETERMINED ON NUTRIENT AGAR FROM OCHRE SAMPLES

<u>SITE</u>	<u>NUMBER OF COMPLEX DEGRADERS<sup>(1)</sup></u>		
	<u>NUMBER ON NUTRIENT AGAR</u>	<u>DILUTION<sup>(4)(5)(6)</sup></u>	
	<u>1</u>	<u>2</u>	<u>3</u>
LLYN COEDTY	>6.2	10.7	NG
PENYGROES 1 (2)	>1.5	< 0.03	11.0
PENYGROES 1 (3)	TN	>13.0	2.0
PENYGROES 5 (2)	TN	2.5	1.5
PENYGROES 5 (3)	TN	>19.8	∅
PENYGROES 7:1 (2)	TN	0.93	0.76
PENYGROES 7:2 (3)	TN	0.0	NG
PENYGROES 7:3 (3)	TN	>8.0	0.0
PENYGROES 7:4 (3)	TN	TN	0.51

(1) NUMBERS OF COMPLEX DEGRADING BACTERIA WERE DETERMINED ON FERRIC AMMONIUM CITRATE MEDIUM

(2) SAMPLES COLLECTED 4.3.83

(3) SAMPLES COLLECTED 9.8.82

(4) THE MAGNITUDE OF DILUTIONS IS EXPLAINED IN THE NOTES ACCOMPANYING TABLE 4.7 (NOTE 4).

(5) NG; NO GROWTH OBSERVED ON BOTH MEDIA. TN: NUMBERS TOO NUMEROUS FOR DETERMINING ACCURATE RESULTS

(6) A RATIO OF 0.0 INDICATES NO GROWTH ON FAC MEDIUM.



indicates that overall a greater number of organisms were encountered on FAC medium suggesting that complex-degrading organisms were more numerous than the total bacterial numbers determined by plating on nutrient agar. However, the variation seen in the results and the possible inhibition of some bacteria by nutrient agar would favour the conclusion that no significant difference existed between the total number of organisms in the sample, as determined by isolating on nutrient agar, and the number of complex-degrading bacteria.

This conclusion confirms the generally accepted view (Section 2.5.2.3) that most bacteria present in the natural environment, especially in soils, are capable of degrading organic complexes by utilizing the organic component. Thus it is to be expected that organisms growing on nutrient agar would also grow on the FAC medium.

#### 4.4 DISCUSSION

At most of the sites examined sheathed filamentous bacteria were observed. In a few sites none or very few filaments were seen. The low numbers encountered at Spanker can be related to the acidity of the site. The optimum pH of the sheathed filamentous bacteria is between 6.0 and 7.0 whereas the pH of the Spanker sites was less than, or just above, 4.0. However, acidity does not explain why filamentous bacteria were not found in the ochre at Penygroes 1 and Penygroes 3 where the pH was 6.4. Similarly at Gallt yr Hulledrem and Morfa Glas, filamentous bacteria were numerous although undried pH values of 4.6 and 4.8 were recorded.

Several types of sheathed filamentous bacteria were seen and most were assigned to the genus Leptothrix spp. Gallionella spp were observed but their occurrence was not as widespread as the filamentous bacteria. In the literature Sphaerotilus spp are commonly associated with ochre but no Sphaerotilus type organisms, characterized by chains of cells within a distinct sheath, were observed during the present study. Not all authors would agree with the classification adopted since some consider

Leptothrix spp to be a type of Sphaerotilus spp (see Section 2.3.1.2)

Scanning electron micrographs of ochre revealed a variety of structures, but filaments and accumulations of ferric iron compounds were predominant. It was shown that the filaments observed during light microscopy were tubular and composed primarily of iron. These tubes or filaments were usually surrounded by ferric particles.

The abundance of filaments, devoid of living cells suggests that the sheathed filamentous bacteria present in ochre are capable of producing "tubes" prolifically. This confirms the observation made by Van Veen et al (1978) that Leptothrix ochracea could produce large masses of empty sheaths within a short time.

The widespread distribution of Thiobacillus ferrooxidans was surprising. T.ferrooxidans was isolated from ochre using autotrophic 9K medium. It is known that this bacterium grows at pH values below 4.5 with an optimum of about 2.5 (Section 2.4.1) but high counts of Thiobacilli were obtained from sites having a much higher pH. For example, the highest number found was  $3.43 \times 10^4$  organisms per gramme oven dried ochre. This was obtained at Hall Farm 1, a site having an undried pH of 6.5. In fact only 2 out of the 14 samples where T.ferrooxidans was detected had a pH value less than 4. This suggests that T.ferrooxidans exists in acidic microenvironments within the drainage system or soil.

It was suggested in section 3.3 that polysaccharide producing acidophilic bacteria were present in some ochre samples on the basis of high ribose : xylose ratios in ochre hydrolysates. The isolation of bacteria from ochre on acidic media strengthens this conclusion although it was not demonstrated that the acidophilic isolates produced extracellular polysaccharide. The incorporation of the anti-fungal agent pimaricin in Harrison's agar (Harrison et al, 1980) gave a particularly useful medium for isolating these bacteria.

Acidophilic heterotrophs were found in 10 out of 17 samples examined. A variety of different organisms were isolated, most of which were also capable of growing on nutrient agar at neutral pH values.

Organisms capable of degrading organic complexes were isolated from ochre using a medium based on ferric ammonium citrate. Growth in the media resulted in the release and subsequent precipitation of ferric iron compounds from the soluble ferric complex. There appeared to be no difference between the number of complex degraders and the total number of bacteria isolated on nutrient agar. This suggests that most organisms in the samples were capable of utilizing the organic component of iron-organic complexes.

The organisms isolated using FAC medium were essentially small, motile, rod shaped bacteria with a tendency to form chains of cells. These chains were similar to the chains associated with filamentous bacteria of the Sphaerotilus - Leptothrix group. However, the later organisms are characterized by a sheath enclosing a chain or filament of cells. Sheaths were not observed in isolates from FAC medium. Although these isolates were capable of releasing iron from complexes, deposition on single cells or chains was not encountered. This suggests that the release of iron is of no significance to these bacteria. They utilize the organic matter of the complex irrespective of the metal component.

It has been claimed that ochre can form as a result of the breakdown of iron-organic complexes by bacteria (Section 2.5.2.3). The presence of such bacteria in ochre samples confirms that this mechanism is feasible. However, the importance of complex degradation as a mechanism of ochre formation depends entirely on the amount of complexed iron in the drainage water. This aspect will be discussed fully in chapter 8.

The chemical and microbial characteristics of 17 ochre samples are shown in table 4.9. The results are derived from tables in Chapters 3 and 4. The data shown can be rationalized by initially examining the extremes. The sites at Spanker can be considered as representing one extreme. Here the pH of the samples was low as was the organic matter content. None or very few filamentous bacteria were found but Thiobacillus ferrooxidans and acidophilic heterotrophs were numerous. The number of acidophilic heterotrophs were high in relation to the total count on nutrient agar. At Spanker 2 the ratio of number of bacteria on acidic plates to the number on nutrient agar was 8.35. The deposits at these sites can be correctly described as pyritic ochre.

At the other extreme, sites such as Park Farm 2 and Park Farm 3 can be considered. These had a higher pH (about 5.5) and a high organic matter content. Filamentous bacteria were numerous but no Thiobacilli or acidophilic heterotrophs were found. These are typical filamentous ochre sites.

Understanding the nature of ochre is made difficult by the numerous samples that fall between the two poles described above. Between the extreme pyritic and filamentous types various combinations can be observed.

Samples from Penygroes 1 and Penygroes 3 represent a slight shift away from purely pyritic ochre. At these sites the pH was about 5.5 and the organic carbon contents of 0.8 and 3.2% respectively were similar to those obtained at Spanker. Filamentous organisms were not found in the ochre samples but they were observed in the drainage water indicating their presence but at low numbers. Thiobacillus ferrooxidans and the related heterotrophs were found at both sites. In the case of Penygroes 1 the number of Thiobacilli was lower than at Spanker but higher numbers were observed at Penygroes 3. The number of acidophilic heterotrophs at Penygroes was lower than at Spanker. The numbers of these organisms were

also less than the total count on nutrient agar showing that their contribution to the total microbial population was less than at Spanker. Similarly, at Hall Farm 4b the number of Thiobacilli and acidophilic heterotrophs were high but the numbers of <sup>acidophilic</sup> heterotrophs were low compared to the total bacterial population. No filamentous bacteria were found at Hall Farm 4b but they were observed in samples from adjacent drain lines.

Several samples could be described as containing a mixture of characteristics representative of pyritic and filamentous ochre types. The most striking example is Hall Farm 1 having a pH of 6.5 and a high organic carbon content of 6.0%. Filamentous bacteria and Gallionella spp were abundant. Additionally the highest counts of Thiobacilli and acidophilic heterotrophs  $3.43 \times 10^4$  and  $1.02 \times 10^8$  organisms per gramme dried ochre respectively, were found here. The number of acidophilic heterotrophs was almost double the count obtained on nutrient agar. Similar combinations of features were seen at Firbeck 2 and Beccles Marsh 3.

Other samples show a closer association with the extreme filamentous ochre type. At Persehall 6  $4.9 \times 10^3$  T.ferrooxidans were found per gramme of dried ochre in addition to filamentous bacteria. At this site, having a pH of 6.5, acidophilic heterotrophs were not isolated. At Penygroes 5 the undried pH was 6.0 Filamentous bacteria and Gallionella spp were observed. Although some acidophilic heterotrophs were isolated they represented a very small proportion of the total microbial population and Thiobacilli were not found.

Penygroes 6, Penygroes 7:1, Llyn Cowlyd and Llyn Coedty represent samples very close to "pure" filamentous ochre. The pH of these sites was about 6.0 and filamentous bacteria were numerous. T.ferrooxidans was isolated but at numbers less than 50 organisms per gramme dried ochre. Acidophilic heterotrophs were only found at Penygroes 7:1 where the ratio of acidophiles to total bacterial numbers was 0.004.

TABLE 4.9 : A SUMMARY OF THE CHEMICAL AND MICROBIOLOGICAL CHARACTERISTICS OF SEVERAL OCHRE SAMPLES

	pH (WET)	pH (DRY)	% ORG CARBON	FILAMENTOUS BACTERIA	NUMBERS/g THIOBACILLUS FERROOXIDANS	DRIED OCHRE ACIDOPHILIC HETEROTROPHS	RATIO TOTAL NUMBERS OF ACIDOPHILIC METERO-TROPHS: COUNTS ON NUTRIENT AGAR
SPANKER 2	3.5	2.4	3.1	-	150	$1.29 \times 10^7$	8.35
SPANKER 4	3.4	2.2	1.1	+ BUT NOT NUMEROUS	530	$1.59 \times 10^7$	0.32
FIRBECK 2	6.2	6.5	5.8	+	$1.01 \times 10^3$	$1.86 \times 10^7$	< 0.12
BURNLEY HALL *(1)	6.5	-	-	-(1)	190	-	0
HALL FARM 1	6.5	7.6	6.0	+	$3.43 \times 10^4$	$1.02 \times 10^8$	1.70
HALL FARM 4b	6.4	7.5	6.1	-	$1.07 \times 10^3$	$1.46 \times 10^5$	0.01
BECCLES 3	6.4	7.3	4.8	+	$1.09 \times 10^3$	$1.40 \times 10^6$	(4)
PERSEHALL 6	6.5	7.5	3.9	+	$4.92 \times 10^3$	-	0
PARK FARM 2	5.6	5.6	*(2)	+	-	-	0
PARK FARM 3	5.4	6.3	12.4	+	-	-	0
PENYGROES 1	5.5-5.8	6.9	0.8	-	30	$4.9 \times 10^5$	0.009
PENYGROES 3	5.4	6.8	3.2	-	$5.2 \times 10^3$	$8.7 \times 10^5$	0.67
PENYGROES 5	5.7-6.2	7.1	1.9	+	-	$1.4 \times 10^6$	0.05
PENYGROES 6	5.7	6.8	6.9	+	20	-	0
PENYGROES 7:1	5.5-6.2	7.1	3.4	+	40	$1.6 \times 10^5$	0.004
LLYN COWLYD	6.1	4.9	4.9	+	40	-	0
LLYN COEDTY	5.8	4.9	5.9	+	20	-	0

FOR EXPLANATORY SHEETS SEE NEXT PAGE

- (1) SAMPLE FROM BURNLEY HALL WAS A WHITE DEPOSIT, APPARENTLY CONTAINING NO IRON
- (2) INSUFFICIENT MATERIAL WAS AVAILABLE FOR ORGANIC CARBON DETERMINATION
- (3) MEAN OF COUNTS ON ACIDIC PLATES
- (4) NUTRIENT AGAR PLATES LOST WHEN TEMPERATURE OF INCUBATOR ACCIDENTALLY RAISED

From the preceding discussion it can be concluded that two extreme types of ochre can be described. Pyritic ochre is characterized by a low pH (less than 3.0 or 4.0), a low organic matter content, no filamentous bacteria but high numbers of T.ferrooxidans and acidophilic heterotrophs. These heterotrophic organisms form a large proportion of the total heterotrophic population. Filamentous ochre on the other hand has a pH of about 6.0 and a high organic matter content as compared to pyritic sites. Filamentous bacteria are numerous whilst acidophilic autotrophs and heterotrophs are absent. Between these extremes many different types of ochre exist. Their pH can vary from 4.0 to over 6.0 as can their organic matter content. Filamentous bacteria, T.ferrooxidans, and acidophilic heterotrophs can be present or absent in any combination. Thiobacilli are discouraged by high pH values but they can be present in ochre samples of high pH by the existence of acidic microenvironments in the drainage system. Conversely filamentous bacteria will be more numerous in the less acidic samples. The acidophilic heterotrophs are also affected by pH. However examination of the different isolates showed that many were capable of growing at neutral pH values. Only one isolate was found exclusively on acid media. A decline in the importance of acidophilic heterotrophs as the samples become more "filamentous" is associated with a reduction in their numbers relative to the total number of bacteria present.

The different types of ochre outlined above can be explained as a transition from pyritic to filamentous ochre. Initially ochre formation would be based on the oxidation of pyrite by T.ferrooxidans. As the amounts of pyrite were reduced the pH would gradually rise and filamentous bacterial would increase in importance eventually replacing the acidophilic autotrophs and heterotrophs. Alternatively the different ochre classes can be described in terms of a coexistence. Pyrite can be present in the catchment area of the drainage system but in quantities that are too low



for the development of extreme acidities resulting from pyrite oxidation by T.ferrooxidans. The amount of pyrite is however sufficient to support significant numbers of Thiobacilli. Under these conditions filamentous bacteria would also thrive resulting in many types of ochre of various compositions.

Whichever of these explanations is correct the transition or coexistence is not dependent upon time only. The results in table 9 show that as much variation is often seen from site to site within an area as is seen between different areas.

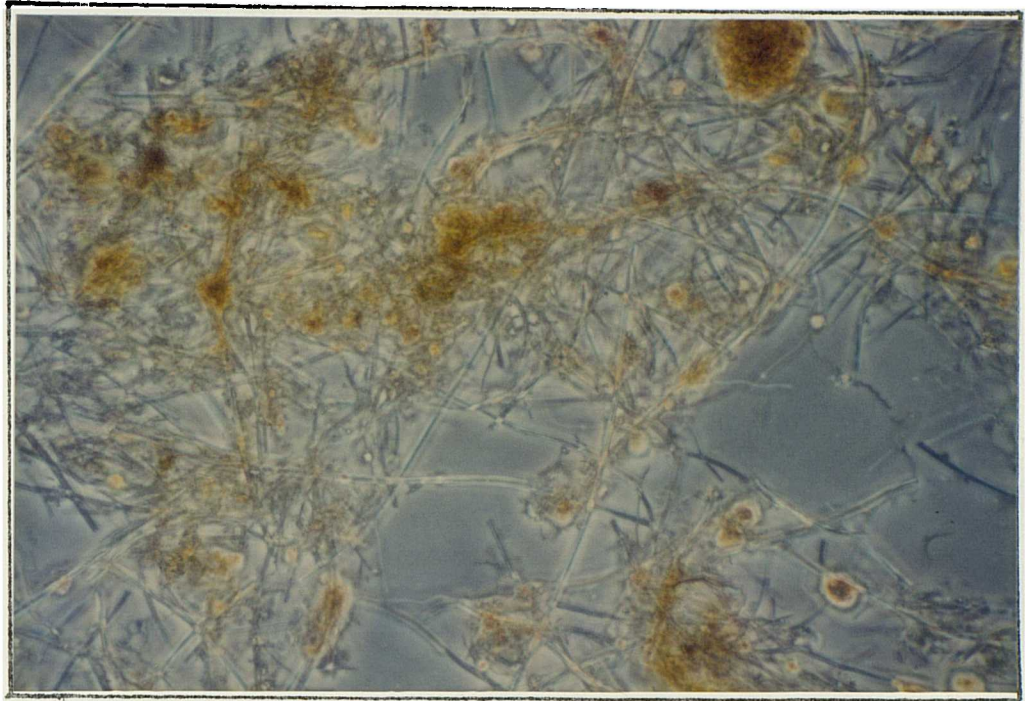


Plate 4:1 Filamentous ochre from Sychnant Pass :  
Phase contrast light microscope X250. Organisms of  
Type 1 and 2 are illustrated.

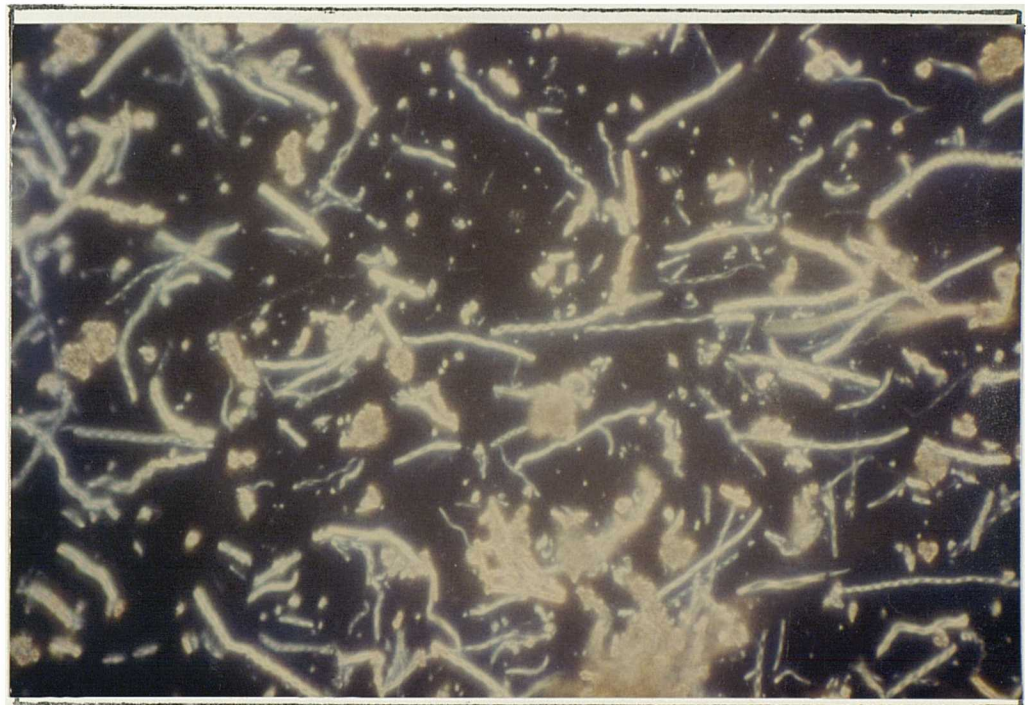
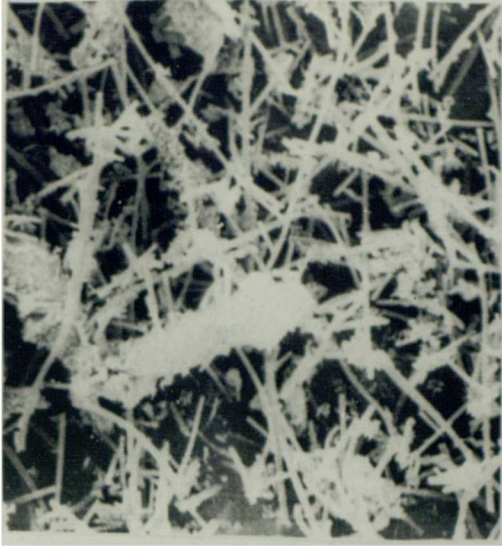
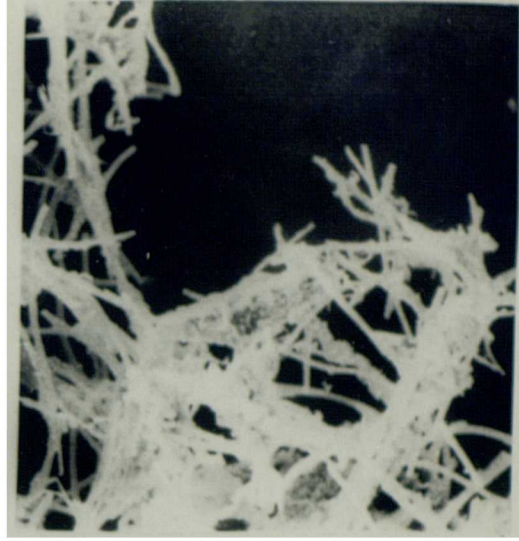


Plate 4:2 Filamentous ochre from Park Farm 7 :  
Phase contrast (dark field) light microscope X250.  
Organisms of Type 4 are illustrated.



4.3

10um



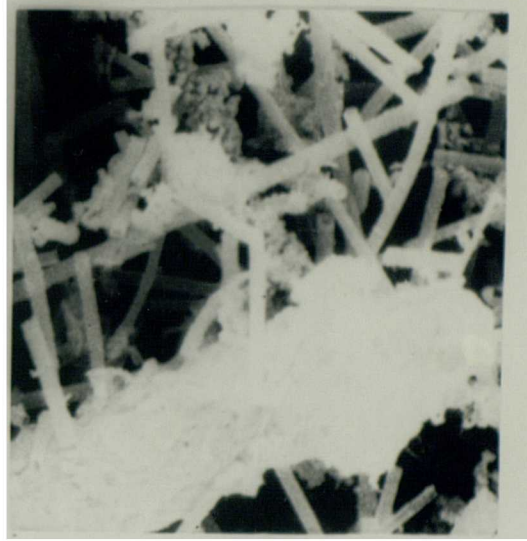
4.4

10um



4.5

2um



4.6

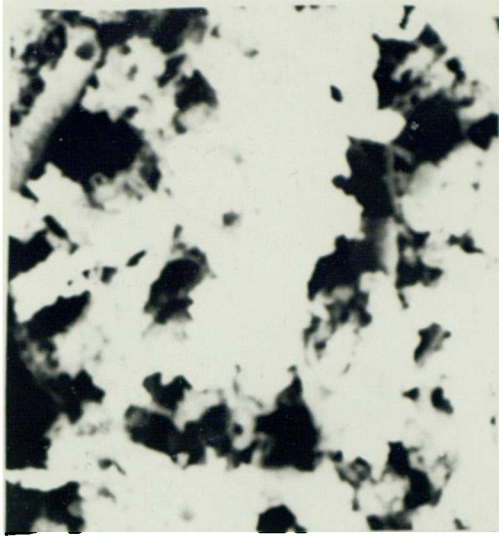
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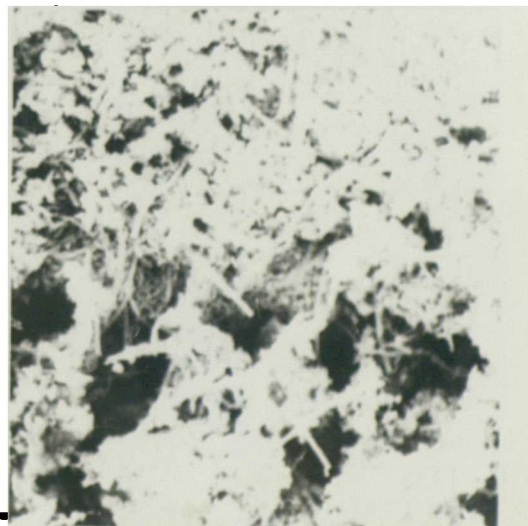
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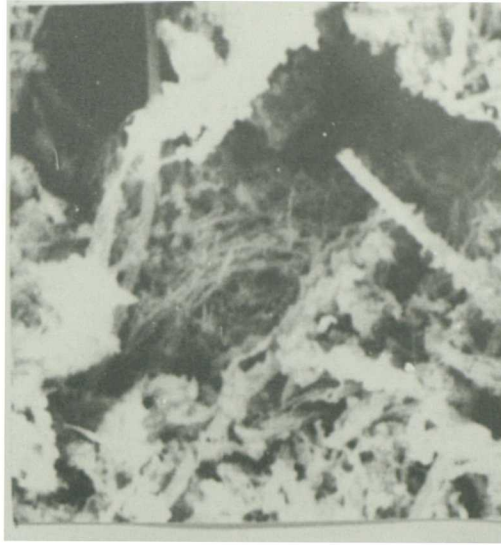
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4.21

5um

Scanning electron micrographs of gold coated ochre samples.

<u>Plate</u>	<u>Site</u>	<u>Magnification</u>
4.3	Donegal	X 700
4.4	Sychnant Pass	X 1000
4.5	Llyn Coedty	X 5000
4.6	Donegal	X 2000
4.7	Donegal	X 3000
4.8	Llyn Cowlyd	X 5000
4.9	Llyn Cowlyd	X 5000
4.10	Llyn Cowlyd	X 2000
4.11	Llyn Coedty	X 700
4.12	Llyn Coedty	X 2000
4.13	Llyn Coedty	X 2000
4.14	Llyn Coedty	X 5000
4.15	Sychnant Pass	X 3000
4.16	Sychnant Pass	X 7000
4.17	Llyn Cowlyd	X 7000
4.18	Llyn Cowlyd	X 7000
4.19	Donegal	X 5000
4.20	Llyn Coedty	X 700
4.21	Llyn Coedty	X 2000

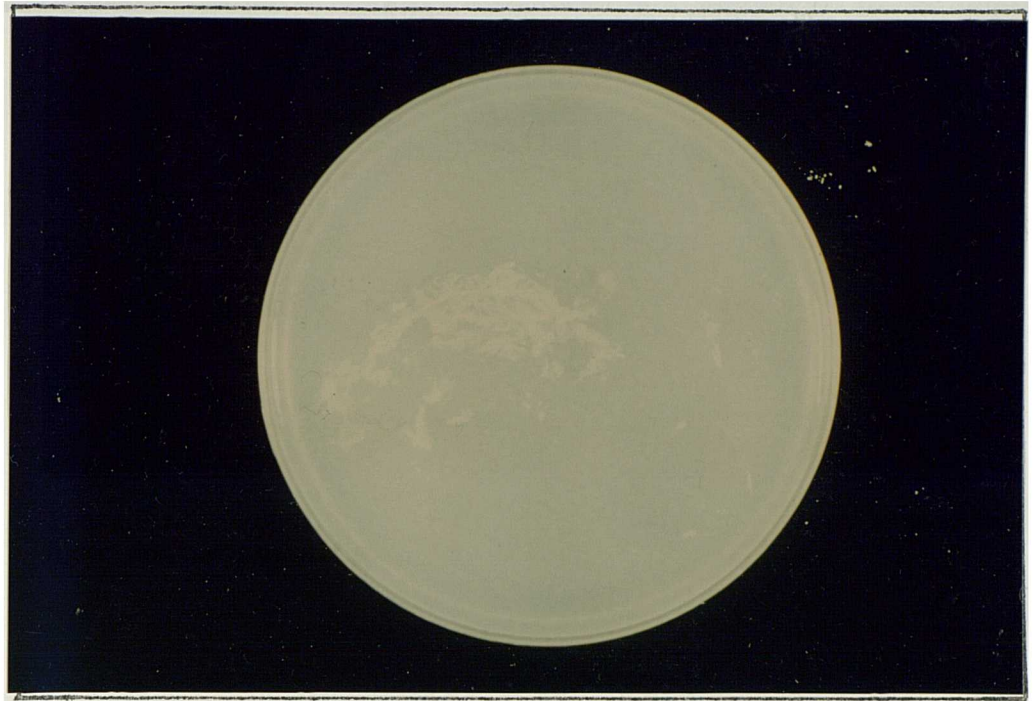


Plate 4:22 Bacterial colonies growing on Harrison's acidic agar medium containing pimarinic.



Plate 4:23 Bacterial and fungal colonies growing on Harrison's acidic agar medium without pimarinic.

## 5. THE CULTURE OF SHEATHED FILAMENTOUS BACTERIA

### 5.1 STANDARD MEDIA

Many media for growing sheathed filamentous bacteria have been described in the literature. Several of the media were investigated as a means of isolating these bacteria from ochre.

