Bangor University

DOCTOR OF PHILOSOPHY

Constructed wetlands for the treatment of British mine drainage waters : a biogeochemical approach.

Dennison, Fiona Eleanor

Award date:
2002

Awarding institution:
Bangor University

Constructed Wetlands for the Treatment of British Mine Drainage Waters – A Biogeochemical Approach.

by

Fiona Eleanor Dennison

Submitted in candidature for the degree of
Doctor of Philosophy

School of Biological Sciences
University of Wales, Bangor
Bangor
Gwynedd

May 2002

Title: Constructed wetlands for the treatment of British mine drainage waters : a biogeochemical approach.
Author Given Name: Fiona Eleanor.
Author Family Name: Dennison
Institution: University of Wales.Bangor,
Issue date: 2002-01-01

Summary

The problem of (acid) mine drainage (AMD) in Britain is widespread and inherent to the mining industry. Ferruginous discharges from abandoned mines contaminate the waterways, through their high metal concentrations and ferric deposits. During this research, three main mine drainage sites have been studied: Ynysarwed, South Wales; Wheal Jane, South England; Mona, North Wales. These sites have similar ferrous iron concentrations ($\sim$250–300mg l^{-1}), although differing pHs – $\sim$6.3, $\sim$3.4 and $\sim$2.9 respectively.

Shake flasks were set up to investigate the factors affecting the rate of iron oxidation (and hence precipitation) as an iron removal mechanism in Ynysarwed and Wheal Jane waters. It was found that the rate limiting factor in both these waters was primarily biological and their biomass was limited by the availability of inorganic nutrients, in particular phosphate. The concentration of phosphate shown experimentally to be most beneficial, was in the order of <0.01M.

Experimental microcosms were used to investigate the effect of soil type and planting on the remediation of Ynysarwed and Mona waters. The results showed, that of the soils tested, mushroom compost was the most beneficial soil in terms of metal removal and alkalinity generation, with an optimal water residence time of four days. Planting was shown to have a detrimental effect on metal removal. However, in terms of sulphate reduction and pH maintenance, plants were beneficial, particularly in peat soil. These soils were shown to have the same remediative effect on several British mine waters, irrespective of their origin and a pilot system was proposed. The mechanisms occurring within planted and unplanted soils were investigated. It was found that competition may be occurring between the plants and microflora for resources such as inorganic nutrients, thus impairing microbial iron removal. Furthermore, the iron formed soluble complexes with the plant derived dissolved organic carbon, preventing removal through precipitation processes.

Acknowledgements

A very big thank you to my supervisor, Dr. Chris Freeman for his guidance, advice and encouragement over the course of my PhD project. For his valuable discussions and support when times were extremely difficult. Also to Dr. Barrie Johnson, for his advice and guidance in the first year of my work.

Thanks also to the Wetland Biogeochemistry group at Bangor- Hojeong, Vicky, Nathalie and Rhian for being great colleagues. To Niall Gilmartin for testing my proposed pilot wetland and to the technical staff for their advice and assistance, especially Glyn Connolly for assisting with the ICP analysis.

Thank you to the Environment Agency who have enabled me to do this research through their sponsorship. To friends at Preston, Warrington, Welsh, Reading and Rotherham Environment Agency Offices for their help in obtaining water samples, advice, encouragement and friendly chats. To Rotherham Environment Agency and Barnsley County Council for advice on water collection sites throughout the north, to Birse especially Trevor Mason for assisting in the collection of water from Ynysarwed and Richard Hemsworth for obtaining the gold mine samples.

I would like to express my appreciation to Dr. Calvin Dytham, Dr. Mike Elliott, Dr. James Allen and Miss. Krysia Mazik for their advice and guidance on the statistical analysis for this thesis. A special thank you to all the staff at York and Hull Universities, for their assistance, use of resources, encouragement, support and guidance particularly in the last couple of years.