1. S medium (Lackey and Wattie, 1940)
2. Stokes' medium (Stokes, 1954)
3. PGY medium (Takiguchi et al, 1978)
4. Armbruster's BOD - Lactate medium (Armbruster, 1969)
5. TCS agar (Farquhar and Boyle, 1971)
6. Heterotrophic medium (Godinho-orlandi, 1980)
7. I medium (Van Veen, 1973)
8. CGY medium (Dondero et al, 1961)
9. SCY medium (Mulder and Van Veen, 1963)
10. A1 and A2 (Mulder and Van Veen, 1963)
11. S and S+ agar (Mulder and Van Veen, 1963)
12. Manganese agar (Mulder and Van Veen, 1963)
13. GG medium (Dondero et al, 1960)
14. L medium (Lackey and Wattie, 1940)
15. Ferric ammonium citrate medium (Rogers and Anderson, 1976)
16. Ferrous sulphide medium (Kucera and Wolfe, 1957)
17. Phaup's medium (Phaup, 1968)
18. Manganous carbonate agar (Van Veen et al, 1978)

All the media were prepared as indicated in the original reference unless stated otherwise. Some of the original chemicals were replaced with an available equivalent.



## INOCULATION OF MEDIA

Where possible the media were inoculated on the day of sampling. They were used on various occasions with ochre collected from several sites. The schemes of dilution and inoculation are described below.

The samples were not homogenized after their initial dilution since it was thought that this would be too severe for the sheathed filamentous bacteria. Additionally, Van Veen (1973) had suggested that normal techniques should be avoided when cultivating sheathed filamentous bacteria since such treatment would liberate a large number of unicellular bacteria from flocs.

### Ochr Cefn Isaf

Liquid media were inoculated using undiluted ochre in a Most Probable Number technique employing 3 inoculations with 1.0 ml aliquots, 3 with 0.1 ml, and 3 with 0.01 ml. An uninoculated control was also prepared.

Solid media were inoculated with 0.5 ml of undiluted ochre per plate. Uninoculated controls were also prepared.

### Sychnant Pass

Ochre was collected from a roadside ditch and 1 ml of undiluted suspension was used as an inoculum.

### Donegal

1.0 ml of ochre was diluted with 200 ml of sterile distilled water and thoroughly shaken. 1.0 ml was withdrawn and added to 9 ml of pre-sterilized distilled water in a sterile test tube. This stage was repeated until 3 tubes (3 dilutions) had been prepared. 1.0 ml of each dilution was used as an inoculum.

### Llyn Cowlyd

2.0 ml of ochre, suspended in drainage water, was diluted in 200 ml of sterile distilled water and thoroughly shaken (dilution 1). 2.0 ml was added to 18.0 ml of sterile distilled water in a conical flask. This process was repeated four times to obtain dilutions 2 to 5. 0.5 ml of each dilution was used to inoculate flasks or plates of the medium.

#### 5.1.1 S medium (Lackey and Wattie, 1940)

	<u>g/l</u> (distilled water)
Na <sub>2</sub> HPO <sub>4</sub>	0.05
NaCl	0.015
KCl	0.007
MgSO <sub>4</sub>	0.005
Peptone	0.1
Dextrose	0.5

S medium or synthetic sewage medium was originally used by Lackey and Wattie (1940) for growing Sphaerotilus natans isolated from sewage sludge. Chang et al (1979) used the medium to investigate the inhibitory effects of different iron compounds on the growth of Sphaerotilus strains isolated from laboratory activated sludge and from the American Type Culture Collection.

Waitz and Lackey (1959), using S medium at room temperature, found that S.natans grew as white, fluffy, cotton-ball like colonies throughout the medium. After about 24 hours growth changed into a gelatinous/strand type.

The medium was inoculated with ochre from Ochr Cefn Isaf. After 8 days the cultures were cloudy with a thin "sediment" in the base of the flasks. The "sediment" contained motile and non-motile rods of various sizes. No growth was apparent in the uninoculated controls. Ochre from Sychnant Pass

yielded similar results. A large number of rod shaped bacteria about 5  $\mu\text{m}$  long were seen. Fungal filaments were also numerous in the cultures.

When S medium was originally used (Lackey and Wattie, 1940) the cultures were continually aerated. To reproduce these conditions 100 ml portions of the medium were inoculated with Donegal ochre. Air was slowly bubbled through the media via sterile plastic tubing. Incoming air was sterilized using a fibreglass filter. Small white flocs developed in the cultures and these formed cotton-wool like growth. These contained branching fungal filaments and many unicellular bacteria. The bacteria included small motile rods, cocci and some chains of rod-shaped organisms. Aliquots from the cultures were transferred onto nutrient agar to characterize the isolates further. Small white-opaque colonies developed within four days. These colonies contained motile rods 4-8  $\mu\text{m}$  long and non-motile chains of rods.

Although bacteria capable of forming chains were isolated from ochre using S medium sheathed chains characteristic of Sphaerotilus spp were not encountered in liquid media or when isolates were grown on agar plates.

#### 5.1.2 Stokes' medium. (Stokes, 1954)

	<u>g/l</u> (Tap Water)
Glucose	1.0
Peptone	1.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2
CaCl <sub>2</sub>	0.05
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.01
Agar	12.5

Stokes (1954) used this medium to isolate Sphaerotilus natans from a variety of aquatic habitats. Colonies of the organism appeared "flat, dull, white and cottony." Microscopic examination revealed chains of rod shaped bacteria within a sheath. According to Waitz and Lackey (1959),

who used Stokes' medium for growing S.natans, growth on solid media was characterized by "outgrowths along the lines of inoculation which had ramose edges and a wavy interlaced centre." Takiguchi et al (1978) used Stokes' medium in a study on growth inhibition of Sphaerotilus and Beggiatoa. Chang et al (1979) used a modification of this medium for maintaining pure cultures of S.natans.

When plates of the medium were inoculated with ochre from Ochre Cefn Isaf colonies formed on all plates within 7 days except on the uninoculated controls. However all the colonies were fungal as confirmed by microscopic examination of prepared slides.

When ochre from Llyd Cowlyd was used as an inoculum fungal colonies were not isolated. Within 6 days growth was seen on plates from the two lowest dilutions. Examination of the colonies revealed motile rods about 20  $\mu$ m long. Purple colonies probably Chromobacterium violacea were also observed.

Colonies similar to those described by Stokes (1954) or Waitz and Lackey (1959) were not seen. Additionally, forms similar to S.natans were not found by examining colonies under the microscope.

### 5.1.3 PGY medium (Takiguchi et al, 1978)

g/l (Tap Water)

Peptone 2.0

Yeast autolysate 1.0

Glucose 0.5

pH was adjusted to 7.0 and to solidify the

medium agar was added at 1.5%

PGY medium was used by Takiguchi et al (1978) to maintain S.natans.

Yoshikawa et al (1979) used liquid cultures and agar plates of PGY to maintain S.natans.

All flasks inoculated with ochre from Ochr. Cefn Isaf developed a dark green colour within 7 days. A gelatinous orange material was seen on the surface and a white film was evident on the sides of the flasks. Prepared slides contained unicellular motile and non motile rods. A similar result was obtained when liquid media were inoculated with ochre from Sychnant Pass. Extensive colony development was seen on agar plates inoculated with ochre from Ochr Cefn Isaf and Llyn Cowlyd. The colonies were composed of motile, sporulating, rod-shaped bacteria up to 10  $\mu\text{m}$  long.

#### 5.1.4 Armbruster's BOD-Lactate medium (Armbruster 1969)

	<u>mg/l</u> (Distilled Water)
Sodium Lactate	100
$\text{NH}_4\text{Cl}$	1.7
$\text{KH}_2\text{PO}_4$	8.5
$\text{K}_2\text{HPO}_4$	21.5
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	34.4
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	22.5
$\text{CaCl}_2$	27.5
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	0.25

This liquid medium was used by Armbruster (1969) to isolate Sphaerotilus. The cultures were incubated at 22-25°C for 5 days.

Portions of the medium were inoculated with ochre from Sychnant Pass and Donegal. The flasks inoculated with Sychnant Pass ochre were examined after 17 days. A red precipitate was seen in the flasks and unicellular rods and fungal filaments were found in solution. The flasks inoculated with Donegal ochre showed no apparent growth after 27 days incubation. Microscopic examination revealed some red particles but no unicellular bacteria. Some filaments were seen in the flasks inoculated with ochre from Sychnant Pass and from Donegal. However, it was concluded that these were derived from the original inoculation. The number of filaments was

very low, they were always associated with accumulations of iron similar to the original sample and the filaments contained no living cells.

5.1.5 TCS agar. (Farquhar and Boyle, 1971)

	<u>g/l</u>
Trypticase Soy	1.0
Agar	15.0

Farquhar and Boyle (1971) used this solid medium for isolating and purifying Sphaerotilus spp and Bacillus spp from activated sludge.

According to the authors colonies of Sphaerotilus "exhibit extensively curled projections growing well out beyond the limits of the main colony".

Plates of TCS were inoculated with ochre from Ochr Cefn Isaf, Llyn Cowlyd and Sychnant Pass. Fungi and motile, rod-shaped bacteria were isolated but sheathed filamentous bacteria were not observed.

5.1.6 "Heterotrophic" medium (Godinho-Orlandi, 1980)

	<u>g/l</u> distilled water
$K_2HPO_4$	0.2
$MgSO_4 \cdot 7H_2O$	0.05
Glucose	0.01
Peptone	0.01
Yeast extract	0.01
$FeCl_3$	4 drops of a 0.01% solution

(The cultures were incubated at 15°C for 10 days).

This medium was found to be the most satisfactory method of isolating filamentous bacteria from lake sediments (Godinho-Orlandi, 1980; Godinho-Orlandi and Jones, 1981). Using "Heterotrophic medium" the filamentous bacteria Leptothrix, Beggiatoa, Vitreoscilla, Pseudonabaena and Flexibacter were isolated. The sterile cellophane was incorporated in

liquid cultures to stimulate growth. It was found that filamentous bacteria did not stand reinoculation in the medium and no further growth was observed.

Liquid portions of "Heterotrophic medium" were prepared. A 2 cm length of 5 mm diameter plastic tubing cut in half lengthways was placed in each flask before autoclaving. 0.2 ml of filamentous ochre from Sychnant Pass was used as an inoculum. All the flasks were disturbed as little as possible during and after inoculation so that the inoculum remained as a distinct red floc in the base of the flask. In this way the inoculum could be identified easily when examining the cultures for new growth. After 10 days the solution surrounding the flocs of inoculum had a cloudy appearance. Filamentous bacteria were found in all the cultures except the uninoculated controls. These organisms were straight or slightly curved. Some were not encrusted with iron whilst others had a slight orange tinge. Some filaments were associated with iron masses. Very few unicellular bacteria were observed.

Despite the apparent success of the medium there remained some doubt as to whether the filamentous bacteria observed were merely part of the original inoculum. In these experiments a large amount of ochre was used to inoculate the cultures. Additionally, no further growth was observed when the flocs were transferred to fresh medium.

In an attempt to prove that filamentous bacteria could grow in "Heterotrophic medium" liquid portions were inoculated with ochre from Llyn Cowlyd. The media were inoculated with a range of dilutions from  $10^2$  to  $10^6$ . In the two highest dilutions no growth was observed. In the flasks inoculated from dilutions  $10^2$  to  $10^4$  a cloudy precipitate formed in the base of the flasks. This was composed of a variety of small rods and cocci. Some spores were evident. A few filaments up to 40  $\mu\text{m}$  long were observed but only in the lowest dilution.

Although "Heterotrophic medium" may support the growth of filamentous bacteria its use for their isolation from ochre is not straightforward. When ochre was diluted filamentous organisms were not isolated. However, if undiluted ochre, or low dilutions, are used it becomes difficult to differentiate between new growths and filaments derived from the inoculum.

#### 5.1.7 I Medium (Van Veen, 1973)

	<u>g/l</u> (Distilled Water)
Glucose	0.15
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.50
Ca(NO <sub>3</sub> ) <sub>2</sub>	0.01
K <sub>2</sub> HPO <sub>4</sub>	0.05
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.05
KCl	0.05
CaCO <sub>3</sub>	0.10
Agar	10.0
Vitamin B <sub>12</sub>	0.01 mg
Thiamine	0.40 mg

Vitamin B<sub>12</sub> and Thiamine were filter sterilized.

Van Veen (1973) and Eikelboom (1975) used I medium for isolating filamentous bacteria, of the Sphaerotilus - Leptothrix group, from activated sludge samples. It was recommended as a medium for isolating "iron bacteria" by Rand et al (1976).

5 ml ochre samples from Llyn Coedty and Donegal were diluted in 50 ml of sterile distilled water in a sterile conical flask (dilution 1). 5 ml of dilution 1 was added to 50 ml sterile distilled water (dilution 2). The process was repeated to obtain two further dilutions. 2 plates were inoculated with 1.0 ml of each dilution for samples from Llyn Coedty and Donegal. Plates of I medium were also inoculated with ochre from Llyn Cowlyd. Growth was seen on most inoculated plates and irregular cream



colonies were dominant. The colonies were composed of 4  $\mu\text{m}$  long rods and cocci. Sheathed filamentous organisms were not observed.

#### 5.1.8 CGY Medium (Dondero et al, 1961)

	<u>g/l</u>
Casitone or Trypticase	5.0
Glycerol	10.0
Yeast autolysate	1.0

15g agar was added to solidify the medium.

Dondero et al (1961) used CGY medium to isolate Sphaerotilus from slime masses in polluted streams, activated sludge, or from slime grown in artificial laboratory troughs or channels. The organism produced colonies with a filamentous edge. The filaments branched in "arborescent fashion." This medium was used for growing pure cultures of Sphaerotilus by Mueller and Litsky (1968) and Venosa (1975).

Agar plates of CGY medium were inoculated with ochre from Llyn Cowlyd and incubated at 25°C for 6 days. Entire, domed/raised, cream colonies 2-4 mm in diameter were found. The colonies showed no filamentous edges.

Microscopic examination revealed motile rods up to 8  $\mu\text{m}$  long and some chains of rods.

#### 5.1.9 SCY medium (Van Veen, 1973)

	<u>g/l</u> (Distilled Water)
Sucrose	1.0
Casitone	0.75
Yeast Extract	0.25
Trypticase Soy Broth without dextrose	0.25
Vitamin B <sub>12</sub>	1 x 10 <sup>-5</sup> g
Thiamine	1 x 10 <sup>-4</sup> g
Agar	10.0

Van Veen (1973) and Eikelboom (1975) used SCY for isolating and purifying cultures of filamentous bacteria from activated sludge. The organisms were initially isolated on I medium (See Section 5.1.7).

Agar plates were inoculated with ochre from Llyn Cowlyd and incubated for 6 days. Cream colonies were observed on the three lower dilutions. The cream colonies contained no sheathed filamentous bacteria and were dominated by motile 8  $\mu\text{m}$  long rods and cocci.

5.1.10 A1 and A2 (Mulder and Van Veen, 1963)

Basal Culture Solution	<u>Mg/l</u> (distilled water)
$\text{KH}_2\text{PO}_4$	27
$\text{K}_2\text{HPO}_4$	40
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	40
$\text{CaCl}_2$	50
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	75
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}^*$	10
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	5
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.1
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.1
$\text{H}_3\text{BO}_3$	0.1
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.05

\*  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  was filter sterilized

A1 To 1 l of basal culture solution were added

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg
Glucose	200 mg
Peptone	200 mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	1 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10 mg

A2 To 1 l of basal culture solution were added

MgSO <sub>4</sub> ·7H <sub>2</sub> O	200 mg
Glucose	200 mg
Peptone	200 mg
MnSO <sub>4</sub> ·1H <sub>2</sub> O	25 mg
FeSO <sub>4</sub> ·7H <sub>2</sub> O	10 mg

These media containing ferrous and manganous sulphate were used by Mulder and Van Veen (1963) to grow filamentous bacteria originally isolated from sewage, activated sludge and iron containing ditch and well water.

Portions of A1 and A2 were inoculated with ochre from Llyd Cowlyd. Red flocs were seen in all the flasks after 3 days. In medium A1, no bacteria were seen except in the lowest dilution. These appeared as small motile rods 4-8 µm long. Similar bacteria were seen in medium A2 in all but the final dilution. Sheathed filamentous bacteria were not observed.

#### 5.1.11 S and S<sup>+</sup> agar (Mulder and Van Veen, 1963)

The Basal Culture Solution described in Section 5.1.10 was diluted tenfold except for the trace element solution which was not diluted.

S medium To 1 l basal culture solution were added

Vitamin B <sub>12</sub>	0.005 mg (filter sterilized)
Peptone	1.0g
Glucose	1.0g
Agar	7.5g

S<sup>+</sup> medium To 1 l basal culture solution were added

Vitamin B <sub>12</sub>	0.005 mg (filter sterilized)
Peptone	5.0g
Glucose	5.0g
Agar	7.5g

Mulder and Van Veen (1963) used these solid media for purifying cultures of organisms from the Sphaerotilus - Leptothrix group especially Sphaerotilus. The bacteria were isolated from sewage, activated sludge and iron containing ditch and well water. On S<sup>+</sup> agar Sphaerotilus and Leptothrix usually produced white, smooth edged colonies. These colonies tended to be filamentous especially when crowded on S medium.

The plates were inoculated with ochre from Llyn Cowlyd and incubated for 6 days. Colonies developed on the 3 lowest dilutions for S agar and on all plates except for the highest dilution on S<sup>+</sup> agar. The most common colonies observed were entire, raised, white-cream colonies up to 8 mm in diameter. These were composed of large motile and non-motile rods up to 8 µm long.

#### 5.1.12 Manganese agar (Mulder and Van Veen, 1963)

	<u>g/l</u> (Tap Water)
Ferrous-ammonium Sulphate	0.15
MnCO <sub>3</sub>	2.00
Beef extract	1.00
Yeast extract	0.075
Na-Citrate	0.15
Vitamin B <sub>12</sub>	0.005 mg
Agar	7.5

Mulder and Van Veen (1963) used this agar for isolating and growing Sphaerotilus and Leptothrix isolated from various sources (see 5.1.10 and 5.1.11). They recorded the formation of filamentous, black-brown colonies of Leptothrix discophora when plates were inoculated from impure cultures in running artificial ditch water. Some isolates produced white colonies becoming brown with time. These appeared as sheathed filamentous organisms under the microscope.

Ochre from Llyn Cowlyd was used to inoculate plates of manganese agar. Growth developed on the three lower dilutions after 6 days. Small white colonies were seen. A few brown colonies having an irregular or rhizoid shape were also observed. These contained motile rods 4-8  $\mu\text{m}$  long and some straight or slightly curved filaments associated with a mineral mass (probably  $\text{MnO}_2$ ). Several brown colonies were transferred onto fresh manganese agar plates, but subsequent growth was not observed.

5.1.13 G.G. (Glycerol Glutamate) medium. (Dondero et al, 1961)

Basal Medium

Glycerol	5.0g
Glutamic acid	0.9g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.1g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.5g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.03g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.03g
Distilled Water	900 ml

pH was adjusted to 7.0 with 10% KOH.

Phosphate Solution

$\text{K}_2\text{HPO}_4$	5.7g
$\text{KH}_2\text{PO}_4$	2.3g
Distilled Water	500 ml

The two solutions were autoclaved separately and mixed in the ratio 1 volume phosphate solution to 9 volumes of basal medium. Agar was added to the basal medium.

Dondero et al (1961) used this medium for growing Sphaerotilus isolated from slime masses in polluted streams, artificial laboratory troughs and activated sludge. Sphaerotilus was identified on the medium by long segments of "cylindrical, non segmented, nonseptate sheaths."

When plates of G.G. medium were inoculated with ochre from Llyn Cowlyd growth occurred within 6 days but only on the plates inoculated from the lowest dilution. Motile and non motile rods 4-8  $\mu\text{m}$  long were seen. These bacteria formed cream-white colonies covering the agar surface. Sheathed cells were not observed.

5.1.14 L Medium. (Lackey and Wattie, 1940)

	<u>mg/l</u>
$\text{NaNO}_3$	25
$\text{MgSO}_4$	10
$\text{K}_2\text{HPO}_4$	10
$\text{KH}_2\text{PO}_4$	15
$\text{CaCl}_2$	45
Peptone	100
Dextrose	500

L Medium was used by Lackey and Wattie (1940) to isolate Sphaerotilus from activated sludge, sewage, and sewage polluted streams.

Flasks of L Medium were inoculated with ochre from Llyn Cowlyd. After 3 days all except the flask inoculated from the highest dilution had a "cloudy" appearance. Motile rods 8-12  $\mu\text{m}$  long were found in solution. These were observed free swimming and as clusters of rods.

### 5.1.15 Ferric Ammonium Citrate Medium (Rogers & Anderson, 1976)

	<u>g/l</u> (Tap Water)
Peptone	1.5
Yeast Extract	1.0
N - 2 hydroxyethylpiperazine - N - 2 ethanesulfonic acid (HEPES)	
- 10 MM	
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2
CaCl <sub>2</sub>	0.05
Ferric ammonium citrate	0.5
MnSO <sub>4</sub> ·H <sub>2</sub> O	0.05
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.01
Agar	13

pH was adjusted to 7.1 with a 3:1 (v/v) mixture of NaOH and KOH.

Rogers and Anderson (1976) used this medium for isolating, purifying and maintaining stock cultures of Sphaerotilus. The basal medium is based on that used by Stokes (1954) (See Section 5.1.2). This medium is not equivalent to the FAC medium described in section 4.3.2.

Plates were inoculated with ochre from Llyn Cowlyd and examined after 6 days. No growth was observed on plates inoculated from the two highest dilutions. On the other plates cream-white, irregular colonies were observed. These were composed of unicellular rods 4  $\mu$ m long. Precipitates of Fe(III) compounds were not observed.

### 5.1.16 Ferrous Sulphide Medium (Kucera and Wolfe, 1957)

#### Basal medium

	<u>g/l</u>
NH <sub>4</sub> Cl	1.0
K <sub>2</sub> HPO <sub>4</sub>	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2

The constituents were sterilized separately in distilled water. The basal solution was dispensed into cotton plugged test tubes (1.0 cm x 7.5 cm sterilized by autoclaving) so that each was approximately half full. Cotton filtered carbon dioxide was then bubbled through each tube for about 5 seconds using a capillary pipette. 0.2 ml sterile ferrous sulphide precipitate was then added slowly and allowed to settle gently to the bottom of the tubes.

This medium was devised by Kucera and Wolfe, (1957) for isolating Gallionella spp. Ivarson and Sojak (1978) used this medium to isolate the organism from filamentous ochre samples.

The tubes were inoculated with Llyn Cowlyd Ochre. Organisms resembling Gallionella had been identified in ochre from this site by light microscopy (See Section 4.1.1). After several weeks the tubes were examined. The ferrous sulphide precipitate had oxidized but no bacteria were observed when the precipitate was examined under the microscope.

### 5.1.17 Phaup's Medium (Phaup, 1968)

	<u>g/l</u> (Distilled Water)
Beef Extract	0.4
Peptone	0.6
KNO <sub>3</sub>	0.15
MgSO <sub>4</sub>	0.2
CaCl <sub>2</sub>	0.05
Agar (When required)	20.0



<sup>h</sup>  
~~Paup~~'s (1968) isolation medium was used to obtain cultures of Sphaerotilus. Entire flocs of the organism were originally placed in the liquid medium. Growths emerging from the flocs were transferred onto agar plates.

Ten 15 ml portions were autoclaved in 30 ml glass vials. Ten agar plates of the medium were also prepared. 1 floc (about 4 drops) of ochre from Sychnant Pass was added to each glass vial using sterile 0.2 ml pipettes. After 5 days the solutions became "cloudy" and small white flocs were evident in the top 1 cm of solution. Motile rods about 4  $\mu$ m long were found by microscopic analysis. Some 10  $\mu$ m rods were also observed. These were motile by gliding.

1 agar plate was inoculated from each vial using a sterile wire loop. After 16 days cream colonies were seen on all plates. A spreading, "leaf-vein" pattern of growth was seen away from the inoculated area. These growths were composed of motile rods and cocci. Ensheathed rods were not seen in the liquid cultures or on the agar plates.

5.1.18 Manganous Carbonate Agar (Van Veen et al, 1978)

	<u>g/l</u>
Glucose	1.0
Peptone	1.0
Manganous Carbonate ( $\text{MnCO}_3$ )	1.5
Agar	15.0

This medium was based on data given by Van Veen et al (1978) for isolating Leptothrix spp.

9 plates were inoculated with ochre from Sychnant Pass. A thin line of undiluted ochre was drawn across the agar with a sterile wire loop. After 5 days colonies had formed along the line of inoculation. These included small, rhizoid purple colonies, white-cream colonies and brown rhizoid

colonies. Some fungal filaments were seen to rise from the agar surface. After a further 14 days many of the brown colonies had developed a filamentous nature, spreading downward into the agar. Several of these colonies were examined. They were composed of fungal filaments associated with brown particles.

## 5.2 MODEL SYSTEMS

Several authors have been able to produce ochre in experimental models of various designs. For example, Talman, 1978; Mulder, 1964; Mulder and Van Veen, 1963; and Ford, 1979(A) and 1979(B).

Two model systems were investigated in an attempt to simulate conditions in field drains and open ditches.

### 5.2.1 TANK MODEL

A 10 l capacity plastic container was fitted with a 3.0 cm diameter domestic grade pipe. Holes were drilled at regular intervals along the pipe. The tank model was filled with gleyed soil (Moretonhampstead) from above a drain pipe in which ochre had been found. Water draining out of the model was reintroduced daily. When water was poured onto the model an odour of hydrogen sulphide could be detected in the drainage pipe as water flowed out. This indicated reducing conditions within the tank.

After 3 months a small amount of orange-organic material accumulated at the outlet. This material contained red aggregates and many microorganisms. Small motile rods were observed under the microscope as were some filamentous bacteria associated with iron particles. Some fungal filaments were also seen. However, after 6 months no extensive ochre formation had occurred in the model.

The tank model was modified to incorporate some of the features of Mulder's Model (1964). (See also Mulder and Van Veen, 1963). This model

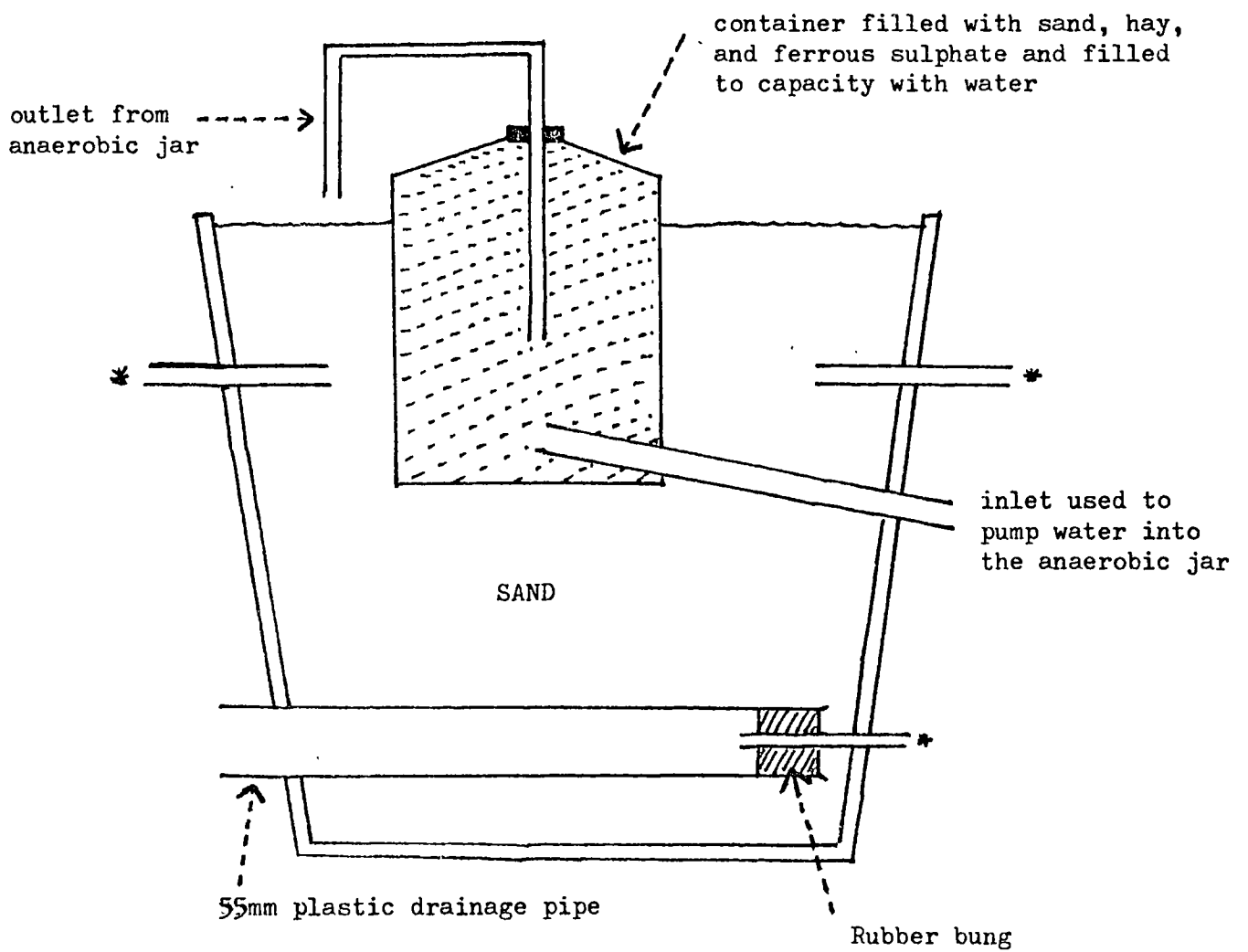
had an iron cylinder containing "ironstone soil" and ferric carbonate. The cylinder was saturated with water and incubated for 3 weeks. The tube served as a source of reduced iron.