A very big thanks you to all my friends and family here in Yorkshire and in Lancashire and Merseyside. For being at the end of the phone when times got tough and helping to make the most of my free-time. I am ever indebted to my parents, Dr. David and Mrs. Gerda Dennison, for all their unfailing love, care, encouragement, support and financial help – without them this could not be possible. For helping me to keep going when life at Bangor was so incredibly hard. Also to my sister, Sarah and my Grandmas Eleanor and Amalia. Without my family, I would have not been able to spend long hours working on my research. I should also like to take this opportunity to thank my parents in-law Nick and Carol Real, my sister in-law Kirsty, and grandparents in-law Walter, Greta and Mildred. For all their support and encouragement before Bangor and beyond.

Finally, a very big thank you to my best friend and husband Alan, whom I love very much. Who always stands by me and has ridden, along with the rest of family, the trials and tribulations of this PhD, whilst undertaking his own doctoral research at York University. Thanks also for teaching me how to use LaTeX, making the job of writing up a little easier!

NEMO ME IMPUNE LACESSIT

(Scottish moto)

To my family.

With much love and thanks.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Acid Mine Drainage

In 1992 a major environmental disaster occurred in the south west of England (Cornwall) causing vast amounts of environmental damage. It was estimated that, over a twenty four hour period, 30,000m^3 of metal rich mine water (Cambridge, 1997) was released into predominantly the Carnon River and its tributaries. The source was a disused tin mine called Wheal Jane, which had been allowed to flood following its abandonment in 1991. In 1992 an adit-plug (adit - a horizontally placed shaft to allow access or drainage) failed allowing the mine water to be released into the surrounding waterways. The consequential ochre plume stretched for kilometres.

The environmental consequences of acid mine drainage have only really come to light in the past thirty years. Increased public awareness of the advantages of an eco-friendly and aesthetically pleasing environment has created scientific and political pressure to minimise this form of pollution. The problem of "acid mine waste" on the aquatic system, was first mentioned as early as 1938 (Lackley, 1938). Since then, industry and science have seen the need for research to enable a better understanding of the processes which are taking place and, as a consequence, the remediation techniques which must be employed.

Mine drainage and in particular acid mine drainage (AMD), is geographically associated with the majority of active, or historically active, mining countries of the world (Dougan, 1987). For example, copper mining in Anglesey, Wales (Mynydd Parys) (Walton and Johnson, 1992); Copper and Sulphur mining in Avoca, S.E. Ireland (Herr and Gray, 1997), Wheal Jane – a tin mine in Cornwall (Lamb *et al.*, 1998) and metal and coal mining in Colorado, USA (Howard *et al.*, 1989). In 1994, 700km of British rivers were affected by mine water from over 300 mine discharges (Younger, 1997), whilst in the United States, over 17,000km are affected (Elliott *et al.*, 1998). This figure is similar to that predicted by Lundgren *et al.* (1972), who suggested that 16000km of American rivers were affected by surface mining alone, with deep mining enhancing these figures considerably. The legislation that is supposedly in place to control the polluting effect of mines varies from country to country, but the general overriding principle is that the ultimate responsibility falls to the offending company and its regulators. In America, discharged water must meet the water quality standards set out by the Surface Mining Control and Reclamation Act of 1977 (Unz and Dietz, 1986). In Britain, even though the Environment Agency battles long and hard with the problem, the 'Polluter Pays Principle' may be their only prosecution strategy when addressing past offences.

The problem of (acid) mine drainage in Britain is widespread and chiefly located in the abandoned mining areas of SW England (Wheal Jane), Wales (Ynysarwed, South Wales) and the North east of England (e.g. Bullhouse discharge). In 1975 the Department of the Environment reported that 366 discharges were released into the waterways of England and Wales, and this amounted to 881,990m^3 of mine water. Although other types of ore mining cause localised problems, the biggest culprit, especially in the UK, is that of the derelict coal mining industry. Characterisation of these waters, is not only by their acidic pH (<4.5), but also their orange/brown colouration of the iron rich sediments or 'ochre', which accumulate and smother the benthic community (figure 1.1). The complex material called 'ochre' or 'yellow boy', as it is known by miners, is associated with the hydrolysis of ferric (Fe^{3+}) iron to form insoluble ferric salts and hydroxides.

ORIGINAL IN COLOUR

Figure 1.1: Typical orange/brown ferric iron rich sediments, associated with acid mine drainage problems.

However, the dominant species at the point of discharge from the mine or adit is the relatively colourless ferrous iron (Fe^{2+}) causing the upper reaches of an acid mine drainage system to appear clear and unpolluted.