The plastic tank described earlier was emptied and fitted with a length of corrugated plastic drainage pipe (55 mm external diameter). A rubber bung was used to close one end of the pipe to ensure that water entered the pipe through the slots. A length of rubber tubing was passed through the bung and out through the back of the model. Two small lengths of 5 mm diameter plastic tubing were inserted through the sides of the tank at two locations about 5 cm from the top of the model.

In order to generate a supply of reduced iron in association with organic matter a plastic container was filled with sand, 20g of chopped hay and 5.0g of ferrous sulphate. The container was then filled to capacity with water. A length of rubber tubing had been fitted to the base of the jar. The jar was placed in the tank model and the rubber tube passed through a hole cut at the side of the tank (Figure 5.1).

5.0 ml of Sychnant Pass ochre was injected into the model using 5.0 ml plastic syringes. Ochre was introduced via the two side inlets and into the drainage pipe through the rubber bung. 5.0 ml of water was then injected through these inlets to force ochre into the sand and drainage pipe. Distilled water was pumped into the anaerobic jar through the lower inlet pipe using a syringe. This was continued until water escaped from an outlet pipe in the lid of the jar. For one month ochre was introduced once a day and water pumped through the anaerobic jar twice a day. Although red stains appeared in the vessel used to collect the outflow no ochre developed in the drainage pipe.

Figure 5.1 : Diagram of the tank model used in an attempt to simulate ochre formation



\* Inlets used to inject ochre into the model

### 5.2.2 OPEN DITCH MODEL

A model simulating an open ditch was made using a 70 cm length of plastic guttering fixed to a wooden stand by plastic support brackets. The model was closed at both ends using proprietary end pieces. At one end an inlet pipe was installed and an outlet pipe at the other end. The model was inoculated with a 15 cm piece of 55 mm diameter corrugated plastic drainage pipe. This pipe had been left in a ditch at Sychnant Pass for several days. During this time ochre had accumulated inside and outside the pipe.

A dilute infusion was prepared by extracting 30g of hay in 600 ml of water. The extract was diluted to 10 l and 5.0g of ferrous sulphate added (Solution A). This solution was diluted further in the ratio 11:9l and allowed to flow slowly through the model. After 5 days 10 l had flowed through the model. A further 10 l was prepared by diluting 1 l of autoclaved 'solution A' to 10 l with water and adding 5.0g of ferrous sulphate. Precipitates of iron formed in the model within a few days. These restricted the flow of water to some extent. Gelatinous deposits were observed in association with the ferric precipitates. Under the microscope it was seen that these were composed predominantly of inorganic particles. Some filamentous bacteria were observed as were fungal filaments.

When iron had precipitated along the entire length of the model the precipitates were examined microscopically. No filamentous bacteria were found at the upper end of the model or at the outlet pipe. Filamentous bacteria encrusted with ferric iron compounds were seen at the centre of the model. After a further 10 days fungal colonies had developed, in the form of red "cotton-wool" like flocs. Under the microscope fungal filaments and iron particles were seen. Motile rod shaped bacteria, 5-10  $\mu\text{m}$  long, were also seen but no filamentous bacteria. Filamentous bacteria were not observed in the model after this time.

### 5.3 EXPERIMENTAL MEDIA

#### 5.3.1 DRAINAGE WATER

Two 150 ml portions of drainage water from Llyn Cowlyd and Llyn Coedty were collected in sterile polypropylene bottles. Care was taken to avoid collecting flocs of ochre. A 100 ml portion of drainage water was filter sterilized on site using a 0.22  $\mu\text{m}$  Millipore filter attached to the apparatus described in section 5.3.4. A brass bicycle pump was used to force air through the system.

In the filtered water iron (III) had precipitated out as a thin layer composed of small flocs. No bacteria were observed in these flocs. At both sites flocs of orange coloured material formed in the unsterilized water. These were composed of red particles associated with sheathed filamentous bacteria. The bacteria were straight or slightly curved organisms some of which were encrusted with ferric particles. Many unicellular bacteria were also observed. The total organic carbon content of these drainage waters is given in Table 5.4.

Growth of filamentous bacteria in drainage water was also observed in samples collected at Penygroes site 1 (See plate 5.1).

It was demonstrated that growth of the filamentous organisms related to the iron content of drainage water. Drainage water was collected from a ditch near the ochre sites at Llyn Cowlyd. No ochre was found in the ditch and iron could not be detected in the water by atomic absorption spectrophotometry (Plate 3.28).

The effect of filtration and dilution on the growth of filamentous organisms in drainage water should also be noted. 1.0 l of drainage water from Llyn Cowlyd was filter sterilized on site using a 0.22  $\mu\text{m}$  Millipore filter. Two 50 ml portions in pre-sterilized 100 ml conical flasks were inoculated with 1.0 ml of diluted ochre on site. The ochre was diluted

1.0 ml of suspension in 100 ml of sterile tap water. The inoculated flasks were examined after several days and a pale yellow precipitate was observed at the bottom of both flasks. Large flocs were not seen and filamentous bacteria were not found in the precipitate.

### 5.3.2 EXTRACTS AND INFUSIONS

In the past, plant extracts have been used to isolate and grow filamentous bacteria. For example, Brown (1903) succeeded in growing the organisms in water containing an infusion of hay and ferric salts. Similarly, Mulder and Van Veen (1963) used a pea-straw extract as an enrichment medium for isolating organisms of the Sphaerotilus - Leptothrix group. The organic compounds present in such extracts are similar to those present in drainage water.

#### 5.3.2.1 Hay Infusions

Liquid and solid media based on extracts of hay were prepared.

Approximately 38g of hay (Perennial ryegrass : clover) was extracted for 15 minutes at 120°C in 300 ml of tap water. Two 120 ml portions of extract were diluted to 500 ml with sterile tap water. 0.3g ferrous sulphate was added to one dilution and 1.0g ferrous sulphate was added to the other dilution. The flasks were inoculated with 0.5 ml of Sychnant Pass ochre and incubated at 25°C for 14 days. Fungal growth was observed in the flask containing 0.3g ferrous sulphate. In the second flask fungi were not evident and the microbial population was dominated by small unicellular bacteria.

Agar plates were prepared by autoclaving 8.0g hay in 250 ml tap water. The filtrate was made up to 250 ml with tap water and 4.0g of agar added. After the solution had been autoclaved and cooled slightly 1.0g ferrous sulphate was incorporated. 3 plates were inoculated with 0.5 ml of filamentous ochre from Sychnant Pass. A further 4 plates were inoculated

with 0.5 ml of ochre diluted 1.0 ml in 100 ml sterile distilled water. An uninoculated control was prepared. No growth occurred on the control plate. Fungal colonies dominated the plates inoculated with undiluted ochre. On the other plates irregular, raised, white colonies were observed. The colonies contained small, motile rod-shaped bacteria.

Filamentous bacteria were not isolated with these hay infusions. It was thought that high organic matter levels were encouraging the growth of fungi and unicellular bacteria at the expense of the filamentous organisms. Media based on diluted hay extracts were prepared. These media, and the problems associated with them, are discussed in section 6.2.

#### 5.3.2.2 Dried grass

A medium based on dried grass was prepared. A similar medium had been used (Kelso, W.I, Personal Communication) for growing filamentous bacteria from ochre samples. 20g of a dried Perennial Ryegrass : Clover Mixture (50:50) was added to 1 l of boiling water and left for 5 minutes. To 250 ml of cooled extract was added 0.4g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and to a further 250 ml was added 0.4g  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ . The pH was adjusted to 6.5 with 2M NaOH. Each flask of medium was diluted so as to contain 30 ml of extract and 50 ml water. 6 flasks of the iron and manganese containing media were prepared and inoculated with 0.1 ml of filamentous ochre from the Vale of Pickering (collected by Fletcher, P.). 2 flasks of each medium were inoculated with 0.1 ml of ochre from Sychnant Pass. The flasks were incubated at 25°C for 22 days. All media showed fungal contamination with fungal "pads" on the surface of the cultures. Fungi were clearly seen under the microscope but no filamentous bacteria.



### 5.3.2.3 Heather extracts

70 g heather was autoclaved in 800 ml distilled water and the extract filtered through Whatman 540 filter paper. The extract had a pH of 4.0 and an organic carbon content of 1.8 g/l (organic carbon was determined by the method described in Section 5.3.5).

1.0 ml of extract was added to various volumes of ferrous sulphate solution (0.5 g/l) and diluted to 100 ml with distilled water. 6 different volumes of ferrous sulphate solution were used, in the range 0 to 25 ml, giving a final Fe(II) concentration between 0 and 25  $\mu\text{g/ml}$ . The pH of each solution was adjusted to 6.5 with NaOH. The solutions were autoclaved, pH was recorded, and the presence or absence of a precipitate noted. The results shown in Table 5.1 demonstrate that up to 20  $\mu\text{g/ml}$  of Fe could be held in solution by 1.0 ml of extract in 100 ml at a pH above 6.0. Since this amount of iron had been held in solution during autoclaving it was assumed that any release of iron in inoculated solutions would be the result of bacterial degradation of the iron-organic complexes.

TABLE 5.1 THE EFFECT OF AUTOCLAVING ON THE pH AND PRECIPITATION OF IRON  
IN A HEATHER EXTRACT CONTAINING FERROUS SULPHATE

<u>Fe(II) CONCENTRATION (<math>\mu\text{g/ml}</math>)</u>	<u>PRECIPITATE (1)</u>	<u>pH</u>
0	-	7.3
5	-	7.1
10	-	6.9
15	-	6.4
20	-	6.4
25	+	4.2

(1) + indicates that a precipitate formed during autoclaving.

Portions of heather extract media with 20  $\mu\text{g/ml}$  Fe(II) were prepared as described above and inoculated with 0.1 ml filamentous ochre. The media were kept at room temperature for 15 days. Red flocs appeared in all the

inoculated flasks whilst they did not develop in uninoculated controls. Microscopic examination of the flocs showed that they were composed of straight filaments associated with ferric particles. Some unattached non-encrusted filaments were also observed.

Although these media were apparently successful they were not investigated further. The composition of the extracts was poorly defined and identifying new growths as opposed to original inoculum was difficult. Additionally, the chemical oxidation effects discussed in section 6.2 with regard to hay extract would make it difficult to confirm that filamentous bacteria were growing in the heather extract media.

### 5.3.3 ORGANIC ACIDS

Ochre often forms in water flowing from an anaerobic zone. It is to be expected that such water would contain organic acids such as lactic, butyric and acetic acid which are characteristically produced in anaerobic environments (Russell, 1961). Ford (1979B) was able to produce ochre in chambers or tubes receiving continuous injections of ferrous iron (2.0 µg/ml) and 1.0 µg/ml of ascorbic acid, tannic acid, lactic acid or sulphonated lignin at pH 6.5. Several media incorporating organic acids and ferrous iron were investigated.

#### 5.3.3.1 Ascorbic acid

Media containing ascorbic acid and 10 µg/ml Fe(II) as ammonium ferrous sulphate were prepared. Some were supplemented with either yeast extract or tryptone soya broth (Oxoid). The composition of these media is shown in Table 5.2.

Table 5.2 The composition of artificial media based on ascorbic acid and ferrous iron

	CONCENTRATION g/l		
	<u>ASCORBIC ACID</u>	<u>YEAST EXTRACT</u>	<u>TRYPTONE SOYA BROTH (OXOID)</u>
1.	0.2	1.0	-
2.	0.2	0.5	-
3.	0.2	-	1.0
4.	0.2	-	0.5
5.	1.0	1.0	-
6.	1.0	0.5	-
7.	1.0	-	1.0
8.	1.0	-	0.5
9.	0.2	-	-
10.	1.0	-	-

EACH MEDIUM ALSO CONTAINED 10 µg/ml Fe(II) AS AMMONIUM FERROUS SULPHATE

100 ml portions of each medium were inoculated with 0.5 ml diluted Synchron Pass ochre (1.0 ml ochre suspension diluted in 100 ml sterile distilled water). The media were incubated at 25°C for 5 days. A red-orange precipitate had formed in all flasks except those containing 1.0 g/l yeast extract or 1.0 g/l tryptone soya broth. Many rod shaped unicellular bacteria were found in all media except number 2 (Table 5.2). These were motile organisms about 4 µm long. Sheathed filamentous bacteria were not observed.

#### 5.3.3.2 Tannic, Citric and Lactic acid

Media containing 0.1 g/l Fe(II) as ferrous sulphate and either tannic acid or citric acid at 1.0 g/l, or 3.0 g/l of 88% lactic acid solution were prepared. Some portions of each medium were supplemented with 2.0 g/l yeast extract. The pH was adjusted to 6.5 with 0.5M KOH and the solutions autoclaved.

0.5 ml suspension of Sychnant Pass ochre was used to inoculate the media. After 12 days at 25°C the flasks were examined. The lactic acid cultures were dominated by fungi with some large rod-shaped bacteria also being present. Motile rods and spherical shaped unicellular bacteria were observed in the tannic acid and citric acid cultures except in the tannic acid media supplemented with yeast extract. In the latter no bacteria were observed. Filamentous bacteria were not observed in these media.

#### 5.3.4 GLUCOSE - IRON MEDIA

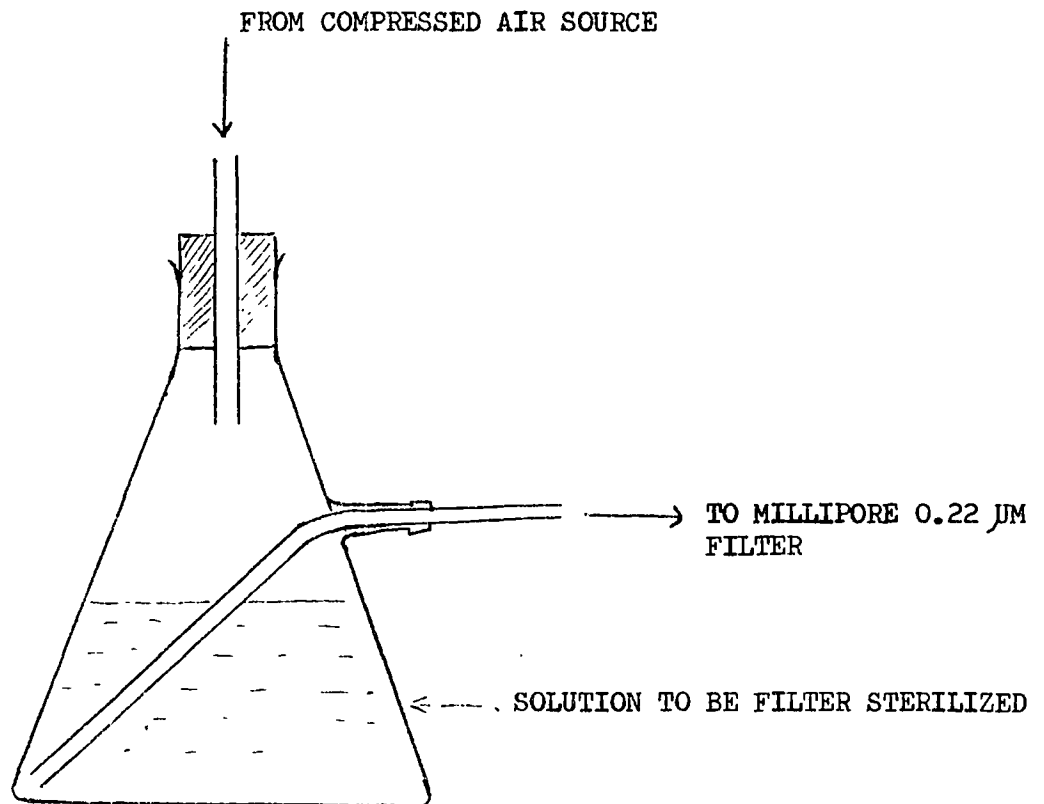
During the present study it was found that sheathed filamentous bacteria would grow in drainage water collected from several sites (5.3.1). It was concluded that filamentous bacteria might grow in artificial media having an organic matter content similar to drainage water samples. Additionally, in drainage water at ochrous sites the rate of Fe(II) oxidation is rapid (Section 6.1 ). Glucose as a source of organic matter does not substantially affect the amount of iron held in solution. In order to avoid precipitation of iron before inoculation a procedure was devised for adding filter sterilized ferrous sulphate to the media.

Compressed air was used to force a ferrous sulphate solution through a 0.22 µm Millipore filter (GS) from a modified conical flask with side arm. The flask is illustrated in Figure 5.2. The rubber bung in the neck of the flask was held in place by hand so that the flow of filtered solution could be stopped immediately by releasing pressure on the bung. In this way aliquots of filtered ferrous sulphate could be added directly to media in 100 ml graduated flasks.

#### Determination of organic matter in drainage water

In order to determine accurately the amount of organic material in drainage water supporting ochre formation, the organic carbon content of several waters was calculated.

FIGURES 5.2 : DIAGRAMMATIC REPRESENTATION OF THE CONICAL FLASK USED FOR FILTER STERILIZING DRAINAGE WATER SAMPLES AND FERROUS SULPHATE SOLUTIONS



150 ml of drainage water was collected in sterile polypropylene bottles. The bottles were sterilized by autoclaving for 15 minutes at 120°C. The drainage water was filter sterilized through a 0.22 µm Millipore filter within 1 hour of sampling.

The apparatus described earlier (Figure 5.1) was used for filtration. A brass bicycle pump was used to pump air into the system. 5 ml of concentrated sulphuric acid was added to the filtrate. The total volume was determined in the laboratory and then aliquots were concentrated as necessary by rotoevaporation under vacuum. The total organic carbon content of the water was determined by the method outlined below (Anon, 1969).

5.0 ml of sample, 0.5 ml mercuric sulphate solution (50g in 225 ml of water and 25 ml concentrated sulphuric acid), 2.0 ml potassium dichromate (7.6605 g/l) and 7.5 ml silver sulphate/sulphuric acid solution (5g silver sulphate in 500 ml 36N H<sub>2</sub>SO<sub>4</sub>) were mixed in a 50 ml round bottom flask. An anti-bump rod was added and the solution heated under reflux for 2 hours. The sides of the flask were then flushed with a little distilled water and 2 drops of indicator solution added (1,10 - Phenanthroline - ferrous sulphate - complex solution 0.025M, BDH). The mixture was titrated against ferrous ammonium sulphate-(9.803g in 400 ml distilled water containing 40 ml concentrated H<sub>2</sub>SO<sub>4</sub>, made up to 2 l with water).

A blank containing 5.0 ml of distilled water was prepared for each batch of samples. The concentration of organic carbon in solution was calculated by multiplying the difference between the sample and control titres by 7.7. The results are given in Table 5.4.

TABLES 5.4 : THE ORGANIC CARBON CONTENT OF DRAINAGE WATER SAMPLES

	<u>µg/ml</u> (1)
Llyn Cowlyd	9
Llyn Coedty	12
Penygroes 1	59
Penygroes 3	20
Penygroes 5	33
Penygroes 7	196 (2)

(1) Mean of two samples

(2) Penygroes 7 was an open ditch containing much organic debris

On the basis of these results two experiments were conducted. In the first set media containing 10 µg/ml of glucose were used. In the second set levels of 50 µg/ml and 100 µg/ml were investigated. These concentrations covered the range of organic carbon levels in the drainage water samples.

#### 5.3.4.1 Glucose at 10 µg/ml

25 ml portions of 20 µg/ml glucose and 0.3 ml 0.05M NaOH were autoclaved. 25 ml of 0.1g/l filter sterilized ferrous sulphate was added. 6 portions were inoculated immediately with Sychnant Pass ochre collected a few hours previously. Ochre was diluted 1.0 ml in 100 ml of sterile distilled water and 1.0 ml was used as an inoculum. It was felt that this dilution would avoid the problems associated with the presence of large amounts of inoculum in the medium when cultures were examined for bacterial growth.

In the glucose media red particles and many filamentous bacteria were seen. The organisms were encrusted inside iron concretions but non-encrusted at the edges of the iron particles. Many motile, rod shaped, unicellular bacteria were also seen. Filamentous bacteria were seen in 5 out of the 6 inoculated flasks.

The growth of filamentous bacteria in the glucose-iron medium was substantiated by inoculating various modifications of the medium with fresh ochre and from previously growing cultures. The following media were prepared:

Glucose 10  $\mu\text{g/ml}$ , Fe(II) 10  $\mu\text{g/ml}$

Glucose 10  $\mu\text{g/ml}$

Glucose 10  $\mu\text{g/ml}$ , Fe(II) 10  $\mu\text{g/ml}$ , Cu 5  $\mu\text{g/ml}$  (as Copper Sulphate)

5 portions of each medium were prepared. Two were inoculated with 1.0 ml of ochre from Sychnant Pass diluted 1.0 ml in 100 ml sterile distilled water. Two were inoculated with 1.0 ml from a thoroughly shaken Glucose-Fe culture from the previously described experiment. The remaining flask was kept as an uninoculated control.

After 8 days large flocs appeared in the Glucose-Fe medium. The flocs in flasks inoculated from the "growing cultures" were, however, smaller. No flocs were seen in the medium containing glucose alone except for small particles of inoculum. In the Glucose-Fe medium containing copper small light orange particles were seen in the inoculated flasks but nothing in the uninoculated control.

Microscopic analysis revealed that in the glucose-iron media inoculated with fresh ochre, inorganic iron particles and filamentous bacteria (encrusted and non-encrusted) had developed. However, in the uninoculated flask and in those inoculated from previous cultures inorganic particles were evident but no filamentous or unicellular bacteria. No filamentous bacteria were seen in any of the media containing glucose alone or in those containing copper.

These results show that the Glucose-Fe medium is satisfactory for growing filamentous bacteria from fresh ochre. The addition of copper to the medium or exclusion of iron prevents bacterial growth. However, the medium does not support the growth of bacteria transferred from previous cultures.



### Supplemented Glucose-Iron Medium

These media were used in an attempt to maintain filamentous bacteria isolated in Glucose-Fe medium.

25 ml portions of 20 µg/ml glucose and 0.3 ml 0.05M NaOH were autoclaved. 25 ml of filter sterilized 0.1 g/l ferrous sulphate was added. The media were inoculated immediately with 1 ml of Donegal ochre diluted 1.0 ml in 100 ml of sterile distilled water. 3 flasks were inoculated. An uninoculated control was also prepared.

After 8 days orange flocs were seen in all the flasks. The flocs were large in 2 inoculated flasks but small in the third. In all inoculated flasks iron encrusted and non-encrusted filaments were observed. The filamentous bacteria were associated with iron masses being encrusted inside the mass and non-encrusted outside. One spiral, encrusted filament was seen. Many unicellular rods about 4.8 µm long were observed. A fungal filament was found in one inoculated flask. No organisms were seen in the uninoculated control. The final pH of the media was 5.0.

Flocs from these cultures were transferred into glucose-iron media containing various supplements.

A basal salts solution was prepared:-

	<u>g/l</u>
$(\text{NH}_4)_2\text{SO}_4$	2.0
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.04
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.8
$\text{K}_2\text{HPO}_4$	0.8

Where required 0.2 g/l of yeast extract was added to the basal salts solution

3 media were prepared:-

Glucose-iron medium with basal salts

Glucose-iron medium with yeast extract

Glucose-iron medium with basal salts and yeast extract.

These media were prepared by mixing 25 ml of 20 µg/ml glucose, 12.5 ml of stock solution containing basal salts, yeast extract or both and 0.3 ml 0.05M NaOH. These were autoclaved for 15 minutes at 120°C. 12.5 ml of 0.2 g/l filter sterilized ferrous sulphate was then added. The final concentrations of glucose and Fe(II) were 10 µg/ml. The final concentrations of salts and yeast extract were a quarter of those used in the basal salts solution.

Four portions of each medium were prepared. One was kept uninoculated whilst the others were inoculated with 1.0 ml of shaken cultures of glucose-iron media containing organisms isolated from Donegal ochre.

After 8 days distinct red flocs were seen in the media containing yeast extract. Unicellular rods 8 µm long were seen in these flocs. Chains of rods associated with iron particles were also observed in one of the inoculated flasks. Filamentous bacteria were not observed.

The pH of the spent media was between 6.5 and 6.8.

#### 5.3.4.2 Glucose at 50 µg/ml and 100 µg/ml

Five media were prepared and tap water was used throughout except when making up the ferrous sulphate solution.

25 ml of glucose solution (with or without yeast extract) and 0.5 ml of 0.05M NaOH was autoclaved. 25 ml of filter sterilized 0.2 g/l ferrous sulphate was added. The media used are listed below:-

1. Glucose 100 µg/ml, Fe(II) 20 µg/ml
2. Glucose 100 µg/ml, Fe(II) 20 µg/ml, Yeast Extract 0.05 g/l
3. Glucose 50 µg/ml, Fe(II) 20 µg/ml
4. Glucose 50 µg/ml, Fe(II) 20 µg/ml, Tannic Acid 10 µg/ml
5. Glucose 50 µg/ml, Fe(II) 20 µg/ml, Ferric Ammonium Citrate 10 µg/ml

2 flasks were inoculated with 1.0 ml Sychnant Pass ochre diluted 1 ml to 100 ml with sterile tap water. 2 portions were inoculated with 1.0 ml of sterile ochre. Sterile ochre was prepared by diluting 1.0 ml of ochre in 100 ml distilled water and autoclaving for 15 minutes at 120°C. One flask was kept as an uninoculated control.

After 3 weeks the flasks were examined. The pH of all the media was between 4.0 and 5.0 and a fine, pale yellow precipitate was found in all the flasks except those containing ferric ammonium citrate where well flocculated, red particles, were observed. In the cultures containing tannic acid small flocs of brown precipitate were seen.

No bacteria were found in any of the uninoculated flasks. Some filaments were observed in all inoculated media but they were also found in the same numbers in the flasks inoculated with sterile ochre. Thus increasing the concentrations of glucose and iron in the glucose-iron medium decreased its efficiency for isolating filamentous bacteria from ochre. This experiment highlighted the problem associated with the presence of original inoculum. Even when ochre is diluted a hundred times and 1.0 ml used to inoculate 50 ml of medium filaments from the inoculum can still be found by examining portions of the solutions with a microscope.

#### 5.4 DISCUSSION

Many different media were utilised in an attempt to isolate sheathed filamentous bacteria from ochre.

None of the twenty standard media obtained from the literature were successful. The only positive results were obtained using Heterotrophic medium (5.1.6) and Manganese agar (5.1.12). In these media filamentous bacteria were observed. However, the isolates did not grow when transferred into fresh medium so that confirmation of growth was not obtained. Since all these media have been used successfully by other authors for isolating the filamentous organisms normally associated with ochre it is worth considering why filamentous bacteria did not grow during the present study. The most probable reasons are listed below.

1. Twelve of the standard media used were designed for isolating Sphaerotilus spp from polluted streams and especially activated sludge. Others had been used for isolating organisms of the Sphaerotilus - Leptothrix group from activated sludge and sewerage. Most of these environments are substantially different to the low organic matter drainage water associated with ochre formation.

The most common organic compounds used in the media were glucose and peptone. 11 media used glucose, 12 used peptone and 10 of these used glucose and peptone. The concentrations of these compounds ranged from 0.15 g/l<sub>n</sub> of glucose and from 0.1 g/l to 5.0 g/l of peptone. The only medium to have lower concentrations was heterotrophic medium (5.1.6) which used 0.01 g/l of glucose and peptone. In six media other organic compounds were used in place of glucose and peptone. These included sodium lactate, sodium citrate, glycerol, sucrose, tryptone soya broth, casitone or trypticase, and glutamic acid. These compounds were used in concentrations ranging from 0.1 g/l to 10 g/l. These concentrations of organic compounds are high in relation to the organic matter levels of drainage water determined, for example, in Table 5.4 where the highest organic matter content was 196 µg/ml.

2. In all the standard media, unicellular bacteria grew well but no filamentous forms were found. Fungi were also isolated from ochre on 5 media. The only exceptions were Armbruster's medium (5.1.4) and the autotrophic ferrous sulphide medium (5.1.16) where no microbial growth was observed. The growth of unicellular bacteria is not surprising since the components used, especially glucose and peptone, are common constituents of biological media.

The unicellular bacteria and fungi competed against filamentous bacteria in the high organic matter media used. The problem of competition by unicellular organisms has been noted by several authors. Godhino-Orlandi (1980) and Godhino-Orlandi and Jones (1981) found that it was difficult to isolate filamentous bacteria on solid media due to rapid and heavy growths of unicellular bacteria. Similarly, Van Veen (1973) noted that isolating filamentous bacteria from bulking sludge was not easy due to the presence of large numbers of rapidly growing non-filamentous bacteria.

3. Many unicellular bacteria were isolated using the standard media. Several workers have noted that filamentous bacteria can undergo changes when isolated in laboratory media. Such changes include the loss of ability to form a sheath (see Section 2.3.1.2). Since most of the filamentous bacteria associated with ochre have not been isolated in pure culture their behaviour and properties in standard media is unknown. However, several authors have recorded the growth of filamentous bacteria as unicellular organisms (Venosa, 1975; Mulder and Van Veen, 1963; Dondero et al, 1961; Van Veen, 1973; Yoshikawa et al, 1979). The tendency to exist as unicellular organisms is more pronounced in media with high nutrient concentrations (Pringsheim, 1949, Yoshikawa & Takiguchi 1979). It is therefore possible that the lack of filamentous isolates in the media does not necessarily mean that filamentous bacteria were absent. It may be that they were isolated, but as unicellular organisms.

4. Although the relationship between filamentous bacteria and iron is not well understood it is known, from observations in nature, that filamentous bacteria often occur in iron rich environments.

Iron was included in 10 of the standard media. The most common form was  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . However, this was only used in trace quantities except in two media (Stokes' medium, 5.1.2, and ferric ammonium citrate medium, 5.1.15) where 0.01 g/l was used. Ferrous sulphate was used in three media. In two of these trace quantities were used but 0.5 g/l was included in G.G. medium (5.1.13). 0.15 g/l of ferrous ammonium sulphate was used in manganese agar (5.1.12) and 0.5 g/l of ferric ammonium citrate in medium 5.1.15. On the whole iron was either absent from the media or present at low concentrations.

It is possible that lack of iron in an easily oxidised form contributed to the poor performance of the media with regard to the isolation of sheathed filamentous bacteria. The importance of iron was shown in section 5.3.1. Drainage water containing iron supported growth of filamentous bacteria. However, in drainage water without iron filamentous bacteria did not grow.

Filamentous bacteria were not isolated using the experimental media based on organic acids or low dilutions of hay or dried grass extracts. In these media unicellular bacteria and fungi grew well but no filamentous bacteria were observed. Although these media were based on results obtained by other workers and on the type of compounds likely to be present in drainage water from anaerobic zones they were not successful in the present study. The reasons for this are probably the same as those discussed above for the standard media. Iron was included in the experimental media but at concentrations that would be held in solution by the extracts or organic acids.

The model systems used yielded poor results. Some growth of filamentous bacteria was detected but it was only small and temporary. The inadequate

performance of the models is related to the difficulties in simulating the conditions under which ochre forms. In the tank model it was difficult in practice to maintain anaerobic and aerobic zones. It was especially difficult to ensure that the boundary between these zones occurred at the drain-soil interface. For example water tended to flow down the sides of the tank rather than through the soil. Thus the edges of the soil block were well aerated and iron was chemically oxidized here leaving a thin precipitate on the side of the tank. The major problem with the open ditch model was the growth of fungi and unicellular bacteria. Although filamentous bacteria did grow in the model they did not compete well against other microorganisms and were eventually replaced by them.

The most satisfactory method found for isolating filamentous bacteria from ochre was a medium based on glucose and iron. The advantage of the glucose-iron combination was that the organic concentrations could be increased without affecting the concentrations of iron held in solution. The medium was composed of 25 ml of 20  $\mu\text{g/ml}$  glucose, 0.3 ml of 0.05M NaOH and 25 ml of 0.1 g/l ferrous sulphate giving 10  $\mu\text{g/ml}$  of Fe(II) in the final solution. A method was developed for filter sterilizing the ferrous sulphate solution directly into the autoclaved glucose solution. The medium was inoculated immediately after adding the iron. Ochre was usually diluted 1.0 ml in 100 ml of distilled water.

It was shown that filamentous bacteria were growing in the medium by the experiment described in section 5.3.5.1 where copper was added before inoculation at 5.0  $\mu\text{g/ml}$ . In the presence of copper no filamentous bacteria were detected, whereas filaments were clearly observed in media without copper. This shows that the glucose-iron medium is suitable for isolating filamentous bacteria. However, the medium as described was not completely satisfactory and two problems remain unresolved.

The first problem is maintaining the isolates. None of the organisms grown from ochre in glucose-iron medium grew when transferred into supplemented or unsupplemented medium. The second problem is confirming that filamentous bacteria are growing. Analytical techniques are not applicable. The determination of glucose cannot be used to indicate the growth of filamentous bacteria since unicellular bacteria can also grow in the medium. Similarly, monitoring ferrous iron levels is irrelevant since chemical oxidation is the main factor causing its precipitation. In normal inoculation techniques it is assumed that bacteria seen in media have grown in the cultures. Since high dilutions of original sample are used the numbers of bacteria from the inoculum would be insignificant in the final medium. However, it was found that if high dilutions of ochre were used then no growth occurred. This was observed both with heterotrophic medium and with drainage water. If drainage water was filtered and inoculated with small volumes of unfiltered water no filamentous bacteria grew. However, they grew well in unfiltered water from the same site. This observation reflects the low numbers of filamentous bacteria in ochre. It has been noted (Section 4.4) that small numbers of these bacteria can produce large amounts of filaments, thus giving a false impression of the number of living filamentous bacteria in the samples.

For the reasons outlined above low dilutions of ochre (1 ml in 100 ml water) were adopted for the glucose-iron medium. At these dilutions the number of filaments in the medium that are derived from the original inoculum is quite high. Consequently the observation of filaments in the glucose-iron medium does not necessarily prove that they have grown there. In the present work this problem was overcome by inoculating glucose-iron medium with ochre and sterile ochre. Ochre was sterilized by autoclaving 1 ml in 100 ml of distilled water. Growth could then be confirmed by comparing the extent of filamentous growth in the inoculated media and their sterile counterparts. It was also noted that the appearance of organisms from the inoculum and in new growth was different under the



microscope. In new growth many filamentous forms were associated with pale orange deposits. The filaments derived from the original inoculum were composed of one or two filaments associated with darker (orange-brown) particles of iron. This difference reflects an aged ferric hydroxide deposit in the ochre samples as compared to freshly precipitated iron in the medium.

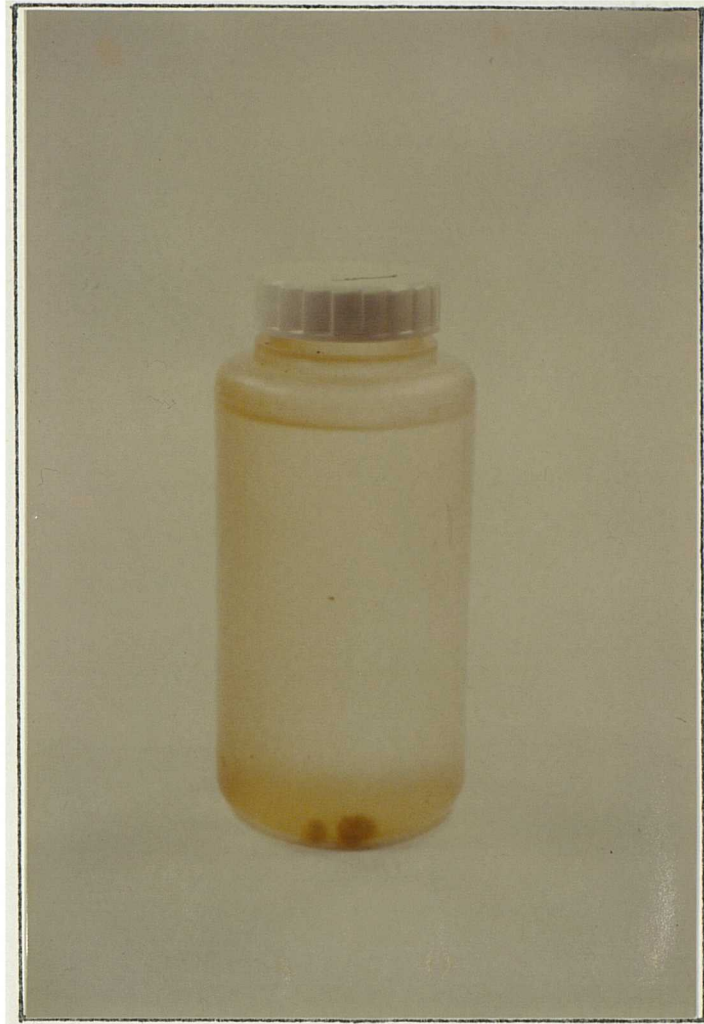


Plate 5:1 Growth of sheathed filamentous bacteria in drainage water from Penygroes site 1.

## 6. CHEMICAL OXIDATION AND THE FORMATION OF OCHRE

### 6.1 CHEMICAL OXIDATION OF FERROUS IRON IN DRAINAGE WATER

During routine collection of drainage water samples it was observed that waters which were clear when sampled developed orange-red precipitates in a few days (Section 5.3.1). Microscopic examination showed that the precipitate was composed of iron particles and filamentous bacteria. Iron precipitates also formed in drainage water filtered through a 0.22  $\mu\text{m}$  Millipore filter but no bacteria were present in these precipitates (5.3.1). These observations show that chemical oxidation was contributing to the removal of ferrous iron from the drainage water. To investigate this further an experimental procedure was developed for on site acidification and filtration of drainage water:

20 ml glass vials were washed in concentrated hydrochloric acid, rinsed several times in distilled water and sterilized by autoclaving at 120°C for 15 minutes. 12 vials were prepared for each site; 1.0 ml of concentrated hydrochloric acid was placed in 8 of the vials.

Drainage water was collected using 10 ml plastic syringes and filtered through 0.22  $\mu\text{m}$  Millipore filters (GSWP) in Swinnex filter units. The samples were then treated in one of three ways:-

1. ACIDIFIED (A): 10 ml of drainage water acidified with 1.0 ml of concentrated HCl.
  2. FILTERED + ACIDIFIED (F+A): 10 ml of drainage water filter sterilized and acidified with 1.0 ml of concentrated HCl.
  3. FILTERED (F): 10 ml of drainage water, filtered but not acidified.
- 4 replicates of each treatment were used at each site.

The vials were left at room temperature for two to three days, after which an orange-red precipitate formed in the filtered (unacidified) treatment (Plate 6.1). 4.0 ml aliquots from treatment F were filtered through 0.22  $\mu\text{m}$  Millipore filters and acidified with 0.5 ml of concentrated hydrochloric acid. Total iron was determined for all the vials on a Pye Unicam SP2900 atomic absorption spectrophotometer. The results were compared to those obtained for standard ferric nitrate solutions in the range 0-12  $\mu\text{g/ml}$ .

The procedure outlined above was used at 12 sites. The results, corrected for dilution by acid, are shown in Table 6.1. At six sites the concentration of manganese was also determined by the same procedure and the results are given in Table 6.2.

TABLE 6.1: THE CONCENTRATION OF IRON IN DRAINAGE WATER SAMPLES

<u>SITE</u>	<u>FOLLOWING ACIDIFICATION AND FILTRATION</u>			TOTAL IRON IN SOLUTION $\mu\text{g/ml}$
	<u>A</u>	<u>F+A</u>	<u>F</u>	
LLYN COWLYD 1	8.4	8.0	0.0	
LLYN COWLYD 2	7.6	7.0	0.0	
LLYN COWLYD 4	8.7	5.7	1.3	
LLYN COEDTY 1	15.2	14.8	3.2	
LLYN COEDTY 2	14.1	14.1	6.8	
LLYN COEDTY 3	5.2	1.6	1.3	
PENYGROES 1	22.4	19.5	0.2	
PENYGROES 5	5.8	5.7	0.4	
PENYGROES 7:2	3.2	2.9	0.1	
PENYGROES 7:3	1.1	1.0	0.0	
PENYGROES 7:4	1.9	1.4	0.0	
PENYGROES 7:5	0.4	0.3	0.3	

TABLE 6.2 : THE CONCENTRATION OF MANGANESE IN DRAINAGE WATER SAMPLES  
FOLLOWING ACIDIFICATION AND FILTRATION

<u>Site</u>	<u>TREATMENT</u>			<u>TOTAL Mn IN SOLUTION <math>\mu\text{g/ml}</math></u>
	<u>A</u>	<u>F+A</u>	<u>F</u>	
PENYGROES 1	3.0	3.0	NR	
PENYGROES 5	1.0	1.0	0.9	
PENYGROES 7:2	2.5	2.5	NR	
PENYGROES 7:3	1.2	1.2	1.0	
PENYGROES 7:4	1.8	1.8	1.7	
PENYGROES 7:5	0.8	0.7	0.6	

NR No result available.

The three treatments A, F+A and F represent, respectively, total iron in the drainage water, total iron in solution at the site of collection, and total iron remaining in solution after oxidation. The total iron in the drainage water ranged from 0.4 to 22.4  $\mu\text{g/ml}$  and the total iron in solution ranged from 0.3 to 19.5  $\mu\text{g/ml}$ . The sites having the highest iron concentrations were Llyn Cowlyd (1), Llyn Coedty (1) and Penygroes 1 where total iron in solution was 8.0  $\mu\text{g/ml}$ , 14.8  $\mu\text{g/ml}$  and 19.5  $\mu\text{g/ml}$  respectively. The concentrations of iron remaining in solution after oxidation were lower at all sites, ranging from 0.0  $\mu\text{g/ml}$  to 6.8  $\mu\text{g/ml}$ . At 4 sites iron was below detection limits after oxidation. The results were analysed statistically by two way analysis of variance. The treatments were used as one factor and the sites were used as the second factor. The overall treatment means were:-

<u>Treatment</u>	<u><math>\mu\text{g/ml Fe}</math></u>
A	7.8
F+A	6.8
F	1.1

Tukey's test was used to determine the significance of the results. A significant difference was found between all treatments at the 1% level (at  $p < 0.01$ , honestly significant difference = 0.68). Most iron in the drainage water on site was in solution and 84% (5.7  $\mu\text{g/ml}$ ) was removed from solution by oxidation.

The concentrations of manganese in the samples were lower than those recorded for iron. The total amount in drainage water ranged from 0.8 to 3.0  $\mu\text{g/ml}$  while the total amount in solution ranged from 0.7 to 3.0  $\mu\text{g/ml}$ . The concentrations in solution after 2-3 days were slightly lower, ranging from 0.6  $\mu\text{g/ml}$  to 1.0  $\mu\text{g/ml}$  in the samples analysed.

The difference between each treatment at each site was also examined statistically by two way analysis of variance. The results are shown in Table 6.3. In 9 out of the 12 samples no significant difference was found between total iron in the drainage water and total iron in solution. A significant difference at the 1% level was only found at Llyn Coedty 3. At Llyn Cowlyd 3 and Penygroes 1 a significant difference was observed at the 5% level. At 7 sites a significant difference (at the 1% level) was observed between total iron in solution before and after oxidation. At Penygroes 7:3, 7:4 and 7:5 the level of iron initially in the water was less than 2.0  $\mu\text{g/ml}$  and a significant difference could not be detected between the treatments but a reduction in iron content after oxidation was observed.

TABLE 6.3 : THE STATISTICAL SIGNIFICANCE OF THE DIFFERENCES BETWEEN  
IRON CONCENTRATIONS IN UNFILTERED AND FILTERED DRAINAGE  
WATER BEFORE AND AFTER OXIDATION

	<u>TREATMENT</u>			
	<u>A</u>	<u>F+A</u>	<u>F</u>	<u>A</u>
LLYN COWLYD 1			**	**
LLYN COWLYD 2			**	**
LLYN COWLYD 4	*	.	**	**
LLYN COEDTY 1			**	**
LLYN COEDTY 2			**	**
LLYN COEDTY 3	**			**
PENYGROES 1	*		**	**
PENYGROES 5			**	**
PENYGROES 7:2				*
PENYGROES 7:3				
PENYGROES 7:4				
PENYGROES 7:5				

Tukey's Honestly significant difference =

2.84 at  $p < 0.05$

3.25 at  $p < 0.01$

\* STATISTICALLY SIGNIFICANT DIFFERENCE FOUND AT 5% LEVEL ONLY

\*\* STATISTICALLY SIGNIFICANT DIFFERENCE FOUND AT 1% LEVEL

## 6.2 THE EFFECT OF OCHRE ON THE RATE OF FERROUS IRON OXIDATION

### 6.2.1 The apparent growth of filamentous bacteria in hay extract media

The use of plant extracts to isolate sheathed filamentous bacteria was discussed in section 5.3.2.

#### 6.2.1.1 Qualitative Observations

Amongst the experimental media used to isolate sheathed filamentous bacteria were dilute hay extracts containing ferrous sulphate. 2.5 g of hay was boiled in 600 ml of water for three minutes. 10.0 ml, 1.0 ml and 0.1 ml aliquots of extract were diluted to 20.0 ml with distilled water giving three dilutions; 1, 2 and 3 respectively. These solutions were autoclaved for 15 minutes at 15 lbs pressure. 1.0 ml of filter sterilized 1.0 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was then added giving approximately 10  $\mu\text{g/ml}$  of Fe(II) in the medium. Ferrous sulphate solutions were filter sterilized by passing through 0.22  $\mu\text{m}$  Millipore filters in Swinnex filter holders. After addition of iron the media had a pH of 4.0. Since one of the major problems associated with the study of filamentous bacteria is the chemical oxidation of ferrous iron at pH values above 5 (Van Veen, 1972; Ehrlich, 1978; Langworthy, 1978) the pH of the experimental media was not initially adjusted.

The media were inoculated with 0.1 ml of undiluted ochre from Llyn Coedty and Donegal. The inoculum was introduced slowly to form an entire floc in the medium. Uninoculated controls were also prepared. The flasks were examined visually after a few days. Whilst the flasks inoculated with ochre from Llyn Coedty showed no apparent increase in the size of the inoculum it appeared as if some growth had occurred in the flasks inoculated with Donegal ochre. For example, in Donegal dilution 1 a thin opaque film was evident on the surface of the medium. Such a film was not seen in any of the uninoculated flasks.



The flocs from the inoculated flasks were transferred into fresh media at the corresponding dilutions. After twelve days wet mounts of the flocs were examined under the microscope. The flocs were composed of straight filaments associated with iron particles. However, such visual and microscopic examination was limited since it was difficult to determine whether or not the organisms observed were part of the original inoculum.

#### 6.2.1.2. Quantitative Observations

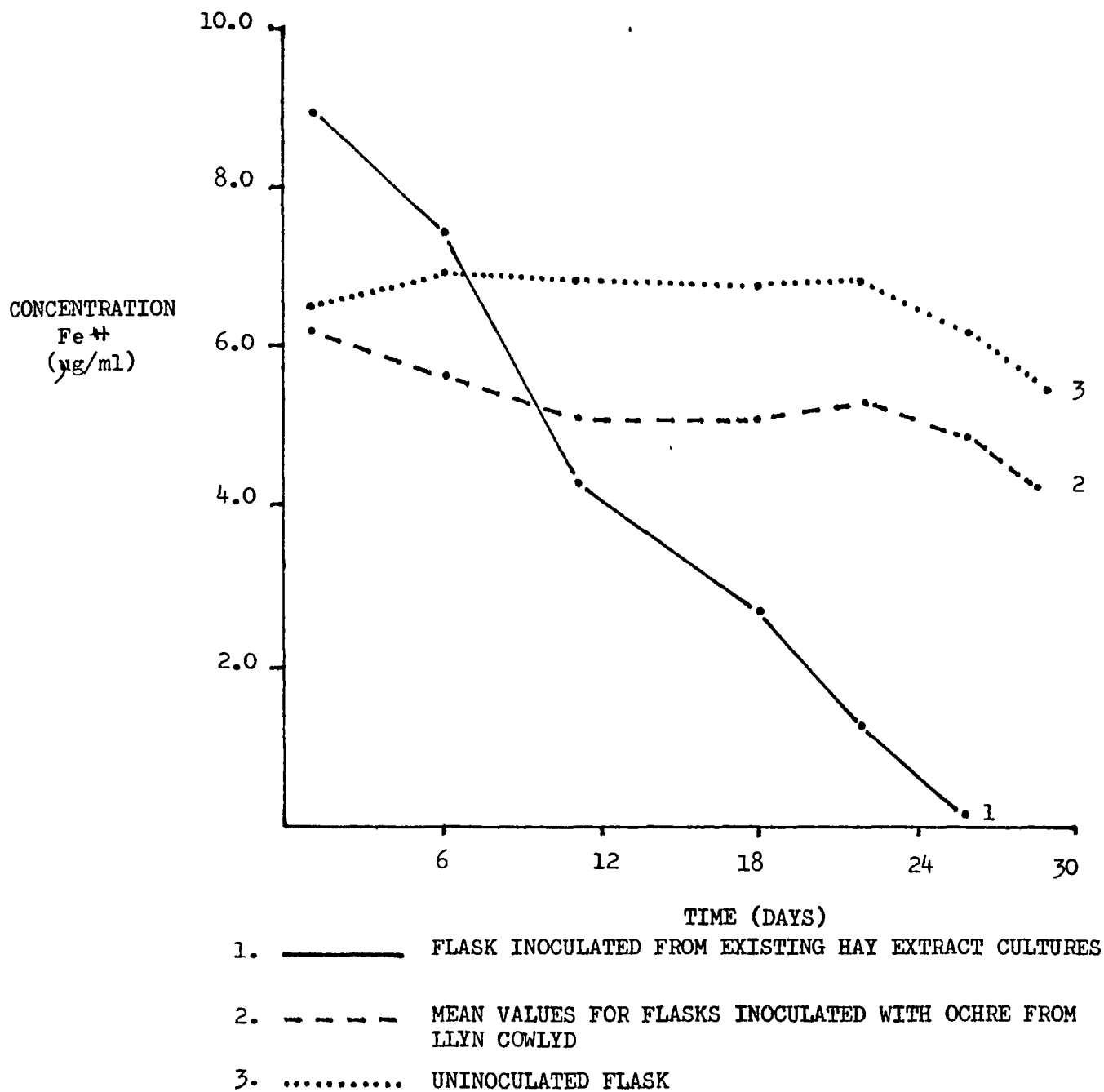
In order to monitor the growth of sheathed filamentous bacteria in the hay extracts the ferrous iron concentration was determined throughout the incubation period.

Four 100 ml portions of medium (dilution 3) were prepared as described earlier. One flask was inoculated with 2.0 ml from a hay extract culture inoculated initially with ochre from Donegal. Two flasks were inoculated with 2.0 ml of filamentous ochre from Llyn Cowlyd and the remaining flask was kept as an uninoculated control.

At regular intervals 5.0 ml was withdrawn from each flask using a sterile pipette. The aliquots were filtered through a 0.22  $\mu$ m Millipore filter and stored in sterile containers. Total iron in solution was determined immediately by atomic absorption spectrophotometry.

The results, shown in Figure 6.1, seem to confirm the conclusion drawn from visual examination of the initial media that filamentous bacteria could grow in the dilute hay extracts. Although a marked decline in Fe(II) concentration was not observed in the flasks inoculated with Llyn Cowlyd ochre all iron had precipitated in the flask inoculated with flocs from the Donegal culture. The drop in iron concentration in the control flask after 22 days was attributed to chemical oxidation.

Figure 6.1    THE CONCENTRATION OF FERROUS IRON IN AN INOCULATED HAY EXTRACT MEDIUM



This experiment was repeated using dilution 2 and dilution 3 hay extract media. Replicate 100 ml portions were inoculated with 5.0 ml of filamentous ochre from Donegal. Two uninoculated controls were also prepared. 5.0 ml of autoclaved ochre was included in one flask to act as a sterile control. 5 ml portions were removed at regular intervals, filtered through 0.22  $\mu$ m Millipore filters, and total iron in solution determined by atomic absorption spectrophotometry. The results, summarized in Table 6:4, suggest that growth had occurred in the inoculated flasks. In these flasks no iron remained in solution after 6 days. Although some oxidation of iron was evident in the uninoculated flasks over 6  $\mu$ g/ml remained in solution after 18 days. However, a rate of oxidation comparable to that observed in the inoculated media was recorded in the culture containing autoclaved ochre. This implies that chemical oxidation of iron in the hay media was occurring. It was also realized that the rate of oxidation was increased in the presence of sterile ochre.

TABLE 6.4 : THE CONCENTRATION OF FERROUS IRON IN HAY EXTRACT MEDIA

	CONCENTRATION OF Fe(II) ( $\mu$ g/ml)			
		<u>TIME (days)</u>		
	<u>1</u>	<u>6</u>	<u>12</u>	<u>18</u>
<u>DILUTION 2</u>				
INOCULATED (2)	8.8	0.3	0.0	1.0
UNINOCULATED (2)	9.4	7.3	6.5	6.1
STERILE CONTROL (1)	8.0	0.6	*	*
<u>DILUTION 3</u>				
INOCULATED (2)	8.8	0.0	*	*
UNINOCULATED (2)	9.6	8.2	7.3	6.8
STERILE CONTROL (1)	9.3	1.6	*	*

\* NO RESULT AVAILABLE

(1) THE STERILE CONTROL WAS INOCULATED WITH AUTOCLAVED OCHRE

(2) MEANS OF IRON CONCENTRATION IN TWO REPLICATES

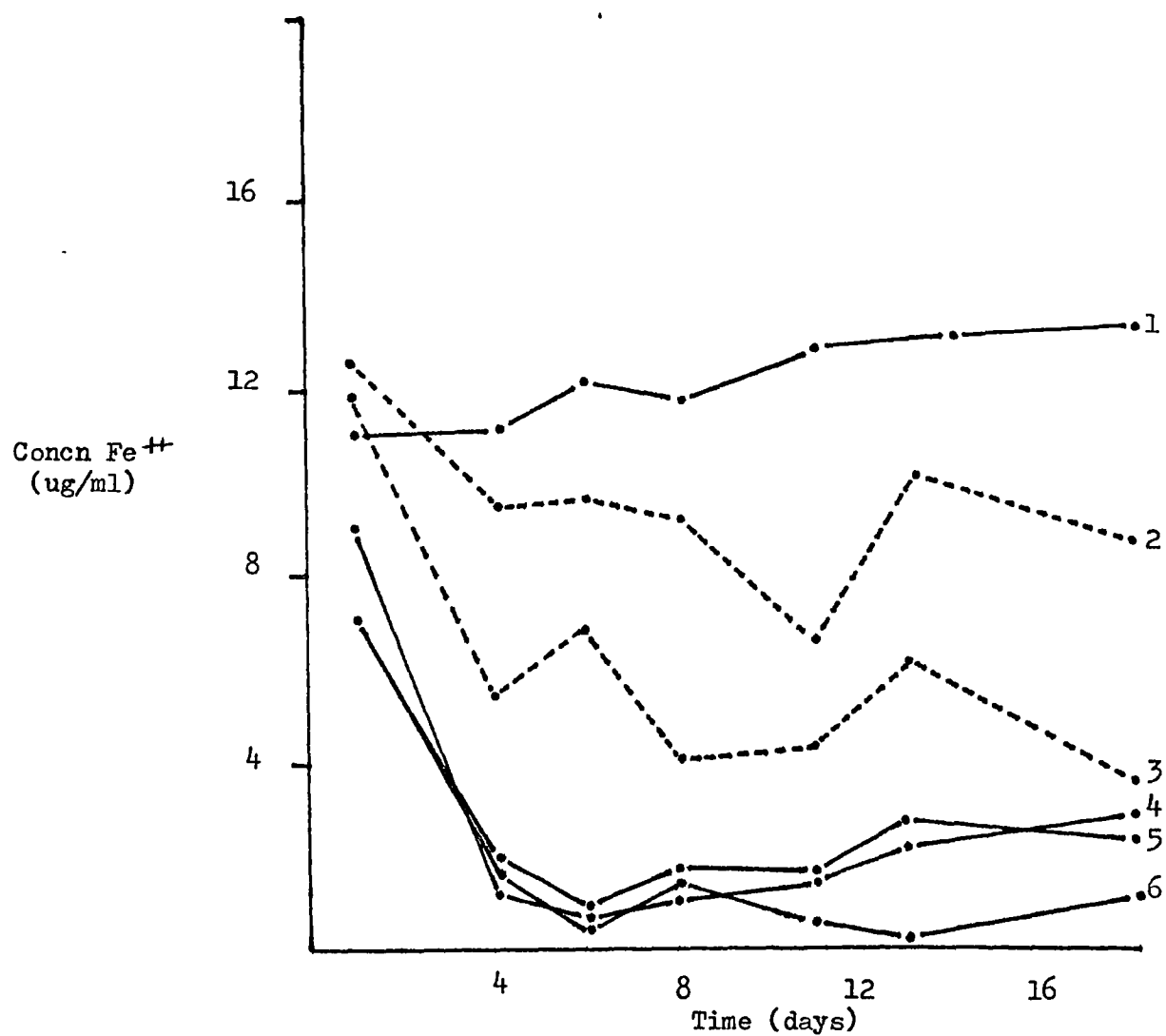
6.2.1.3 The effect of sterilized ochre on the concentration of iron in hay extract medium and distilled water

The effect of sterile and non-sterile ochre on the concentration of ferrous iron in hay extracts and water were determined as follows. Two solutions were used: firstly, some portions of hay extract medium (dilution 2) were prepared as described earlier (6.2.1.1); secondly, some portions of distilled water were autoclaved and 5.0 ml of filter sterilized ferrous sulphate solution (1.0 g/l) added. Replicate flasks of each medium were inoculated as shown below.

<u>FLASK</u>	<u>INOCULUM</u>
1	1.0 ml of ochre from Donegal
2	1.0 ml of autoclaved Donegal ochre
3	1.0 ml of Donegal ochre and 25 mg of Sodium Azide
4	1.0 ml of ochre from Llyn Cowlyd
5	1.0 ml of autoclaved Llyn Cowlyd ochre
6	Uninoculated

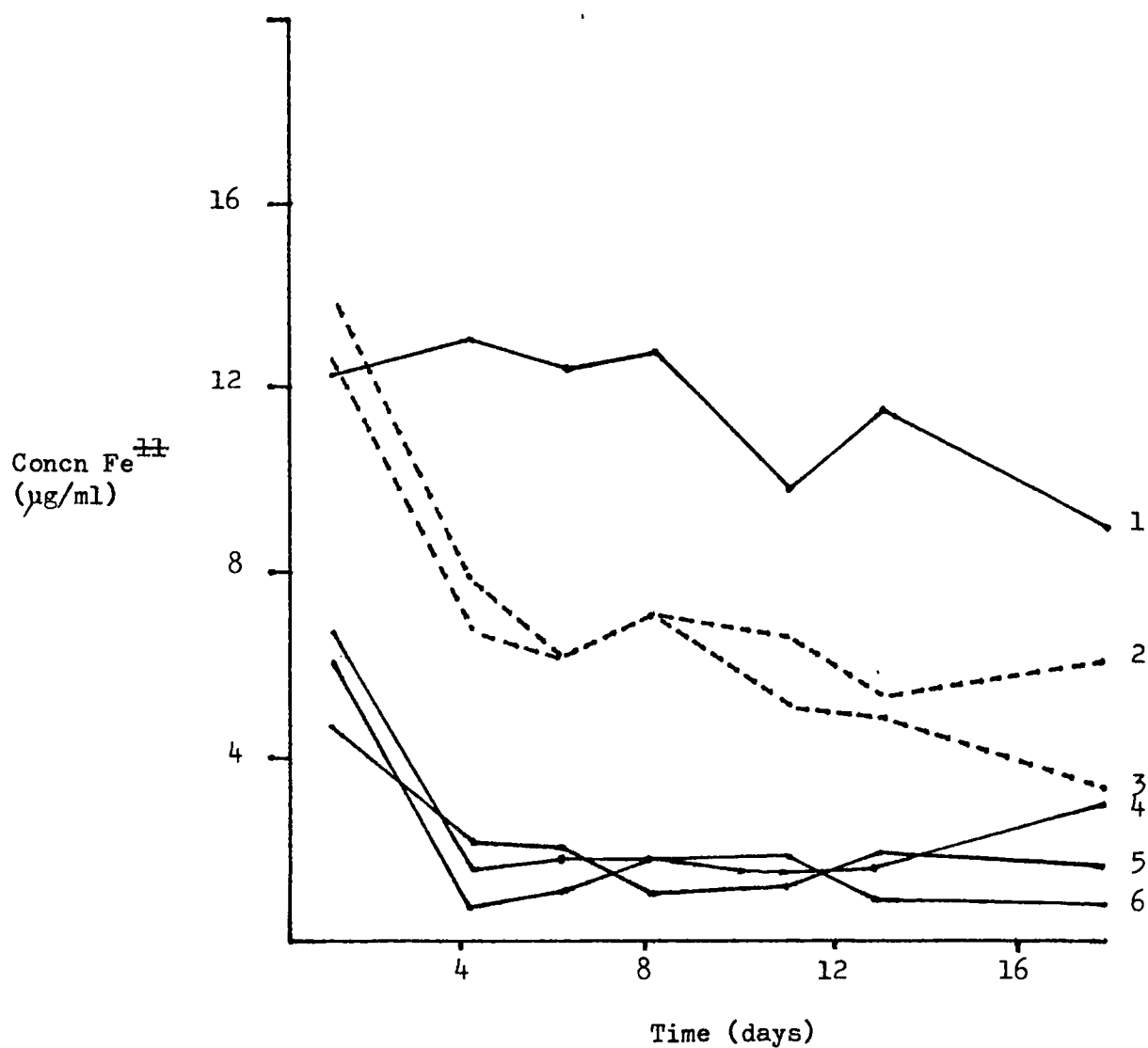
Total iron in solution was determined at regular intervals by atomic absorption spectrophotometry of filtered (0.22  $\mu$ m) aliquots. The results for the hay extract and water media are shown in graphs 6.2 and 6.3 respectively. It can be seen from the graphs that the concentration of ferrous iron is substantially lower in all the inoculated flasks than in the uninoculated controls. After 4 days incubation the concentration of iron in the control flasks remained above 11  $\mu$ g/ml and 9  $\mu$ g/ml in the hay extract and water medium respectively. During the same time period the concentration in the inoculated flasks did not rise above 10  $\mu$ g/ml and 8  $\mu$ g/ml in the hay and water media. The flasks containing unsterilized Donegal ochre had a slightly lower iron concentration at the end of the experiment. However, the difference was no greater than that recorded between the flasks containing ochre sterilized by autoclaving and by sodium azide. It appears from Figure 6.2 that more oxidation had occurred

Figure 6.2 The concentration of ferrous iron in hay medium inoculated with sterilized and non-sterilized ochre samples



- 1 CONTROL
- 2 LLYN COWLYD - AUTOCLAVED
- 3 LLYN COWLYD - NON STERILIZED
- 4 DONEGAL - SODIUM AZIDE
- 5 DONEGAL - AUTOCLAVED
- 6 DONEGAL - NON STERILIZED

Figure 6.3 The concentration of ferrous iron in water medium inoculated with sterilized and non-sterilized ochre samples



- 1 CONTROL
- 2 LLYN COWLYD - AUTOCLAVED
- 3 LLYN COWLYD - NON STERILIZED
- 4 DONEGAL - SODIUM AZIDE
- 5 DONEGAL - AUTOCLAVED
- 6 DONEGAL - NON STERILIZED

in the flask containing unsterilized ochre from Llyn Cowlyd. At the end of the experiment the concentration of iron in the sterile treatment was  $8.8 \mu\text{g/ml}$ , whereas a concentration of  $3.8 \mu\text{g/ml}$  was recorded in the flask containing unsterilized ochre. This suggests that although chemical oxidation, at a rate increased by the presence of ochre, does occur microorganisms are also contributing to the oxidation of ferrous iron in hay medium. However, growth of sheathed filamentous bacteria in the hay medium is difficult to confirm by monitoring Fe(II) levels since chemical oxidation also causes a reduction in Fe(II) concentration.

It was concluded from these experiments that the apparent growth of filamentous bacteria in the hay extract - ferrous iron medium could be attributed to a chemical oxidation of iron caused by the presence of ochre.

#### 6.2.2 The effect of air dried ochre on the concentration of Fe(II) in a ferrous sulphate solution

In the experiments described in section 6.2.1 employing concentrations of iron less than  $15 \mu\text{g/ml}$ , filtration, and the undefined hay extracts, the extent of experimental error was quite large. This is revealed by the fluctuations in iron content from one sampling date to another (see figures 6.2 and 6.3). The effect of ochre on the concentration of Fe(II) in solution under standard conditions was studied by a series of experiments employing air dried ochre and ferrous sulphate solutions.

##### 6.2.2.1 The effect of air dried ochre on the rate of oxidation of Fe(II) in a ferrous sulphate solution

1.0 g samples of air dried ochre (Persehall 2) were weighed into 16 polypropylene bottles (150 ml) and 50 ml of 5 g/l  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  solution added. The initial concentration of Fe(II) in this solution was 0.96 g/l as determined by permanganate titration. 16 bottles containing 50 ml of ferrous sulphate were used as controls. The bottles were shaken on an

end-over-end shaker for  $3\frac{1}{2}$  hours. Every 30 minutes 2 control and 2 ochre containing bottles were removed. The contents of these bottles were filtered through Whatman No 1 filter paper and the concentration of Fe(II) was determined by titrating 5.0 ml of filtrate and 5.0 ml of 1M  $H_2SO_4$  against  $3.0 \times 10^{-3}M$  (0.5 g/l) potassium permanganate. The latter was standardized by titrating against  $5 \times 10^{-3}M$  ammonium ferrous sulphate. The pH of the filtrate was recorded throughout the experiment.

The results, shown in figure 6.4, clearly demonstrate the effect of air dried ochre. All the ferrous iron was oxidized in the presence of ochre, whereas no significant change was observed in the Fe(II) concentration of the controls. The reduction in Fe(II) concentration was accompanied by an increase in pH. The pH of the controls remained constant at pH 4.1 - 4.2 throughout the experiment while the pH in the ochre containing solution rose from an initial pH of 3.9 to a pH of 5.4 immediately after ochre was added. After  $3\frac{1}{2}$  hours the pH had risen to 6.8. Since the chemical oxidation of Fe(II) increases rapidly as pH rises above 5.0 the observed decrease in ferrous iron concentration in the presence of ochre can be attributed to the concurrent increase in pH.

The importance of oxygen in this reaction was shown by repeating the experiment using unshaken conical flasks. In this case, as shown in figure 6.5, 0.5 g/l of ferrous iron remained in solution after 7 hours in the presence of ochre.

#### 6.2.2.2 The effect of various weights of air dried ochre on the concentration of Fe(II) in ferrous sulphate solutions

50 ml of 5 g/l  $FeSO_4 \cdot 7H_2O$  solution was added to replicate samples of air dried ochre in 150 ml polypropylene bottles. The initial ferrous iron concentration of the solution was 0.96 g/l (determined by permanganate titration). Various weights of ochre were used in the range 0.0 g to 1.4 g in 0.2 g intervals. The bottles were shaken on an end over end shaker for



Figure 6.4 The effect of air dried ochre on the ferrous iron concentration and pH of a Ferrous Sulphate Solution

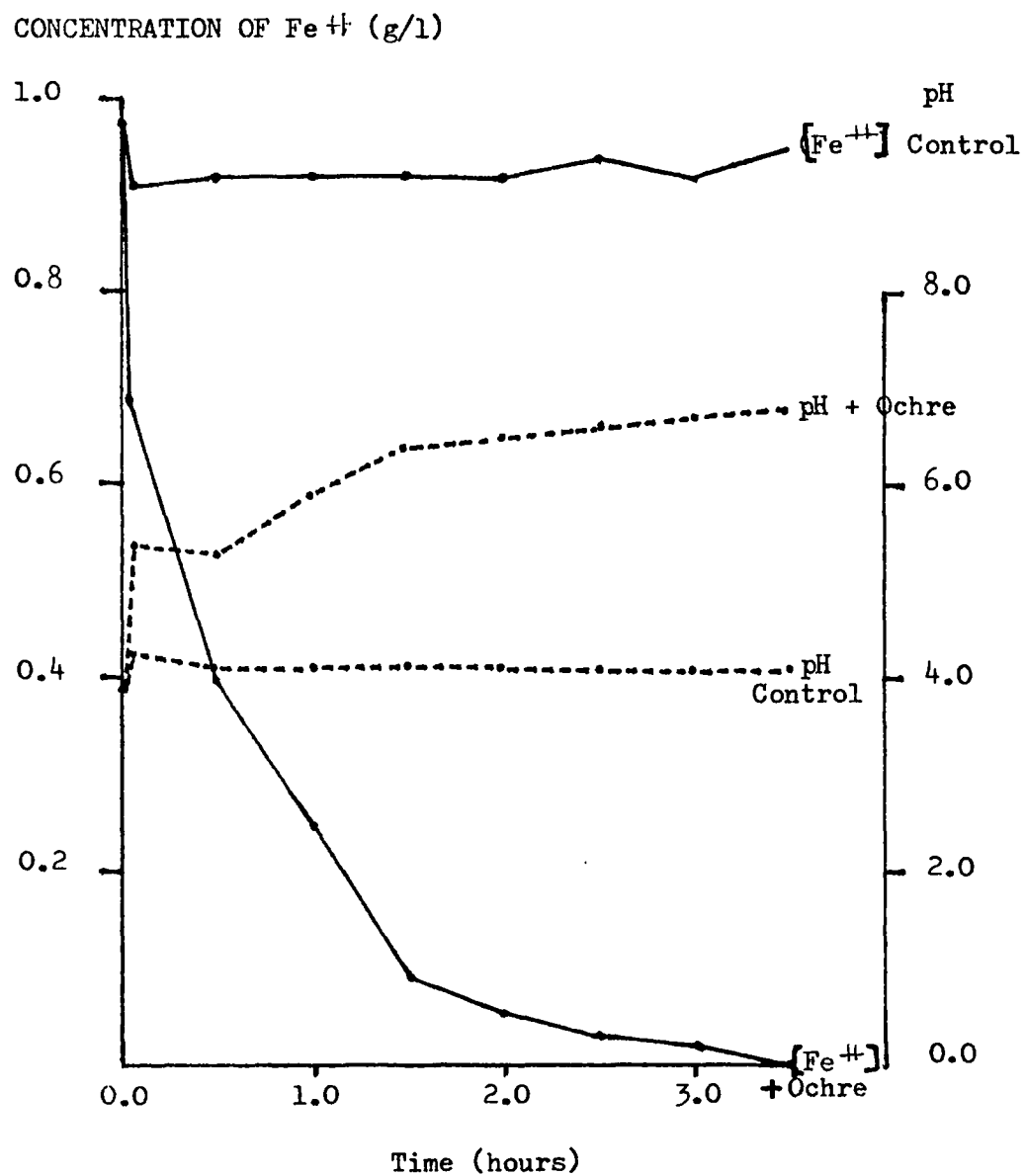
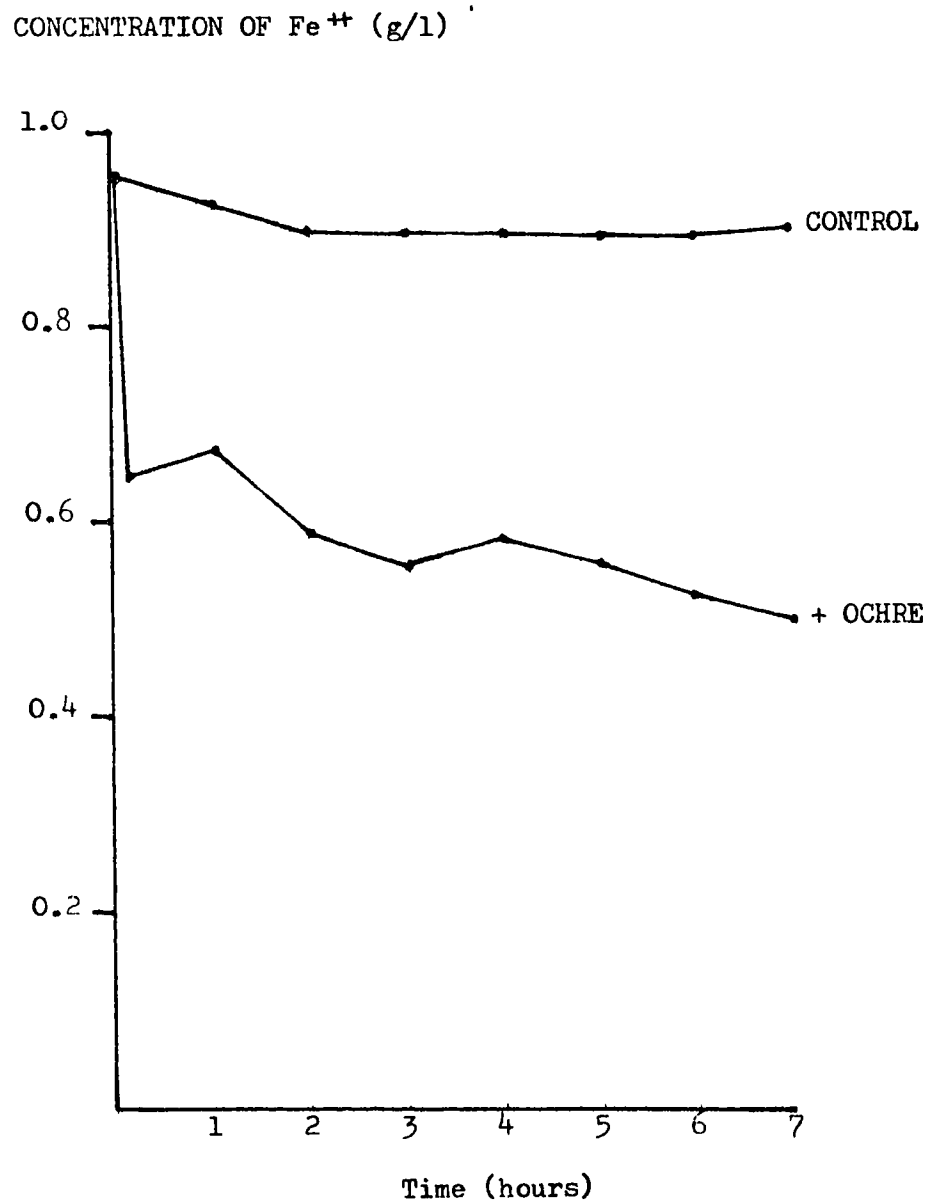


Figure 6.5 The effect of Air dried ochre on the Ferrous iron concentration of a Ferrous Sulphate solution under oxygen limiting conditions

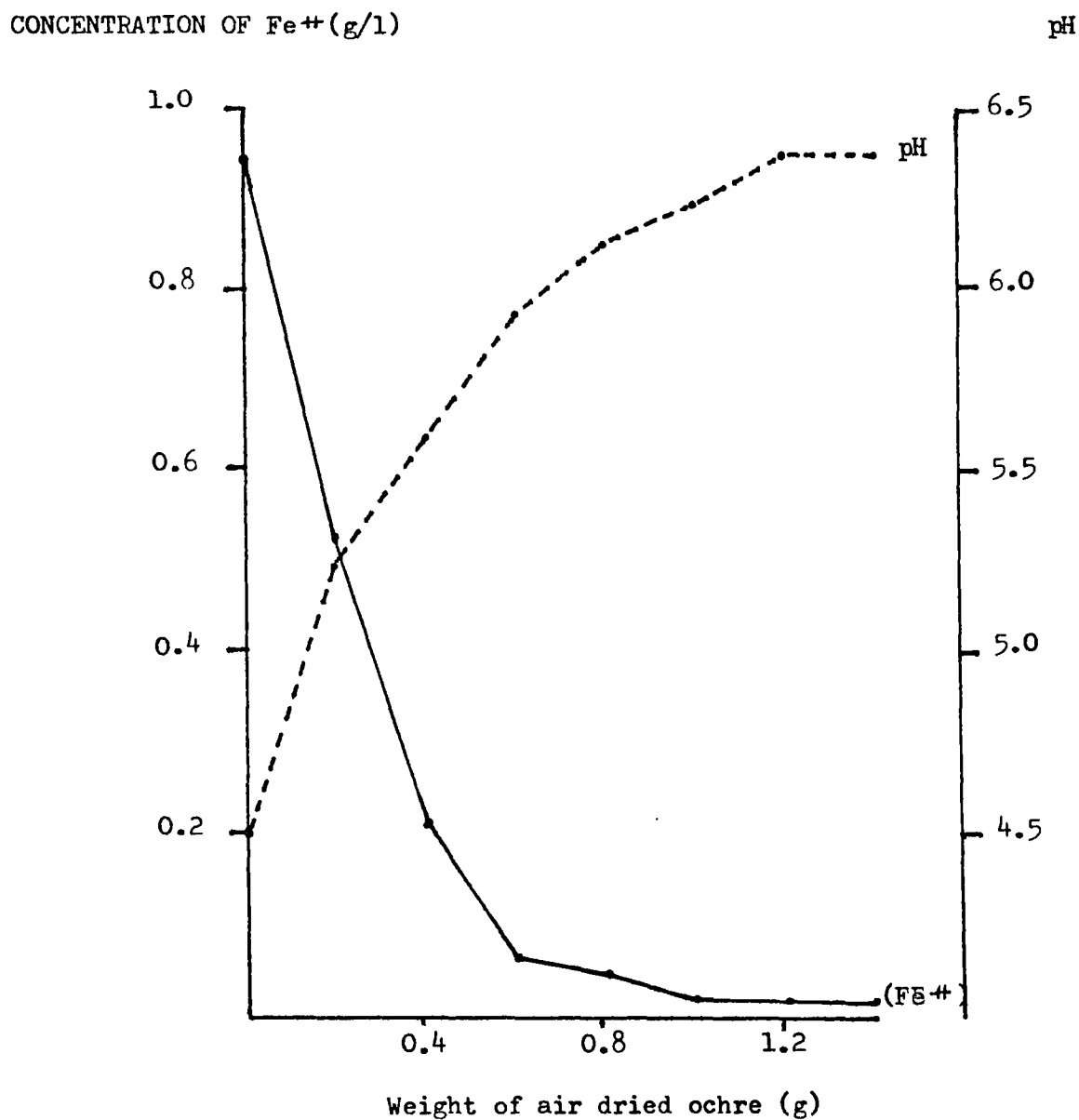


2 hours, and after this time the contents were filtered through Whatman No 1 filter paper. The ferrous iron concentration of the filtrate was determined by permanganate titration and the pH of the filtrate was also recorded. The results are shown in figure 6.6. It can be seen that air dried ochre has a marked effect on both ferrous iron concentration and pH. The regressions of weight on pH and iron concentration are both significant at 1.0%. Weights of ochre greater than 1.0 g resulted in an almost complete removal of iron from solution and caused the pH to rise from 4.5 to over 6.25. Additionally, these results reveal that most of the variation observed in iron concentration can be explained by the increase in pH. A correlation coefficient of 0.97 was recorded between these two variables.

6.2.2.3 The effect of air dried ochre on the Fe(II) concentration of ferrous sulphate solutions in a continuous flow apparatus

5.0 l of ferrous sulphate solution (5.0 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}/\text{l}$ ) was allowed to flow continuously by gravity through a column containing 8 g of air dried ochre. The column was made from a 20 ml syringe with a 200  $\mu\text{l}$  Pipetman tip attached at the outlet. A 100  $\mu\text{M}$  mesh disc was placed at the base of the syringe to prevent ochre from entering and blocking the tip. The solution dripping from the column was collected in a 5 l container over 2.5 days. After this time some ferrous sulphate remained in the upper container below the outlet, and this solution and the solution in the receiving vessel were examined. The concentration of ferrous iron was determined by filtering the solutions through Whatman 541 filter paper and titrating three aliquots of filtrate against standard potassium permanganate as described in section 6.2.2.1. The pH of the filtrate was also recorded. The experiment was repeated using the same column for a further 2.5 days. The results are given in Table 6.5.

FIGURE 6.6 The effect of air dried ochre on the ferrous iron concentration and pH of a ferrous sulphate solution



The results show that ochre caused an initial rise in the pH of the ferrous sulphate solution accompanied by a decrease in ferrous iron concentration. The initial ferrous iron concentration of the solution was 0.96 g/l at pH 4.3. After 2.5 days the ferrous iron concentration of this solution had fallen to 0.89 g/l. In the solution that had passed through the column the ferrous iron concentration had fallen to 0.79 g/l and the pH had risen to 4.5. The results for the second run show that the ability of ochre to raise the solution pH is reduced as the volume of ferrous sulphate passing through increases and its buffering capacity is exceeded. After five days the concentration of Fe(II) in the upper flask was 0.95 g/l and in the receiving vessel the concentration was 0.89 g/l.

TABLE 6.5 : THE FERROUS ION CONCENTRATION AND pH OF A FERROUS SULPHATE SOLUTION PASSED THROUGH AIR DRIED OCHRE IN A CONTINUOUS FLOW APPARATUS

TIME (DAYS)	<u>UPPER CONTAINER</u>		<u>RECEIVING VESSEL</u>	
	<u>CONCENTRATION Fe(II) g/l</u>	<u>pH</u>	<u>CONCENTRATION Fe(II) g/l</u>	<u>pH</u>
<u>FIRST RUN</u>				
1	0.96	4.3	-	-
2.5	0.89	4.1	0.79	4.5
<u>SECOND RUN</u>				
2.5	1.01	4.0	-	-
3.0	0.97	3.9	0.91	5.1
5.0	0.95	3.9	0.89	4.5

6.2.3 The effect of air dried ochre on the concentration of iron in a citric acid - ferrous sulphate solution

The experiment described in section 6.2.2.1 was repeated using air dried ochre (Persehall 2) and a solution of citric acid and ferrous sulphate at pH 6. It was thought that such a solution would be a better representation of natural drainage waters, which contain organic compounds, than a ferrous sulphate solution alone.

0.1 g citric acid and 0.05 g ferrous sulphate were dissolved in distilled water and made up to 1 l. The pH of the solution was adjusted to 6.0 with 1M NaOH. 1.0 g of air dried ochre was weighed into 16 150 ml polypropylene bottles. 50 ml of the citric acid - Fe(II) solution was added to these bottles and to 16 empty bottles used as controls. The bottles were shaken in an end over end shaker for  $3\frac{1}{2}$  hours. Every 30 minutes 2 controls and 2 bottles containing ochre were removed. The contents were filtered through Whatman 541 filter paper. The pH of the filtrate was recorded. A portion of each filtrate was passed through a 0.22  $\mu$ m Millipore filter to remove all iron precipitates before total iron was determined by atomic absorption spectrophotometry. The initial iron concentration of the solution was 11.9  $\mu$ g/ml.

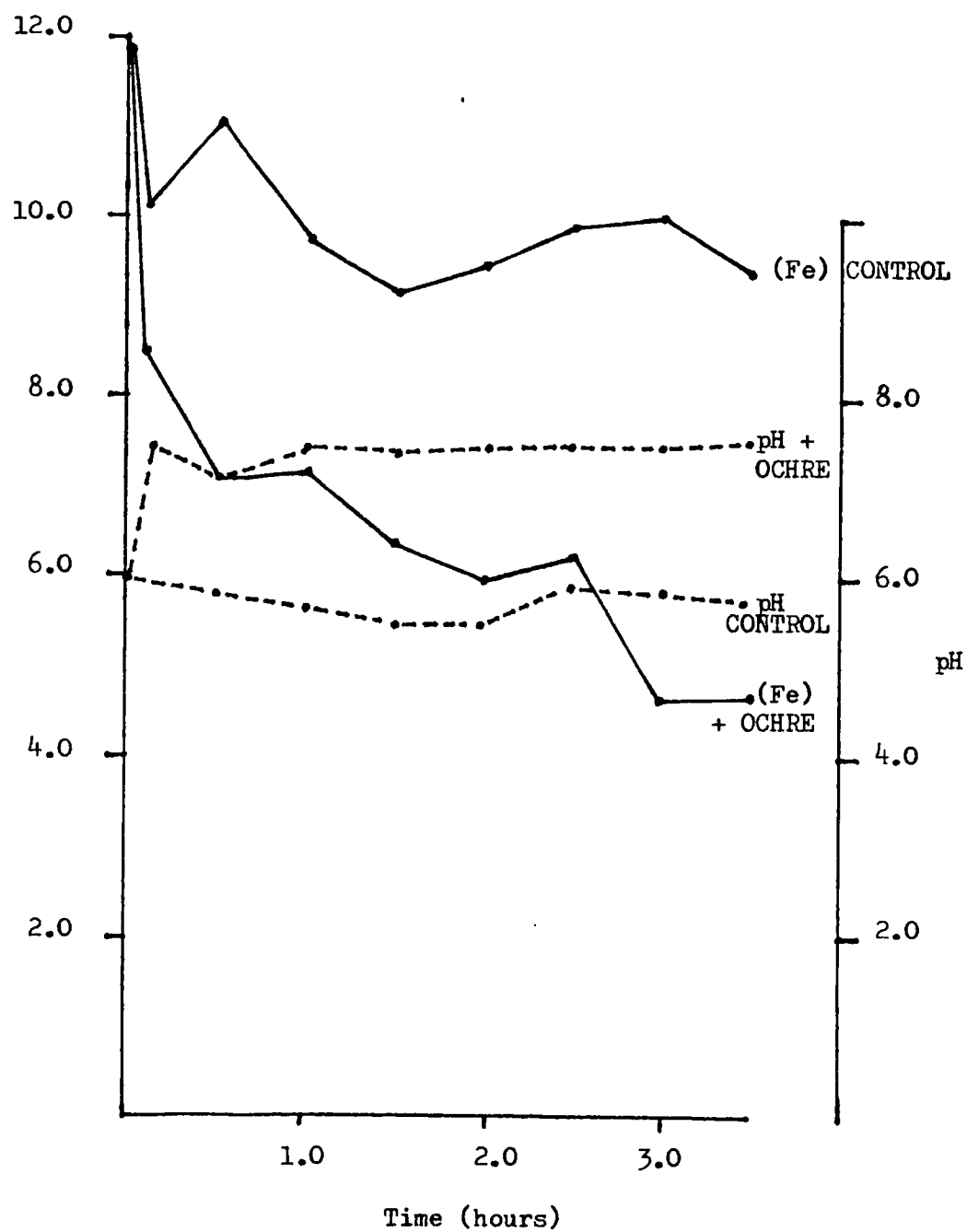
The results, shown in figure 6.7, are comparable to those obtained in section 6.2.2.1 (figure 6.4) where a ferrous sulphate solution was used. The bottles containing ochre showed a decrease in iron concentration from 11.9  $\mu$ g/ml to 4.8  $\mu$ g/ml after  $3\frac{1}{2}$  hours. In the control solutions 9.5  $\mu$ g/ml of iron remained in solution after  $3\frac{1}{2}$  hours. Although an initial increase in pH, from 6.0 to 7.5, was recorded after adding ochre no further increase was observed during the experiment. The results show that iron associated with organic compounds is also subject to an increased rate of oxidation in the presence of air dried ochre.

#### 6.2.4 The effect of sterilized ochre on the concentration of iron in drainage water

Filamentous ochre was collected from Sychnant Pass. The pH of the sample, as determined in the laboratory, was 6.2. 15.0 g portions of this ochre (0.18 g oven dried ochre) were weighed directly into 4 150 ml polypropylene bottles. The bottles were sterilized by autoclaving for 15 minutes. 4 bottles containing 15.0 ml of distilled water were also autoclaved.

Figure 6.7 The effect of air dried ochre on the pH and concentration of iron in a citric acid - ferrous sulphate solution

CONCENTRATION OF IRON  
IN SOLUTION ( $\mu\text{g/ml}$ )



Two bottles containing ochre and two control bottles were filled on site with approximately 150 ml of drainage water from Llyn Cowlyd and Llyn Coedty.

It had been found that the contribution of microorganisms to the extent of iron oxidation in drainage water was negligible and chemical oxidation could account for the removal of iron from drainage water (section 6.1) Consequently the water samples were not sterilized in this experiment but to confirm that microbial iron oxidation was not affecting the results replicate aliquots of filtered (0.22  $\mu\text{m}$ ) and unfiltered water were collected at each site.

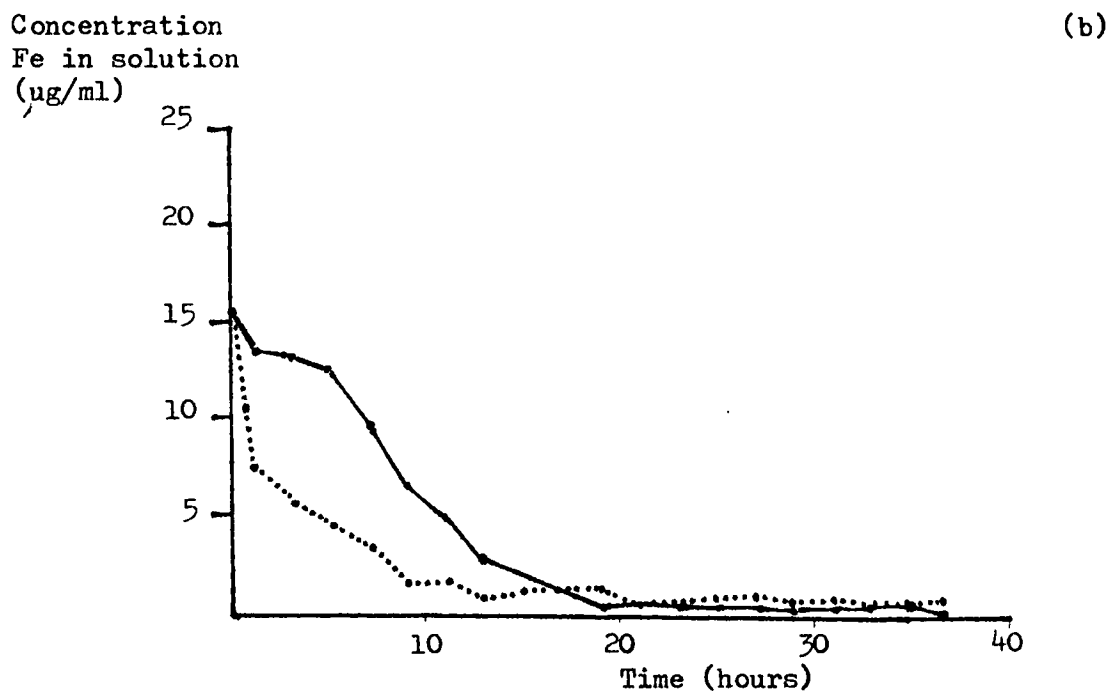
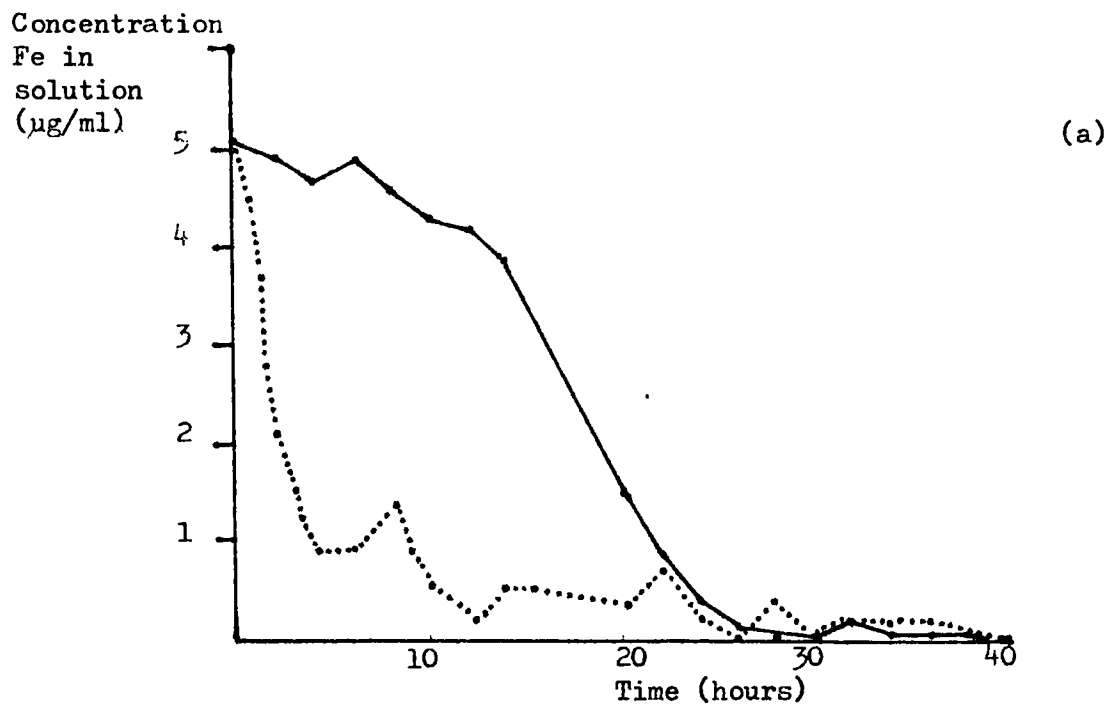
The initial iron concentrations of drainage water samples were determined by acidifying 10 ml samples with 1.0 ml of concentrated hydrochloric acid on site. At regular intervals 3.0 ml aliquots were withdrawn from each polypropylene bottle, filtered through 0.22  $\mu\text{m}$  Millipore filters, and acidified with 0.5 ml of concentrated hydrochloric acid. The acidified aliquots were stored in pre-sterilized glass vials. This procedure was continued for 38 hours. Total iron in solution was determined for the acidified aliquots by atomic absorption spectrophotometry with ferric nitrate standards in the range 0 to 12  $\mu\text{g/ml}$  of iron.

The experiment outlined above was repeated using three drainage waters collected at Penygroes 1, 5 and 7:1. In this case 10 g portions (0.12 g oven dried ochre) of sterilized Sychnant Pass ochre were used.

The results are shown in figures 6.8 and 6.9 and are summarized in Table 6.6. The results are also shown as log-time plots in figure 6.10. To avoid negative logarithms in the latter iron concentrations were expressed in mg/ml.



Figure 6.8 The effect of sterilized Ochre on the concentration of iron in drainage water from (a) Llyn Cowlyd and (b) Llyn Coedty



————— WITHOUT OCHRE  
 ..... WITH OCHRE

Figure 6.9 The effect of sterilized ochre on the concentration of iron in drainage water from Penygroes

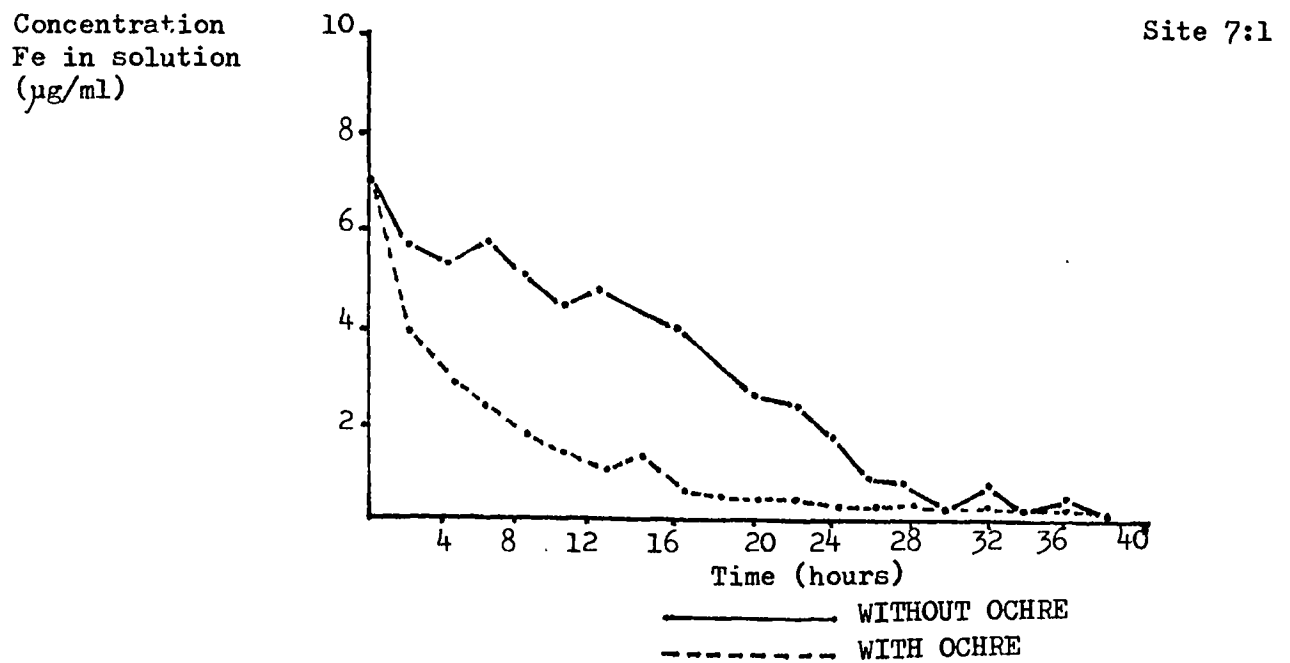
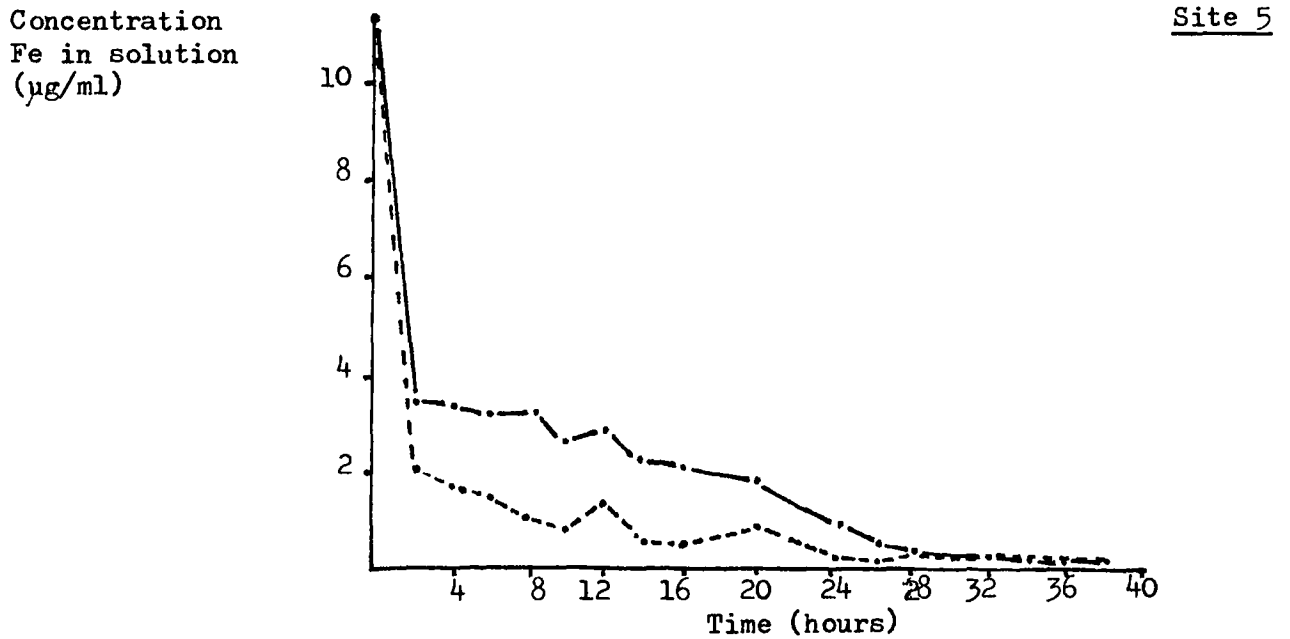
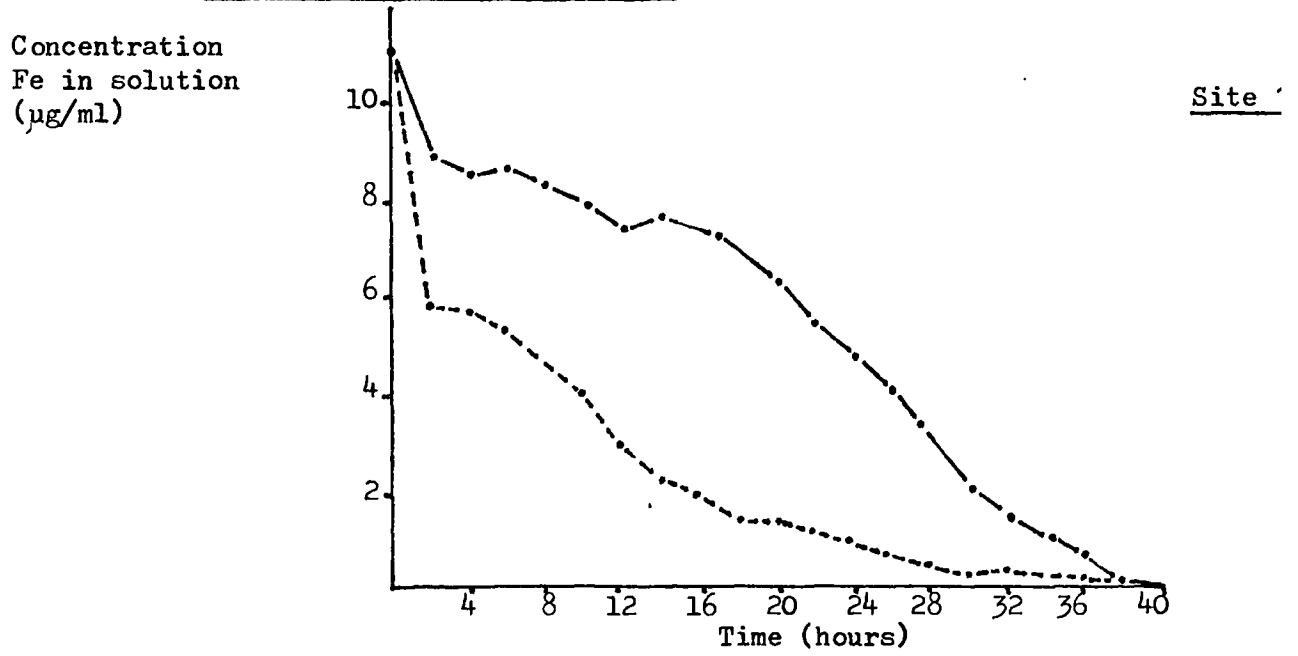
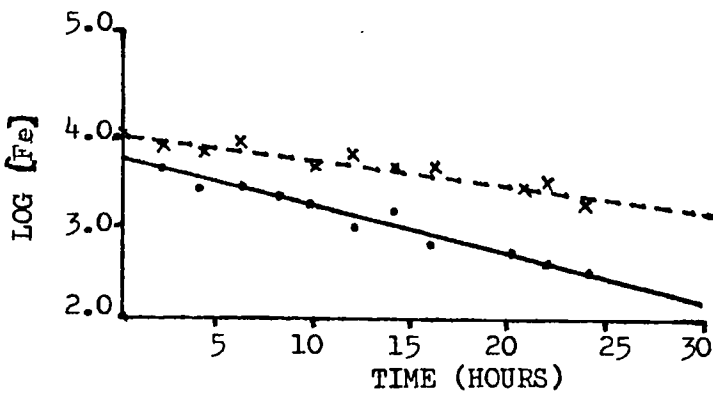
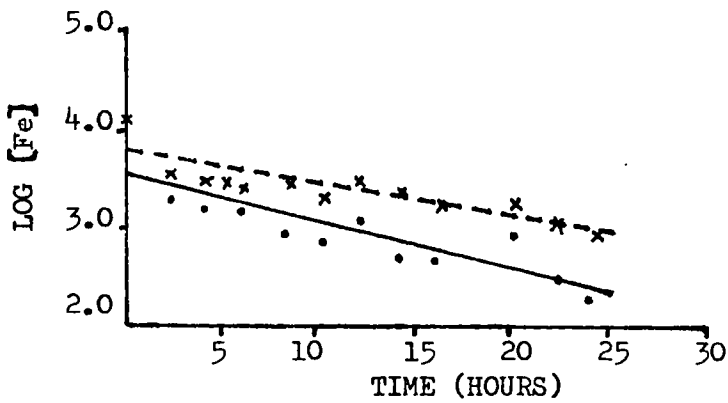
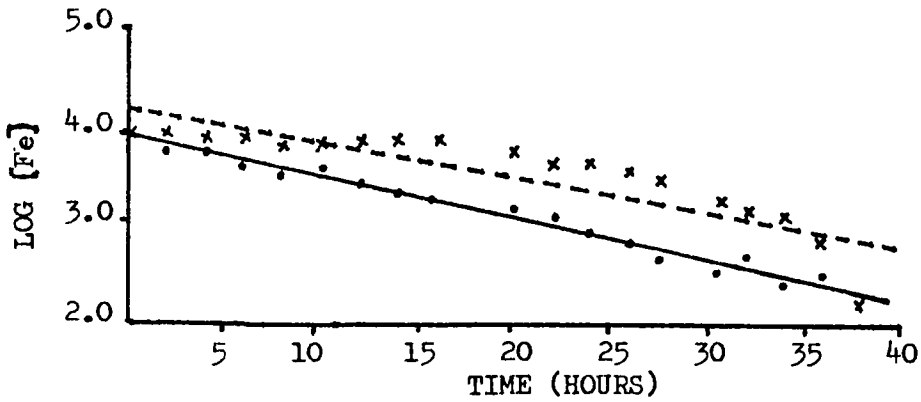
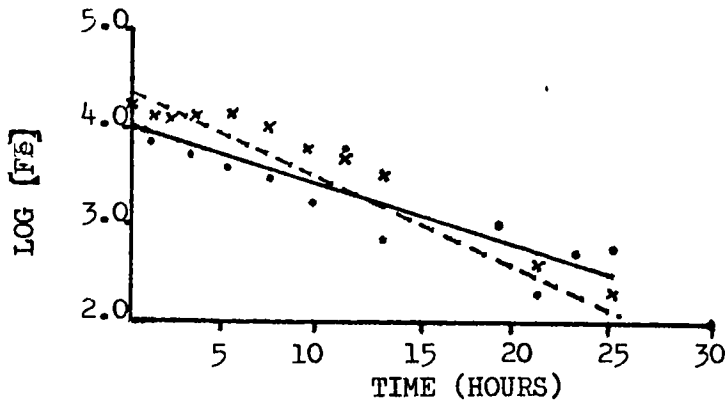
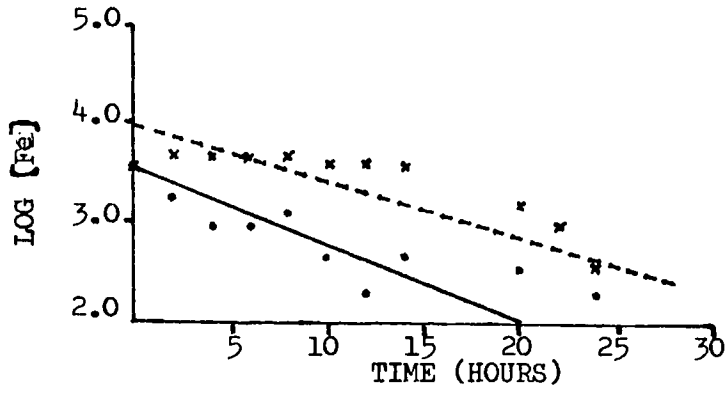


Figure 6:10 LOG (Fe)/Time Plots Showing the Effect of Sterilized Ochre on the Concentration of Iron in Drainage Water (key on page 250)





At all sites the oxidation of iron was complete within 38 hours. In the samples containing sterilized ochre the rate of oxidation was greatly increased. After two hours 57% of the iron initially in solution had been oxidized in the bottles containing ochre. The corresponding value for the controls was 25%. Similarly, after 12 hours 86% and 46% iron had been oxidized in the bottles with and without ochre respectively.

At all sites a sudden decrease in iron concentration was observed during the first two hours. The reason for this is not known but one explanation is that during this time the samples were transported to the laboratory and were subject to increased agitation and aeration. This does not explain why, for example, the initial decrease was only 2  $\mu\text{g}/\text{ml}$  in the control bottles from Llyn Coedty but about 8  $\mu\text{g}/\text{ml}$  in the Penygroes 5 control.

In the samples without added ochre the rate of oxidation after the first two hours was slow and the concentration of iron fell by only 1-2  $\mu\text{g}/\text{ml}$  within the first twelve hours. This is substantiated by the almost horizontal distribution of points on the log plots during this period. After an initially slow rate of oxidation in the controls the rate increased rapidly until no iron remained in solution.

The oxidation of iron in the bottles containing ochre contrasts sharply with the pattern described for the controls. In this case an initial period of slow oxidation was not observed. Iron was oxidized quickly from the start of the experiment, the rate gradually decreasing as the amount of iron in solution diminished. In the log plots a downward sloping distribution of points was observed without the initial horizontal pattern seen in the controls.

TABLE 6.6 : THE EFFECT OF STERILIZED OCHRE ON THE CONCENTRATION OF  
IRON IN DRAINAGE WATER

THE AMOUNTS OF IRON ARE EXPRESSED AS A PERCENTAGE OF TOTAL IRON INITIALLY PRESENT IN SOLUTION. THE PERCENTAGES ARE MEAN VALUES CALCULATED FROM THE DATA SHOWN IN FIGURES 6.8 AND 6.9

% iron oxidized		
<u>TIME (hours)</u>	<u>WITH OCHRE</u>	<u>WITHOUT OCHRE</u>
0	0	0
2	57	25
4	68	29
6	72	28
8	76	34
10	83	43
12	86	46
14	88	51
16	90	54
20	91	70
22	93	77
24	96	83
26	96	75
28	94	91
30	97	95
32	96	93
34	98	97
36	97	97
38	98	99

The iron concentration in filtered and unfiltered aliquots collected on site are shown in Table 6.7. The results show that the oxidation recorded in figures 6.8 to 6.10 can be attributed to chemical mechanisms. If biologically mediated oxidation was occurring to a significant degree then the concentration of iron in the unfiltered samples would have been less

than that recorded in the filtered aliquots. In all cases the iron concentration in filtered samples were less than or equal to the concentration in unfiltered aliquots.

TABLE 6.7 : THE CONCENTRATION OF IRON IN SOLUTION IN FILTERED AND UNFILTERED DRAINAGE WATER AFTER 48 HOURS

	Fe ( $\mu\text{g/ml}$ )	
	<u>FILTERED</u>	<u>UNFILTERED</u>
LLYN COWLYD	0.1	0.4
LLYN COEDTY	0.9	2.1
PENYGROES 1	0.0	0.0
PENYGROES 5	0.0	0.0
PENYGROES 7:1	<u>0.0</u>	<u>0.1</u>
MEAN	0.2	0.5

### 6.3 DISCUSSION

The drainage waters analysed had a range of iron concentrations from 0.3  $\mu\text{g/ml}$  to 19.5  $\mu\text{g/ml}$ . Ochre was present at all the sites showing that it can form in drainage waters containing large or small quantities of iron.

In drainage water iron can be present in solution or as Fe(III) precipitates in suspension. The amount of iron in suspension depends upon the degree of aeration of the water. The significance of oxygen in the removal of iron from solution was shown in section 6.2.2.1 where lack of oxygen reduced the rate of ferrous iron oxidation. In some sites a significant amount of iron was found in suspension in the drainage water, for example, in Llyn Cowlyd 4 and Llyn Coedty 3, which were some distance away from the source of iron containing water, the proportion of oxidized iron was high. At Llyn Cowlyd (1) and Llyn Coedty (1) where water reached the ground surface the amounts of iron in solution were 95% and 97% of

total iron respectively. At Llyn Cowlyd 4 and Llyn Coedty 3 the percentage in solution had fallen to 66% and 31% respectively. The results show that most of the iron in the drainage water was in solution. At all sites, except Llyn Coedty 3, the amount in solution was greater than that in suspension. Taking the mean of all samples the amount of iron in solution was 83%. The percentages in solution at each site are given in Table 6.8.

At the near neutral pH values associated with most ochre sites it is to be expected that any ferrous iron would be oxidized rapidly. The amounts of iron in solution at sampling and after 2-3 days are shown in Table 6.8. Taking a mean of all sites it was found that 76% of the iron initially in solution was chemically oxidized within 2-3 days. At four sites 100% oxidation was recorded.

The manganese levels in drainage water from Penygroes (Table 6.2) were much lower than that recorded for iron and the highest concentration was 3.0 µg/ml (Penygroes 1). Manganese remains in solution at pH values higher than those required for the oxidation of Fe(II) and very little oxidation of manganese could be detected in the drainage water samples.

The results have not revealed a new mechanism for ochre formation but they have shown the importance of chemical oxidation in that it can account for most iron precipitation. Its importance in relation to the other mechanisms of ochre formation will be discussed in chapter 8. The results have also shown that the autocatalytic effects described in the literature, where the rate of Fe(II) oxidation is increased in the presence of Fe(III), is also relevant to ochre formation.

The rate of oxidation of Fe(II) was increased by ochre under standard conditions. In ferrous sulphate solutions, initially at pH 4, the addition of air dried ochre caused complete oxidation of iron (6.2.2). It was also shown, using a citric acid - ferrous sulphate solution (6.2.3), that ochre could cause the precipitation of iron even when there was



sufficient organic complexing agent to prevent the normal oxidation of Fe(II) at pH 6.0.

The catalysis of ferrous iron oxidation was also observed in drainage water samples. Sterile ochre did not increase the amount of iron oxidation but it did increase the rate of oxidation. In drainage water containing sterile ochre 50% of the iron initially present in solution was oxidized in less than two hours. The corresponding time for the control solutions was over 12 hours (Table 6.5). In the ferrous sulphate solutions (and to some extent the citric acid - Fe(II) solutions) it was found that the effect of ochre on the oxidation of Fe(II) could be explained in terms of pH. Ochre raised the solution pH causing iron precipitation. This explanation is probably too simple with regard to the drainage water experiments. No pH difference was found between the drainage waters with and without ochre in samples from Llyn Cowlyd and Llyn Coedty. At Llyn Cowlyd a final pH of 5.8 was recorded in the drainage water with and without sterilized ochre. Similarly, for Llyn Coedty samples the pH in drainage water with added ochre was 5.9 and without ochre 5.8.

In the literature the oxidation of Fe(II) is described in terms of two mechanisms (see section 2.5.2.1). Fe(II) is firstly oxidized to Fe(III) and further oxidation of Fe(II) is catalysed by ferric hydroxide. Both these mechanisms were observed in the drainage water experiments. In water without added ochre the oxidation of Fe(II) was initially slow with the rate increasing with time. The increased rate resulted from the catalysis of Fe(II) oxidation by ferric hydroxide. The rate of oxidation was greatly increased by adding sterile ochre to the drainage water. Since ferric hydroxide was initially present the "catalysed oxidation" mechanism operated from the start of the experiment and an initial slow phase was not observed.

TABLE 6.8 : THE PROPORTIONS OF IRON IN DRAINAGE WATER IN SOLUTION AND  
SUBJECT TO CHEMICAL OXIDATION

<u>SAMPLE</u>	<u>% Fe IN SOLUTION<sup>(1)</sup></u>	<u>% CHEMICALLY OXIDIZED<sup>(1)</sup></u>
LLYN COWLYD 1	95	100
LLYN COWLYD 2	92	100
LLYN COWLYD 4	66	77
LLYN COEDTY 1	97	78
LLYN COEDTY 2	100	52
LLYN COEDTY 3	31	19
PENYGROES 1	87	99
PENYGROES 5	98	93
PENYGROES 7 : 2	91	97
PENYGROES 7 : 3	91	100
PENYGROES 7 : 4	74	100
PENYGROES 7 : 5	75	0

(1) CALCULATED FROM RESULTS IN TABLE 6.1

$$\% \text{ Fe in solution} = \frac{(F+A)}{A} \times 100 \quad \% \text{ chemically oxidized} = \frac{(F+A) - F}{F+A} \times 100$$

These considerations are relevant to the formation of ochre at sites with high and low pH values. Water entering drains and watercourses at pyritic sites are characterized by a high Fe(II) and  $\text{SO}_4^{2-}$ , and pH values between 3 and 4. These conditions are similar to those found in the ferrous sulphate solutions, where ferrous iron could be precipitated by adding air dried ochre. In these solutions no oxidation could be detected if ochre was not added. Ochre forming at pyritic sites would therefore encourage the further oxidation of ferrous iron in the drainage water despite its low pH. At filamentous ochre sites where the pH is normally 5.5 - 6.5 the effect of existing ochre deposits would be to increase the rate at which uncomplexed or loosely complexed iron is chemically precipitated. The increased rate of precipitation would result in complete removal of iron

from solution in a shorter period of time. In terms of drainage systems this would cause deposition of iron within a smaller area, thus increasing the severity of the problem.



Plate 6:1 The effect of acidification and filtration of drainage water from an ochreous site (Llyn Coedty)

## 7. THE CONTROL OF MICROORGANISMS ASSOCIATED WITH OCHRE DEPOSITION

### 7.1 THE EFFECT OF COPPER ON SHEATHED FILAMENTOUS BACTERIA

Copper is a well known bacteriocide and its use as an ochre preventative has been described in the literature (Section 2.6.2.6). In the present study the effect of copper on sheathed filamentous bacteria in artificial media and drainage water samples was investigated.

#### 7.1.1 The effect of copper on sheathed filamentous bacteria in glucose-iron medium

Thirty 50 ml portions of glucose-iron medium were prepared as described in Section 5.3.4.1.

Solutions containing 510, 255, 127 and 51  $\mu\text{g/ml}$  of  $\text{Cu}^{++}$  as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were prepared. 1.0 ml of each solution was filter sterilized (0.22  $\mu\text{M}$  Millipore filter) into six portions of glucose-iron medium giving final concentrations of 10, 5, 2.5 and 1.0  $\mu\text{g/ml}$   $\text{Cu}^{++}$ . 1.0 ml of filter sterilized distilled water was added to a further six portions of medium giving controls containing no copper. 0.3 ml 0.05M NaOH was used to adjust the pH of media containing 0.0 or 1.0  $\mu\text{g/ml}$   $\text{Cu}^{++}$  and 0.4 ml 0.05M NaOH was used at the remaining copper concentrations. The average pH of the spent media was 5.3.

At each copper concentration replicate portions of media were treated as follows:

1. Uninoculated
2. Inoculated with 1.0 ml diluted ochre (Llyn Coedty ochre diluted 1.0 ml to 100 ml with sterile distilled water)
3. Inoculated with 1.0 ml sterile diluted ochre (Llyn Coedty ochre diluted 1.0 ml to 100 ml with distilled water and autoclaved)

After ten days a pale orange, poorly flocculated, precipitate was found in most media, but the media with no added copper contained large, pale orange flocs. Portions of all media were examined by light microscopy. In the uninoculated media sheathed filamentous bacteria were not observed. Pale orange or grey ferric iron particles were seen in all uninoculated flasks.

In the media inoculated with sterilized ochre only a few sheathed filamentous organisms were observed and these were encrusted with small amounts of dark orange material. The flasks inoculated with unsterilized ochre and containing no copper contained many large, bright orange particles associated with sheathed filamentous bacteria. In the media containing copper only a few filaments were observed.

#### 7.1.2 The effect of VC-17 antifouling paint on sheathed filamentous bacteria in drainage water

It was demonstrated in Section 5.3.1 that sheathed filamentous bacteria will grow in drainage water collected from ochre sites.

150 ml polypropylene bottles were washed in dilute hydrochloric acid, rinsed in distilled water and sterilized by autoclaving for 15 minutes at 120°C. VC-17 (Extensor AB, Box 323, S-181 03, LIDINGO, SWEDEN) is a marine antifouling paint containing finely ground copper. VC-17 was prepared by stirring 4.8 g of copper dust into 30.0 ml of paint. 3.5 cm lengths of 8.0 mm diameter glass tubing were coated with VC-17 by immersing in the paint for a few seconds.

Drainage water was collected at 3 sites, Penygroes 1, Penygroes 5 and Penygroes 7:1. Care was taken to avoid collecting any ochrous flocs. One coated glass rod was placed in four bottles at each site. Four other bottles were used as controls containing no VC-17.

After 7 days light orange precipitates and numerous small, dark orange flocs were observed in all the bottles. The precipitate from each bottle was examined using a phase contrast light microscope. When the precipitates were taken up by Pasteur pipette during slide preparation a difference emerged between the samples. On the prepared microscope slides the flocs from bottles containing VC-17 were small and too numerous to count. Conversely, flocs from samples without VC-17 were large and distinct and only 5 to 10 were visible on the slide. This difference was observed in water collected at Penygroes 1 and 5 but not at Penygroes 7:1 where large flocs were observed on all slides irrespective of treatment with VC-17.

Microscopic examination of the precipitates revealed that at all sites VC-17 had a marked effect on the growth of filamentous bacteria. In all the untreated bottles irregular iron particles in association with filamentous bacteria were seen. The filaments were mostly straight or slightly curved and encrusted and non-encrusted filaments were found. Spiral organisms similar to the bacteria described as Type 4 in Section 4.1.1 were observed in some of the bottles containing water from Penygroes 1 and 5. In samples containing VC-17 the precipitates were not associated with filamentous bacteria and tended to be uniform or angular in shape. The particles formed circular or rectangular accumulations. Sheathed filamentous bacteria were observed in some of the bottles containing VC-17 but in each case only one or two filaments were found on the microscope slide.

The pH of the drainage water samples at the end of the experiment was recorded. The results are shown in Table 7:1. The samples had a pH ranging from 5.8 to 6.2 and the inclusion of VC-17 did not affect the final pH.

TABLE 7:1 : THE FINAL pH OF DRAINAGE WATER SAMPLES WITH AND WITHOUT  
ADDED COPPER AFTER IRON OXIDATION

<u>SITE (1)</u>	<u>REPLICATES</u>				<u>MEAN</u>
	1	2	3	4	
PENYGROES 1	5.8	5.7	5.8	5.8	5.8
PENYGROES 1 +	5.7	5.8	5.8	5.8	5.8
PENYGROES 5	6.1	6.0	6.1	6.2	6.1
PENYGROES 5 +	6.2	6.0	6.1	6.1	6.1
PENYGROES 7	6.0	6.2	6.1	6.0	6.1
PENYGROES 7 +	6.2	6.1	6.1	6.2	6.2

(1) '+' indicates the inclusion of VC-17 in the drainage water samples.

#### 7.2 THE EFFECT OF BIOCIDES ON THIOBACILLUS FERROOXIDANS

9K medium (4.2) was prepared and dispensed in 20 ml aliquots into sterile 100 ml conical flasks.

Various dilutions of Panacide (BDH), Acrolein (BDH) and copper sulphate were prepared. 6.56 g of Panacide solution (40% W/W was diluted to 25 ml with distilled water. 3.088 ml of 0.85 g/ml acrolein solution was diluted to 25 ml with distilled water and 10.32 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was dissolved in 100 ml of distilled water. The stock solutions were diluted and incorporated into 9K medium by adding 1.0 ml of each dilution to replicate portions of medium giving final concentrations of 10, 100, 1000 and 5000  $\mu\text{g/ml}$  of acrolein and panacide. The final concentrations of copper were 10, 100, 1000 and 1250  $\mu\text{g/ml}$ . Controls containing 1.0 ml of filter sterilized distilled water were also prepared.

Two strains of T.ferrooxidans were used to inoculate the media:

1. Type A - NClB 11820 (Johnson + Kelso, 1983)
2. Type B - T.ferrooxidans from acid mine drainage water (Section 3.1.2)

1.0 ml from growing cultures of each type in 9K medium were used as an inoculum.



After 10 days the flasks were examined visually. In some cultures a distinct orange colour had developed indicating oxidation of Fe(II) by the bacteria. Growth of T.ferrooxidans was confirmed by titrating the 20 ml medium against 0.01M KMnO<sub>4</sub>. 20.0 ml of 1.0M H<sub>2</sub>SO<sub>4</sub> was added to the cultures before titration. Titres less than 5.0 ml were taken as positive results indicating that over 90% of the Fe(II) initially present in the medium had been oxidized. The results are shown in Table 7.2. Acrolein inhibited T.ferrooxidans at concentrations above 10 µg/ml but at 10 µg/ml ferrous iron oxidation was unaffected. Panacide was effective at all the concentrations used but copper had no apparent effect on T.ferrooxidans even at concentrations above 1000 µg/ml. The copper tolerance of T.ferrooxidans is well known (2.4.1).

TABLE 7.2 : THE EFFECT OF PANACIDE, ACROLEIN AND COPPER ON FERROUS IRON OXIDATION BY THIOBACILLUS FERROOXIDANS IN 9K MEDIUM

	<u>TYPE A</u>	<u>TYPE B</u>
INOCULATED CONTROL	+	+
UNINOCULATED CONTROL	-	-
ACROLEIN 10 µg/ml	+	+
100 µg/ml	-	-
1000 µg/ml	-	-
5000 µg/ml	-	-
PANACIDE 10 µg/ml	-	-
100 µg/ml	-	-
1000 µg/ml	-	-
5000 µg/ml	-	-
COPPER 10 µg/ml	+	+
100 µg/ml	+	+
1000 µg/ml	+	+
1250 µg/ml	+	+

+ indicates that over 90% of the iron initially present in the medium had been oxidized by T.ferrooxidans (after 10 days).

### 7.3 THE EFFECT OF ANTI-BACTERIAL COMPOUNDS ON COMPLEX DEGRADING

#### HETEROTROPHIC BACTERIA IN FERRIC AMMONIUM CITRATE MEDIUM

The decomposition of organic-iron complexes has been described as a mechanism by which ochre can form (2.5.2.3). Bacteria capable of utilizing ferric citrate were isolated from ochre as described in section 4.3.2.

##### 7.3.1 The effect of copper on complex degrading heterotrophs in ferric ammonium citrate medium

9.0 ml portions of ferric ammonium citrate (FAC) medium (4.3.2) were transferred into McCartney bottles. Seven solutions containing between 0.1 mg/l and 1000 mg/l of  $\text{Cu}^{++}$  as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were prepared. 1.0 ml of each solution was added to replicate portions of FAC medium before autoclaving. Portions of FAC containing 1.0 ml of distilled water were also prepared.

One bottle of FAC medium at each copper concentration was inoculated, using a sterile wire loop, from a growing FAC culture. The organisms had been isolated from ochre using FAC medium.

After three weeks the bottles were examined visually. In the uninoculated controls the solutions had an orange colour with no precipitate. In the inoculated media containing less than  $5.0 \mu\text{g/ml}$   $\text{Cu}^{++}$  a distinct precipitate was observed with the solution being colourless. At  $5.0 \mu\text{g/ml}$   $\text{Cu}^{++}$  or above no precipitate was seen. These observations were confirmed by filtering 1.0 ml of each medium through a  $0.45 \mu\text{m}$  Millipore filter. The aliquot was then diluted to 50 ml with distilled water and total iron determined by atomic absorption spectrophotometry. The results are shown in Table 7.3.

In the uninoculated controls copper had no effect on the amount of iron in solution. This remained constant at between 0.75 and 0.90 g/l. In the inoculated flasks copper had no effect on iron precipitation at concentrations less than  $5.0 \mu\text{g/ml}$  with no iron remaining in solution in the cultures containing between 0.0 and  $1.0 \mu\text{g/ml}$  of copper. As the

amount of copper was increased up to 100  $\mu\text{g/ml}$  the amount of iron in solution also increased. At 100  $\mu\text{g/ml}$  Cu the amount of iron in solution is comparable to the levels observed in the uninoculated flasks.

TABLE 7.3 : THE EFFECT OF COPPER ON THE GROWTH OF COMPLEX DEGRADING HETEROTROPHIC BACTERIA IN FAC MEDIUM

<u>Cu <math>\mu\text{g/ml}</math></u>	<u>TOTAL IRON IN SOLUTION (g/l)</u>	
	<u>INOCULATED</u>	<u>UNINOCULATED</u>
0.0	0.00	0.77
0.01	0.00	0.87
0.1	0.00	0.88
0.5	0.00	0.86
1.0	0.05	0.75
5.0	0.67	0.87
10.0	0.70	0.86
100.0	0.83	0.89

7.3.2 The effect of acrolein and panacide on complex-degrading heterotrophs in ferric ammonium citrate medium

1.0 g/l stock solutions of biocide were prepared. 1.176 ml of acrolein (0.85 g/ml) solution or 2.5g of 40% W/W panacide was diluted to 1 l with distilled water. The stock solutions were diluted to give 9 dilutions containing 0.0 to 1000  $\mu\text{g/ml}$  of biocide.

1.0 ml of each dilution of biocide was added to 4 9.0 ml portions of FAC medium (4.3.2) in McCartney bottles. Two portions were inoculated from a FAC culture containing organisms isolated from ochre.

After one month the flasks were examined and growth of bacteria was indicated by the presence of a clear solution above a dark orange-red precipitate. The results are shown in Table 7.4.

TABLE 7.4 : THE EFFECT OF PANACIDE AND ACROLEIN ON THE GROWTH OF COMPLEX-DEGRADING HETEROTROPHIC BACTERIA IN FAC MEDIUM

<u>BIOCIDES</u> <u>CONCENTRATION</u> <u>µg/ml</u>	<u>PANACIDE</u>		<u>ACROLEIN</u>	
	<u>INOCULATED</u>	<u>UNINOCULATED</u>	<u>INOCULATED</u>	<u>UNINOCULATED</u>
0.0	+	-	+	-
0.01	+	-	+	-
0.05	+	-	+	-
0.10	+	-	+	-
0.50	+	-	+	-
1.00	+	-	+	-
5.00	+	-	+	-
10.00	+	-	+	-
100.00	+	-	+	-

+ IN THESE SOLUTIONS A CLEAR LIQUID ABOVE AN ORANGE-RED PRECIPITATE WAS OBSERVED. THIS INDICATES REMOVAL OF IRON FROM SOLUTION RESULTING FROM COMPLEX DEGRADATION BY BACTERIA.

- A CLEAR ORANGE SOLUTION WAS OBSERVED WITH NO FERRIC PRECIPITATES.

At the concentrations used neither panacide or acrolein had any effect on the growth of the complex-degrading heterotrophs. No precipitate appeared in any of the uninoculated bottles. However, a precipitate was observed in all the inoculated bottles irrespective of biocide concentration from 0.01 to 100 µg/ml.

### 7.3.3 The effect of antifouling points on complex-degrading heterotrophs

#### 7.3.3.1 VC-17

Short lengths of glass tubing were coated with VC-17 as described in section 7.1.2.

A glass rod was placed in replicate portions of FAC medium (4.3.2). The media were inoculated from a FAC culture containing organisms isolated from Donegal ochre (3.1.3). After 12 days a distinct precipitate was seen in the untreated media but in the portions containing VC-17 no growth was apparent, the solutions having a clear orange colouration.

#### 7.3.3.2 U644, W492 and W319 antifouling paints

16 25 ml portions of FAC medium (4.3.2) were prepared. Four 3.5 cm lengths of glass tubing (1.0 cm diameter) were coated with antifouling paint and allowed to dry overnight. 3 antifouling paints were used, U644, W492 and W319 (obtained from International Paint, Stonygate Lane, Felling, Gateshead, Tyne and Ware, NE100SY).

The coated glass tubes were placed in the media and left for 23 days before inoculation to ensure that the media had not been contaminated when the glass rods were introduced. Two portions containing each antifouling paint were inoculated with bacteria from a growing FAC culture. Four untreated portions of medium were also prepared and two of these were inoculated. The media were incubated at 22°C for five days.

A distinct precipitate formed in the inoculated controls but no precipitate was observed in the uninoculated flasks. A precipitate was not observed in any of the inoculated or uninoculated media containing antifouling. However, a thin orange film was seen on the surface of inoculated cultures containing U644 antifouling suggesting some release of iron from the ferric-citrate complex.

In order to examine the continued effect of the antifouling paints some of the flasks were reinoculated after five days. The results in Table 7.5 show that no growth was detected in any of the flasks containing W319 antifouling<sup>paint.</sup> The uninoculated controls containing W492 had no precipitate after 5 days but in the reinoculated portions containing W644 and U492 some bacterial growth was evident. No distinct precipitate was observed but red flocs were seen at the base of the cultures.

The coated rods were removed from the FAC cultures, washed, and left to stand in distilled water for about five weeks. After this time they were placed in 20 ml portions of freshly prepared and autoclaved FAC medium. Two flasks containing each antifouling were inoculated and two were left uninoculated. Inoculated and uninoculated portions of untreated FAC were prepared as controls.

After 20 days a precipitate had formed in the inoculated, but not in the uninoculated control. In all flasks containing U644 and W492 a precipitate was clearly seen indicating growth of bacteria. No difference was seen between the inoculated and uninoculated media. No distinct precipitate had formed in the flasks containing W319 but an orange precipitate was seen on the surface of both inoculated and uninoculated cultures. This indicates that W319 prevented complete release of iron from solution but did not totally inhibit the complex degrading bacteria.

TABLE 7.5 : THE EFFECT OF THREE ANTIFOULING PAINTS ON THE GROWTH OF COMPLEX-DEGRADING HETEROTROPHIC BACTERIA IN FERRIC AMMONIUM CITRATE MEDIUM

AFTER 5 DAYS

<u>ANTIFOULING</u>	<u>INOCULATED</u>		<u>UNINOCULATED</u>	
	<u>REPLICATE 1</u>	<u>REPLICATE 2</u>	<u>REPLICATE 1</u>	<u>REPLICATE 2</u>
U644	*	*	-	-
W492	-	-	-	-
W319	-	-	-	-

AFTER 15 DAYS

<u>ANTIFOULING</u>	<u>INOCULATED</u>		<u>UNINOCULATED</u>	
	<u>REPLICATE 1</u>	<u>REPLICATE 2</u>	<u>REPLICATE 1</u>	<u>REPLICATE 2</u>
U644	*	*	*	-
W492	*	-	*	-
W319	-	-	-	-

\* NO DISTINCT PRECIPITATE BUT RED/ORANGE MATERIAL SEEN ON SURFACE

- NO PRECIPITATE WAS OBSERVED

□ THESE CULTURES WERE REINOCULATED ON DAY 5

#### 7.4 THE EFFECT OF ANTIFOULING PAINTS ON ACIDOPHILIC HETEROTROPHIC BACTERIA

The isolation and importance of acidophilic heterotrophic bacteria in ochre was discussed in chapter 4.

22x50 mm glass microscope cover slips were coated with various antifouling paints. The cover slips were immersed to half their length in the antifouling paint. Four paints were used, VC17, U644, W319 and W492 (see 7.12 and 7.3.3.2 for details). The cover slips were allowed to dry for several hours, rinsed in sterile distilled water and placed on agar plates of Harrison's glucose medium (4.3.1). Three plates containing each antifouling paint were prepared. To ensure that the media were not contaminated by the coated cover slips the plates were inverted and maintained at room temperature for ten days. After this time no bacterial growth could be detected on any of the plates containing antifouling paint.

The plates were inoculated by transferring small quantities of inoculum from colonies grown on Harrison's glucose agar (4.3.1). The plates were inoculated using three species of acidophilic heterotrophs.

1. NC1B 11746
2. NC1B 11745
3. NC1B 11822

After 5 days the three types of acidophilic heterotrophs had grown well on untreated control plates. However, very little growth was evident on the plates containing antifouling paint.

After 15 days no growth was found on any plates containing U644. VC-17 was effective in preventing the growth of type 1 and 3 but some colonies of type 2 developed on VC-17 treated plates: W319 antifouling prevented growth of types 2 and 3 but colonies of type 1 were observed. W492 prevented growth of type 1 but not types 2 and 3. It is worth noting that the majority of colonies surrounded the untreated side of the cover slips indicating that the antifouling paints had some bacteriocidal activity although growth of the acidophilic heterotrophs was not completely prevented except by U644.

## 7.5 THE RELEASE OF COPPER FROM VC-17 ANTIFOULING PAINT

### 7.5.1 Release of copper from VC-17 in different volumes of distilled water

VC-17 (7.1.2) was prepared by mixing 4.8g of copper dust with 30 ml of antifouling paint. Ten 35 mm lengths of 8.0 mm diameter glass tubing were weighed, dipped in antifouling, allowed to dry and then reweighed. One glass rod was placed in several volumes of distilled water from 50 ml to 250 ml (50 ml increments) in 250 ml conical flasks. Two replicates were prepared for each volume of water.

At regular intervals up to 72 days 5.0 ml aliquots were withdrawn and replaced with 5.0 ml distilled water. The concentration of copper in the aliquot was determined by atomic absorption spectrophotometry. In order to relate the release of copper to the initial amount on the coated rods, the copper content of the dry antifouling was determined by extracting known weights of VC-17 in concentrated nitric acid at room temperature for several days and then by boiling vigorously for five minutes. The extract was diluted with distilled water, filtered and the copper concentration determined by atomic absorption spectrophotometry. The mean concentration of copper in the antifouling paint was 62%. The total amounts of copper in solution at each sampling date expressed as a percentage of the amount originally present on each coated glass rod are shown in figure 7.1. The amounts of copper removed in the 5 ml aliquots were taken into account in these calculations.

The results show that there was an increase in copper release with time (significant at  $p < 0.001$ ). The amount of copper released after one day was <0.1%, whereas after 72 days 3.4% and 9.8% copper had been removed in 50 ml and 250 ml respectively. The release of copper was also related to the volume of water. The mean percentage copper released was 1.9, 3.9, 4.1, 6.1 and 5.6 at 50, 100, 150, 200 and 250 ml respectively, representing a quadratic response (significant at  $p < 0.043$ ) with a maximum release of copper in 200 ml distilled water.



The release of copper from VC-17 in a continuous flow of tap water was recorded by placing a coated glass rod in a 10 ml syringe connected to a tap by a piece of rubber tubing. After 47 days the copper remaining on the rod was extracted in 75 ml concentrated nitric acid for several days. The copper concentration in the filtered and diluted extract was determined by atomic absorption spectrophotometry. The initial weight of copper on the glass rod was estimated to be 10.2 mg and the amount remaining after 47 days was 8.9 mg. The release of copper under a continuous flow of water was therefore 12.7%, approximately double the amount released in 200 ml and 250 ml distilled water over the same time period.

#### 7.5.2 The effect of pH on the release of copper from VC-17 antifouling ...

4 glass rods were coated with VC-17 as described in section 7.5.1.

The glass rods were placed in solutions of distilled water adjusted to pH 2.0, 4.0, 6.0 and 8.0 with dilute  $H_2SO_4$  and NaOH. At regular intervals 5.0 ml aliquots were withdrawn from each solution and replaced with 5.0 ml of distilled water adjusted to the appropriate pH. The copper concentration of the aliquots was determined by atomic absorption spectrophotometry.

The amount of copper in solution at each sampling date expressed as a percentage of the amount originally present on each glass rod are shown in figure 7.2. The amounts removed in the 5.0 ml aliquots were also taken into account. At pH 4.0 to 8.0 less than 4.0% copper had been removed after 43 days. However, at pH 2.0 over 95% of the copper had been removed. 90% of the copper had been removed at pH 2.0 within 7 days, whereas only 0.5 - 1.0% had been removed within the same time at pH 4.0 - 8.0.

Figure 7:1    The percentage of copper removed from VC-17 antifouling paint in different volumes of distilled water

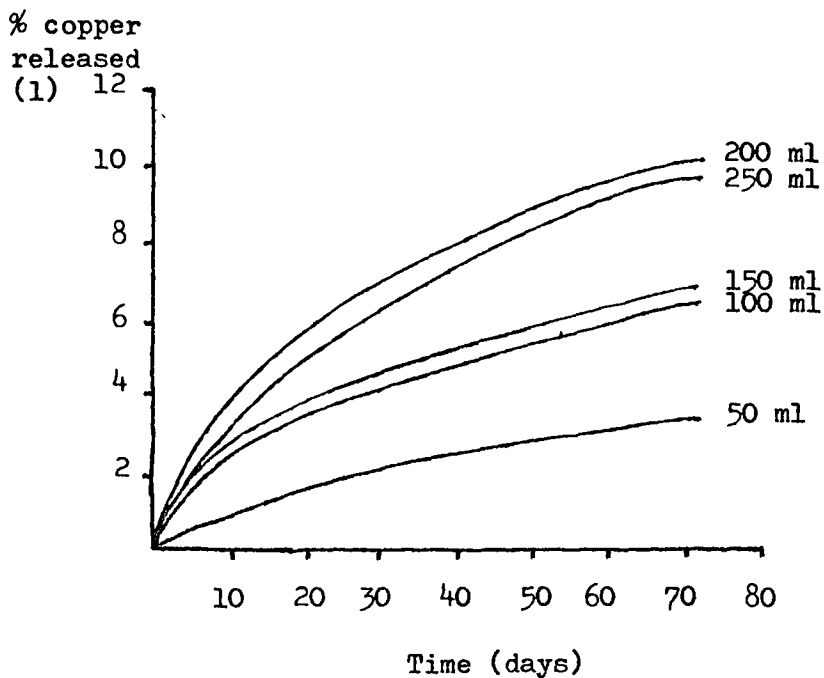
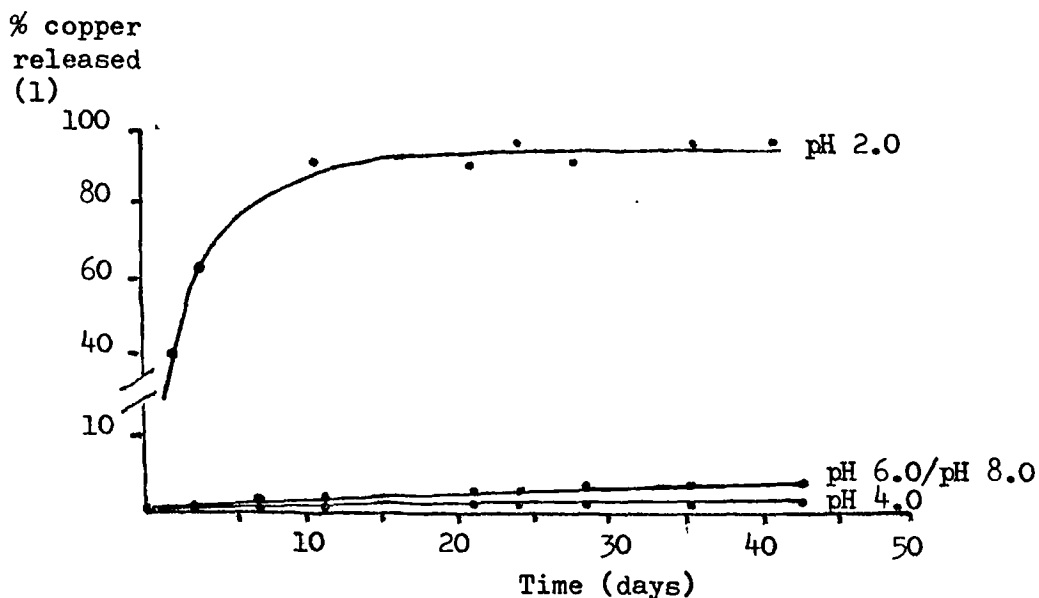


Figure 7:2    The effect of pH on the percentage of copper removed from VC-17 antifouling paint



(1) Total amount of copper released expressed as a percentage of the initial weight on each coated glass rod.

## 7.6 DISCUSSION

The results described in this chapter do not provide a comprehensive examination of the effects of biocides on the microorganisms associated with ochre deposits. It was not possible, in the time available, to study more than a few compounds. However, the results illustrate the susceptibility of "ochre bacteria" to biocides. They also demonstrate the possibilities and problems associated with the use of anti-bacterial compounds to control ochre formation.

Generally the effect of each biocide was assessed by a positive/negative approach. For example, positive growth was determined on agar by the appearance of colonies and in ferric ammonium citrate medium by the presence of a distinct red precipitate. Similarly, in 9K medium over 90% ferrous iron oxidation indicated growth of T.ferrooxidans. This method gives a maximum concentration of biocide at which bacterial growth is unaffected.

Panacide and acrolein were chosen as examples of readily available anti-microbial compounds. Both biocides were effective against T.ferrooxidans in 9K medium. It was found that concentrations of acrolein above 10 µg/ml were required to prevent complete ferrous iron oxidation. Panacide prevented complete oxidation at all the concentrations used, in the range 10 to 5,000 µg/ml. This confirms the results of Le Roux et al (1973) who found that panacide, at 200 µg/ml, stopped the growth of T.ferrooxidans almost instantaneously. Neither acrolein or panacide had an apparent effect on the complex degrading heterotrophs at concentrations up to 100 µg/ml.

Copper controlled the growth of sheathed filamentous bacteria very effectively. In artificial media few filaments were seen in the presence of 1 µg/ml or more of copper as copper sulphate. Copper had little effect on complex-degrading bacteria in ferric ammonium citrate medium at 10 µg/ml, but at 5.0 µg/ml and 10 µg/ml only 21% and 12%, respectively, of the iron present in uninoculated controls had been precipitated. However, copper had no effect on ferrous iron oxidation by T.ferrooxidans even at

concentrations above 1.0 g/l. The tolerance of this organism to copper is well known as it is used commercially to extract copper from low grade ores (2.4.1).

In drainage systems a method of applying the biocide is required. The compound has to be fixed so as to allow a slow but constant release. With this in mind a copper-containing marine antifouling paint, VC-17, was investigated. This is used to prevent the accumulation of marine organisms on the underside of boats.

The paint was effective against sheathed filamentous bacteria. In 150 ml volumes of drainage water no filaments formed when VC-17 coated glass rods were present. VC-17 also prevented the growth of complex-degrading bacteria in FAC medium and was effective against acidophilic heterotrophs growing on Harrison's agar.

Three other antifouling paints were investigated. These controlled the growth of acidophilic and ferric citrate complex-degrading heterotrophs, but it was shown that their effect on the latter was not prolonged. After transferring, washing and soaking, the antifouling had little effect on bacterial growth in FAC medium.

The results show that it is possible to control the organisms associated with ochre by using biocides or antifouling paints. However there are numerous obstacles to their successful use for controlling ochre in drainage systems.

The biocide has to be applied both economically and safely. However, biocides such as panacide and acrolein as laboratory grade chemicals are expensive and in practice it would be difficult to maintain the required concentration in flowing water and in the presence of organic complexing agents, silts and clays.

Another problem is the danger of polluting streams and water courses receiving water from the treated drainage systems. Biocides are effective not only against the microorganisms associated with ochre but also against all forms of aquatic life. For example, it has been found that 1-20 µg/ml

of panacide will strongly inhibit the growth of green and blue-green algae. If the period of contact is lengthened then the inhibitory concentration is reduced (Gupta and Saxena, 1974; Sanena et al, 1978). Similarly, McIntyre (1978) found that copper at 10 to 1000  $\mu\text{g}/\text{l}$  had significant effects on all levels of an experimental aquatic food chain. In certain situations it is possible that drainage water would be diluted in open ditches and streams, thus preventing pollution. Aldrich (1977) found that no significant pollution occurred following sulphur dioxide treatment of ochreous drains provided the outflow was diluted sufficiently. However, in situations where dilution is limited, such as low lying areas or coastal sites below sea level, biocide could pollute watercourses. In such an area Gosling and Baker (1980) recorded acid pollution of ditches resulting from pyrite oxidation in drained soil. This led to the death of fish, freshwater muscles and many macrophytes.

Some of the problems outlined above could be avoided by using antifouling paints. The anti-bacterial effects of these has been discussed. The most effective treatment was VC-17 antifouling which contained copper. The other paints were initially effective but lacked continued bacteriocidal activity. VC-17 provided a means of incorporating copper onto the surface of drainage pipes. The copper is released slowly from the compound in stationary solutions but at a faster rate in running water. It was found that 13% copper was removed within 47 days in continually flowing water. It might be necessary to improve the retention of copper if VC-17 was to be used to prevent ochre in drainage systems. It may be possible to achieve this by incorporating copper dust into plastic drainage pipes as an alternative to coating the pipes with paint.

The retention of copper by VC-17 is also affected by pH. At pH values below 4.0 the rate of release was dramatically increased and at pH 2.0 over 95% copper was released into solution within 43 days (7.5.2). The effect of pH limits the use of VC-17 to sites of moderate acidity. At low pH, pyritic, sites the sudden release of copper would make the treatment

ineffective and cause a pollution hazard. Even if the release of copper at low pH could be controlled, its effects on ochre formation at these pH values is unknown. The bacterium most commonly associated with ochre at acidic sites is T.ferrooxidans and this microorganism is highly tolerant of copper. The acidophilic heterotrophs associated with ochre at low pH are susceptible to inhibition by copper. The effect of copper on ochre formation would depend upon the importance of the heterotrophs in the composition of ochre deposits. It has been argued (Johnson and Kelso, 1980) that these organisms increase the tenacity of ochre. If this is the case then copper might reduce the severity of clogging by acidic ochre by preventing the growth of heterotrophic organisms.

In solutions of pH greater than 4.0, copper in a slowly released form, such as VC-17 or its equivalent, could be used to control the majority of bacteria associated with ochre. Copper was effective against sheathed filamentous bacteria, ferric citrate complex-degrading bacteria and acidophilic heterotrophs. The success recorded in laboratory experiments should be confirmed by conducting trials in the field. Field trials are also required to monitor the duration of copper release and the concentrations of copper in the outflow with regard to pollution. The most important consideration however is the effect of copper on ochre formation.

Copper can prevent the growth of ochre bacteria but cannot prevent the oxidation of Fe(II). Puustjarvi and Juusela (1952) argued that copper will increase the severity of ochre by catalysing ferrous iron oxidation. It was shown in section 6.1 that most of the soluble iron present in drainage water will oxidize chemically within a short period of time. Thus the presence of copper will not increase the amount of iron precipitates although it may increase the rate of precipitation.

In an experimental model Ford (1979A, 1979B) found that chemically precipitated iron, without bacteria, lacked an ability to adhere and did not result in the formation of ochre. The extent to which chemical

precipitates can accumulate to form ochre in field drains must be monitored with regard to the use of biocides. It is also necessary to determine the effect of biocide on drainage performance since ochre can form, not only in the drainage pipe, but also in the backfill or soil around the drain. Having a system to control ochre formation inside the pipe is worthless if ochre in the soil or backfill prevents water from entering the drain. The use of biocides can only be justified if they prevent the accumulation of ochre in drain pipes and if they improve the performance of the drainage system as compared to untreated controls.

## 8. DISCUSSION AND CONCLUSION

### 8.1 Characteristics

Ochre is a general term used to describe a range of iron containing deposits that form in agricultural drainage systems. Ochre has been described in various ways - raglike, a sludge, mare's tail, a slime, a mischief and a menace (Ford, 1978; Ivarson and Sojak, 1978; Bloomfield and Coulter, 1973; Ford, 1979; Brown, 1903; Denison, 1856; Hope, 1981; Pliny the Elder). It is composed of iron and organic matter in the form of an aqueous orange, yellow or dark red deposit. These deposits vary widely in their composition, their appearance, their rate of formation and where they occur.

46 samples were collected from a variety of sites in England and Wales. Their appearance in situ varied from small flocs of dark orange-red material (plate 3.25) to hard, gelatinous, orange deposits (plate 3.27). The colour of the deposits varied but they all had the characteristic red-orange colour associated with ferric iron minerals (Schwertmann and Taylor, 1977). Most of the samples had a pH between 5.0 and 7.0 in the undried state (3.2.2.1) but some samples were characterized by a low pH. A notable example was the Spanker site (3.1.12) where the ochre had a pH between 3.4 and 4.2.

As expected from the colour of the deposits they all had a high iron content (3.2.2.3), half the samples having a total iron content (oven dry sample) of between 20% and 40%. All the samples analysed contained varying quantities of organic matter. The organic carbon content varied from 0.8% to 13.2% (3.2.2.4) whilst the percentage loss on ignition ranged from 10.6% to 52.6% (3.2.2.2).

During sample collection it was noted that ochre formed over a range of sites. Ochre was found in the fen peats of Norfolk, reclaimed open cast coal mine sites, low lying brown earths derived from marine or river alluvium, and upland peats. Ochre formation required iron in drainage water but was not highly dependent on the iron concentration. Samples of drainage water from drains and ditches containing ochre were analysed and the total amount of iron in



solution varied from 0.3 ug/ml to 19.5 ug/ml (5.3.5, Table 5.4). Differences among sites and the related differences in iron concentration in the drainage water influence both the rate at which ochre forms and the time taken for complete blockage by the deposits. At some sites it appeared as if ochre deposition had been initially severe but did not present a long term problem. For example, drains at Hall Farm (Site 4b, 3.1.7) had blocked within 12 months and the land was redrained. At the time of sampling the second set of drains were functioning satisfactorily with little evidence of ochre deposition. Similarly at an upland site in North Wales drainline ochre had been found by a farmer who removed it by rodding. When the site was examined during the present study no ochre could be found suggesting an initial, short term accumulation of ochre.

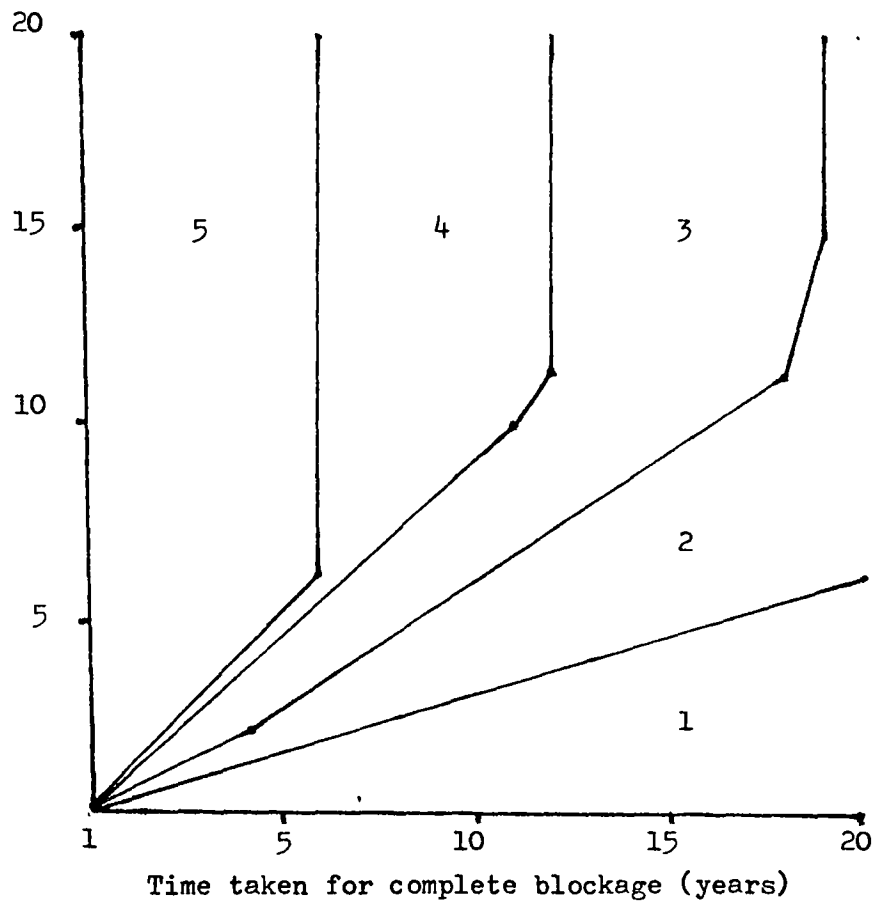
At other sites a different type of problem was found. At Gallt yr Huddrem (3.1.11) for example drains had been operating for over ten years despite the accumulation of ochre at their outlets. Here ochre deposition was long term or permanent but was not severe enough to seriously impair the performance of the drainage system.

The worst ochre problem occurred where deposition was both intense and long term. At Llyn Coedty (3.1.4) ochre accumulation had occurred for many years and there was no indication that the deposition was declining. The total iron content of drainage water at this site was high at 15 µg/ml (Section 6.1). Similarly at Spanker the accumulation of ochre was a constant, severe problem aggravated by low pH.

The effect of ochre "intensity" (the time taken for complete blockage of a drainage system) and the duration of ochre deposition on the severity of the problem is illustrated in figure 8.1. Drainage Schemes are normally expected to function for at least 20 years but 10-15 years are usually taken as a reasonable period for accounting purposes (Brown, 1984; Jackson, 1984). In figure 8.1 the economic effect of ochre is examined over a 15 year period. It

Figure 8.1 The effect of ochre intensity and duration of deposition on the severity of ochre.

Duration of ochre deposition (years)



- 1 = 1 to 10% of potential economic return lost
- 2 = 11 to 20% of potential economic return lost
- 3 = 21 to 30% of potential economic return lost
- 4 = 31 to 40% of potential economic return lost
- 5 = 41 to 50% of potential economic return lost

(Economic return calculated over 15 years)

is assumed that drainage doubles the "Gross Margin" so that the potential economic yield from the drained land can be defined as 100%, whereas in the absence of drainage 50% of this potential return is lost. This level of financial return from drainage is unlikely to be obtained in practice so that the severity of ochre is probably underestimated in figure 8.1. However, the figure shows that the most severe ochre sites (41 to 50% of potential yield lost) are those where blockage is rapid occurring in less than 5 years. Where it takes longer than 5 years for complete blockage of the drainage system the severity of ochre deposition is also influenced by the duration of ochre deposition. The problem becomes more severe as the duration of deposition increases.

## 8.2 Formation

The factors governing the formation of ochre are complex but there are two prerequisites. Firstly, a source of iron and conditions at the source leading to the solution of iron. Secondly, ochre formation requires conditions leading to the precipitation of iron from solution. Thus ochre can form readily in field drains and surface ditches since they are at an interface where water from an anaerobic zone enters an oxidizing environment. The basic process involved in ochre formation is therefore the oxidation of iron and this can occur by chemical or microbial processes.

It was demonstrated (Section 6.1) that the precipitation of iron from drainage water can be accounted for by chemical oxidation. In sterile samples of drainage water it was found that on average 80% of the total iron in solution was oxidized within about 48 hours. At 4 sites 100% oxidation was recorded over this time period (Table 6.1). These results indicate the importance of chemical oxidation as a mechanism of ochre formation. It was also shown that autocatalysis of ferrous iron oxidation by ferric precipitates, which has been demonstrated in chemical solutions (e.g. Tamura et al, 1976) is relevant to drainage water. The addition of ~~air dried~~<sup>sterile</sup> ochre to drainage water samples containing iron in solution increased the rate of ferrous iron oxidation

although not the total amount oxidized. On average it took less than 2 hours to oxidize 50% of the iron initially in solution in samples containing ~~air dried~~<sup>sterile</sup> ochre. The corresponding time for samples without ~~air dried~~<sup>sterile</sup> ochre was over 12 hours. It is suggested that ochre deposits can catalyse the oxidation of ferrous iron in drainage water. The result of this catalysis is to concentrate the ochre problem within a smaller area thus increasing its severity.

Although it was shown that chemical oxidation could explain the formation of ochre the involvement of microorganisms is also an important factor.

Filamentous bacteria were observed by light microscopy in most samples in association with orange-red particles of ferric compounds. These organisms were distinguished from fungi by their narrower filaments and lack of branching. These organisms have an optimum pH between 6.0 and 7.0 and were not numerous at Spanker (Table 4.1) where the pH did not rise above 4.2. At some other sites where the pH was around neutral, filamentous bacteria were not observed. These bacteria were not found in samples of ochre at Penygroes 1 and 3 (Table 4.1) although the pH of the drainage water was 6.4. In the majority of samples however filamentous bacteria were observed and most were assigned to the genus Leptothrix. When samples were examined by scanning electron microscopy and EDAX (4.1.2) it was found that the filaments were composed primarily of iron and were coated with iron particles. Under light microscopy the sheaths appeared as empty "tubes" containing no cells. Lack of cells within the many filaments present in ochre samples suggests prolific sheath generation by a small number of organisms. This was noted as a characteristic of Leptothrix spp by Van Veen et al (1978) (2.3.1.4).

Gallionella spp were also observed in some samples but their distribution was not widespread. In the literature Sphaerotilus spp are often associated with ochre but during the present study these organisms were not observed.

Sphaerotilus spp are identified as filaments composed of rod shaped cells within a colourless sheath - no organisms fitting this description were observed by

light microscopy and they were not isolated in liquid and agar media that have been used by other authors to isolate Sphaerotilus spp. The problems associated with the taxonomy of Sphaerotilus and Leptothrix and the related difficulties in naming the organisms associated with ochre were discussed in section 2.3.1.2.

In addition to the filamentous bacteria, other microorganisms were isolated from ochre samples. Thiobacillus ferrooxidans (2.4.1) is a chemolithotrophic bacterium deriving its energy from the oxidation of Fe(II) and utilizing atmospheric carbon dioxide as its sole carbon source. These bacteria grow at a pH below 4.5 and have an optimum pH between 2.0 and 2.5. In the literature they are commonly associated with pyritic ochre deposits of low pH, but in the present study the distribution of T.ferrooxidans was surprisingly widespread. These organisms were isolated from the acidic Spanker site but the highest number,  $3.43 \times 10^4$ /g oven dried ochre, was found in a sample from Hall Farm 1 where the pH was 6.5 (Table 3.3 and 4.2). T.ferrooxidans were isolated from 14 of the 17 samples examined and of these 14 only Spanker 2 and Spanker 4 had a pH below 4.0. The other samples had a pH between 5.5 and 6.5. The isolation of T.ferrooxidans in ochre at pH greater than 4.0 suggests that they survive in microenvironments of low pH within the soil and drainage system contributing to ochre formation at sites which, on the basis of pH, would be described as filamentous.

Heterotrophic organisms capable of growing on artificial media at low pH were also isolated from ochre samples. These organisms were found in 10 out of the 17 samples analysed. A variety of organisms were isolated, most of which were also capable of growing on nutrient agar at neutral pH values. Johnson (1979) isolated similar organisms from acidic mine drainage water and found that they produced large quantities of polysaccharide. Hydrolysates of the bacterial slime contained high concentrations of ribose giving ribose : xylose ratios greater than 10 as compared to the normal ratios of less than 0.1 encountered in soils. The ribose : xylose ratios of ochre shown in Table 3.8 reveal that

values greater than 10 were not found in the hydrolysates of ochre samples but all, except 2, had ratios greater than those normally found in soils. The role of these heterotrophic organisms in ochre formation is unclear but they would increase the bulk and tenacity of the deposits as a result of their extracellular polysaccharide component.

Heterotrophic bacteria capable of degrading iron-organic complexes were isolated from ochre in a ferric ammonium citrate medium (4.3.2). There appeared to be little difference between the numbers of organisms capable of degrading the ferric citrate complex and those isolated on nutrient agar suggesting that most of the heterotrophic organisms in the ochre samples were able to degrade iron-organic complexes. Light microscopy revealed that the iron released from the complexes was not deposited on the bacterial cells suggesting that the release of iron was incidental. Complex degradation has been suggested as a mechanism of ochre formation (2.5.2.3). The presence of organisms capable of degrading iron-organic complexes confirms that this mechanism is feasible. However the importance of the mechanism depends on the amount of complexed iron present in the drainage water. When iron-organic complexes are present heterotrophic organisms, such as those commonly found in soil, will degrade the complexes releasing iron for chemical oxidation.

The formation of ochre has been discussed in terms of chemical oxidation and the isolation of several bacterial species. The presence of bacteria in iron deposits suggests a connection between these organisms and iron oxidation. The catalysis of ferrous iron oxidation by T.ferrooxidans is well documented (2.5.2.2) the organism being able to derive energy from the conversion of Fe(II) to Fe(III). In contrast the precipitation of iron resulting from the breakdown of organic-iron complexes by bacteria is incidental. These bacteria utilize the organic portion of the complex and in so doing release iron for subsequent chemical oxidation and precipitation. Thus, although via different mechanisms, both T.ferrooxidans and the complex-degrading bacteria are actively responsible for

the precipitation of ferric iron and hence ochre deposition. In their absence iron would probably remain in solution either because of low pH or chelation.

The role of the filamentous bacteria in ochre formation is unclear. There is no evidence to suggest that these organisms derive energy from the oxidation of ferrous iron but in nature they are commonly found in iron bearing waters and microscopic examination reveals that they are often encrusted with ferric deposits. It would appear (2.3.1.6) that iron oxide/hydroxide accumulation by filamentous bacteria is a passive, non-biological process where chemically oxidized ferric compounds are deposited on the organisms' sheaths. This is supported by the growth of these bacteria in water of pH 5.0 to 7.0 where the rate of chemical ferrous iron oxidation is rapid. Although the filamentous bacteria do not appear to cause the oxidation of iron they probably have an important role in ochre formation in that they stabilise and increase the tenacity of the deposits, the sheaths or filaments providing a template and fixture for the accumulation of iron precipitates. Active metal binding as observed in *E.coli* where cell wall peptidoglycan can bind metals including ferric iron (Hoyle and Beveridge, 1984) has not been demonstrated in the filamentous bacteria.

Leentvaar and Rebhun (1983) found that the addition of a polymeric coagulant caused an increase in the strength and size of ferric hydroxide flocs and it is likely that the organic sheaths of the filamentous bacteria would have a similar effect in drainage water. This is confirmed by the observations described in Section 5.3.1 where distinct differences were found between ferric precipitates in sterile and non sterile drainage water. In sterilized water the precipitates formed as a thin layer of ferric oxide/hydroxide particles, whereas in non-sterile water, in the presence of filamentous bacteria, distinct flocs of precipitate were observed.

### 8.3 Types of Ochre

The deposits known collectively as ochre are inherently variable in their appearance and composition, both chemical and microbial. This variability is related to many factors including site and time. It is possible to conclude that each ochre deposit is unique but it is important to rationalize the variation in ochre deposits into broad groups or types.

Ochre found at sites of low pH is commonly described as pyritic ochre and represents one extreme type of ochre characterized by pH values less than 4.0 and low organic matter contents. T.ferrooxidans and acidophilic heterotrophs are found in these deposits but no filamentous bacteria. Filamentous ochre, at the other extreme, is typically found in water of pH 6.0 to 6.5 and has a relatively high organic matter content. No T.ferrooxidans or acidophilic heterotrophs are found but filamentous organisms are numerous and the deposits contain a large population of unicellular, heterotrophic bacteria capable of degrading iron-organic complexes if they are present.

The majority of ochre samples will fall between these extremes and will have various combinations of filamentous bacteria, Thiobacilli and heterotrophic organisms. Chemical oxidation will be less important in ochre formation at low pH sites where most iron oxidation would be the result of biological catalysis by T.ferrooxidans. However, at pH values around 6.5 chemical oxidation probably accounts for a large proportion of iron precipitation. Where organically complexed iron is present it will be released by complex degrading organisms and subsequently oxidized chemically. The chemically precipitated ferric compounds can combine with filamentous bacteria to form ochre deposits. In some sites of near neutral pH (for example, Penygroes 1 and 3 section 3.1.1) the number of filamentous organisms may be small.



#### 8.4 Control

The results in chapter 7.0 demonstrated that the bacteria associated with ochre deposits are susceptible to biocides. For example, Panacide and Acrolein controlled the growth of T.ferrooxidans and iron-complex degrading bacteria effectively but these types of compounds are of little use in the field since they are extremely toxic to all forms of life. Copper was used at 1.0 µg/ml to control the growth of filamentous bacteria in drainage water samples and at 5.0 µg/ml to control complex degrading bacteria in ferric ammonium citrate medium.

In order to control "ochre bacteria" in drainage systems the biocide must be applied, probably to the drainage pipes, in a form that allows a slow and constant release. This is necessary to reduce the risk of polluting watercourses and to maximise the period over which bacterial growth is actively controlled. Marine antifouling paints provide a means by which compounds with biocidal activity can be used to treat drainage systems. The effect of several antifouling paints (7.3.3.2 and 7.4) on filamentous bacteria, acidophilic heterotrophs and complex degrading bacteria was investigated and it was found that most were effective in the short term but lost their inhibitory effect when repeatedly washed with distilled water. However, VC-17, a marine antifouling paint containing copper dust, was used to control the growth of filamentous bacteria in drainage water and heterotrophic organisms in artificial media (7.1.2 and 7.3.3.1). It was found that copper was released slowly except at pH less than 4.0 when the dissolution of copper was rapid. It is suggested that copper applied as an antifouling paint or incorporated in pipes by some method could be used in drainage systems to control the growth of filamentous bacteria and other heterotrophic organisms associated with ochre formation. In acidic sites the method could not be used since T.ferrooxidans can tolerate large concentrations of copper and the release of copper at low pH is likely to be high leading to pollution.

Despite the laboratory described effect of copper on the microorganisms associated with ochre there are two problems which must be solved before this method can be adopted in drainage systems. These problems are relevant to all solutions proposed for ochre control. Firstly, the solution must be effective in terms of ochre formation in the field. It has been shown that copper can control the growth of heterotrophic bacteria associated with ochre. However it has also been demonstrated that biological factors are only one facet in ochre formation which is also related to chemical oxidation. The success of biological control hinges upon the importance of chemical oxidation and the relationship between bacteria and iron in ochre formation. If it is accepted that filamentous bacteria act as a template for iron accumulation rather than contributing directly to iron oxidation then the success of biocides depends upon the extent to which chemically precipitated iron, in the absence of bacteria, will form precipitates similar to ochre. Ford (1979 A) suggested that in the absence of microorganisms chemically precipitated iron did not form drain type ochre since it did not adhere and could be washed easily from experimental drainlines. The difference in the type of iron precipitate from drainage water in sterile and non-sterile conditions was described earlier. There is some ground to believe therefore that controlling the growth of bacteria may change the form of iron precipitates reducing their clogging potential. However this has to be substantiated by further laboratory work and field trials. At Penygroes 1 and Penygroes 3 ochre deposits were found in drain lines but no filamentous bacteria could be observed in the samples suggesting that ochre was formed chemically, in the absence of these bacteria. In view of this observation the effect of any preventative method based on biocides must be demonstrated in relation to ochre production in the field.

The second problem associated with ochre prevention is that any method, when proved effective, must also be economic. The biological and chemical factors associated with ochre are scientifically interesting in themselves but ochre,

and especially its control, must be viewed in economic terms. When Pliny described various types of ochre in "Natural History" he was concerned with their economic value and quoted a price for each. Similarly ochre is important in agriculture since it causes drainage failure, reduces the economic viability of the drainage schemes and financial penalties are incurred. When assessing the effectiveness of ochre control the financial implications must be considered. The cost and benefits of prevention must be balanced against the financial loss caused by ochre.

The economic viability of ochre prevention will depend upon the effectiveness of the control method, its cost, the intensity of ochre accumulation (i.e. the time taken for complete blockage) and the duration of ochre deposition. The benefits of drainage will also influence economic viability, the larger gross margins obtained for cash crops would allow a larger expenditure in ochre prevention than for drainage schemes in low grade pasture land. When ochre is a temporary problem lasting less than 5 to 10 years it is possible to justify prevention whether the problem is severe or slight in terms of the number of years taken for complete blockage. It becomes more difficult to justify expenditure on control where the ochre problem is a permanent one especially if the rate of accumulation is not rapid.

Another fundamental and obvious problem is that in most cases the ochre prevention method would be incorporated as part of drainage installation at a time when the type, duration and intensity of ochre deposition are uncertain. Local features such as deposits in old field drains and ditches can be used to identify high risk sites but accurate prediction methods are not available. Prediction based on soil water iron content (for example Ford, 1982) might indicate the intensity and likelihood of ochre but cannot gauge its duration.

Chemical precipitates of iron in association with bacteria form an aqueous iron-organic deposit that can impair the flow of water in agricultural drainage systems. There is a need for an efficient and economic method by which ochre can be prevented or at least controlled. In the absence of adequate controls predictive techniques are needed to assess whether ochre is likely to be so severe as to make drainage uneconomic. Similarly prediction is needed in conjunction with control measures to determine whether the likely severity of ochre accumulation warrants the cost of prevention. The development of predictive tools and preventative measures must be based on a sound understanding of the nature of ochre, types of ochre, the factors influencing its formation and especially its variability.

(Denison, 1856)

Since Mangon wrote about ochre in 1856, large changes have occurred in agricultural drainage systems. Drainage work has also been improved by mechanisation, the introduction of new materials and modern techniques for drainage scheme design. We now have a better understanding of the characteristics, chemistry and microbiology of ochre but both the problem caused by it and the objective of preventing it remain the same.

"This is the most serious mischief which can happen to works of drainage; and unless it can be prevented, it is vain to attempt the improvement of lands liable to be so affected, by draining"

Mangon 1856

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