The chemical composition of other water bodies is extremely variable due to the influence of the surrounding geology, as is that of mine drainage, which is influenced by mine type. Usually, waters of pH 4.5 are buffered by the "CO_2-bicarbonate-carbonate-buffering system" (Geller *et al.*, 1998). However, waters classed as acidic (<4.5) have very little or no bicarbonate content and this affects the buffering capacity. This low pH has subsequent implications for the solubility of metals. According to Stumm and Morgan (1981) at low pH, metal solubility is increased causing the concentrations of aluminium and iron to be elevated. It was also found that the different species of iron, namely the ferric iron and its hydroxides, had buffering capacities that were comparable to that of the aluminium and carbonate systems, which aid the stabilisation of waters with values between pH 2 and 4 (Geller *et al.*, 1998). Many iron oxides, such as ferrihydrite (amorphous ferric hydroxide), have been identified in acid mine drainage sediments, using X-ray diffraction (Ferris *et al.*, 1989a). These iron oxides were also affected by acidic pH values, in that they showed a marked increase in concentration (Schwertmann and Murad, 1983; Zhang and Evangelou, 1996). It was found that the bacterial cells became encrusted, to varying degrees, in ferrihydrite and other iron oxides, possibly forming templates for iron deposition (Ferris *et al.*, 1989a).

1.1.1 Acid Mine Drainage Genesis

A prerequisite for the formation of acid mine drainage is the presence of sulphide minerals such as pyrite and marcasite (Johnson, 1998; Hustwit *et al.*, 1992). Pyrite (FeS_2), an insoluble crystalline substance found in ores and bituminous coals, is formed when ferrous sulphide and sulphur react (Madigan *et al.*, 1997). It is the oxidation of the sulphide minerals to sulphate, free metal and hydrogen ions, that are responsible for the acidic conditions (Singer and Stumm, 1970). The rate of this acidification is governed by the mineral concerned (Singer and Stumm, 1970). The mechanisms of sulphide mineral oxidation can be either chemical, electrochemical (Mustin *et al.*, 1992) or biological/biochemical (Sand *et al.*, 1995) and are well documented elsewhere (e.g. Evangelou (1995)). Oxidation is known to be dependent upon environmental conditions such as pH (Sand *et al.*, 1995; Nicholson *et al.*, 1995) and the ability to control the rate of iron oxidation in mine waters, is a valuable tool in the development of remediation strategies. The general consensus is that for biological and chemical oxidation, the major oxidant is ferric iron (Sand *et al.*, 1995). This reaction is as follows:

$$FeS_2 + 6Fe(H_2O)_6^{3+} + 3H_2O \rightarrow Fe^{2+} + S_2O_3^{2-} + 6Fe(H_20)_6^{2+} + 6H^+ \quad (1.1)$$

This equation can also be re-written to include sulphate and elemental sulphur because in acidic conditions, thiosulphate hydrolyses due to the fact that it is extremely unstable:

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \quad (1.2)$$

As the ferric iron is consumed during this reaction, oxygen is required for its regeneration (see equation below) which can only occur for a limited time in anoxic conditions. This re-oxidation is slow, although spontaneous (Johnson, 1998), and is as follows:

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O \qquad (1.3)$$

The rate of this reaction may be increased by 10^4 and 10^6 times by the presence of bacteria such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, which are chemolithotrophic bacteria and obligate acidophiles. By promoting the growth of these bacteria at low pH, the rate of mineral weathering can be manipulated (Strömberg and Banwart, 1995).

The acidity of acid mine water, in the context of ferric iron as a source of hydrogen ions, was discussed earlier. It should also be noted that, the hydrolysis of dissolved metals such as aluminium and manganese also produce hydrogen ions, as follows (Clarke, 1995):

$$Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+ \qquad (1.4)$$

$$4Mn^{2+} + O_2 + 6H_2O \rightarrow 4MnOOH + 8H^+ \qquad (1.5)$$

Therefore, according to Hedin *et al.* (1994), the total acidity of acid mine water can be calculated as follows taking into account these extra parameters:

$$\text{Acidity}_{\text{Calculated}} = 50\left\{\frac{2\text{Fe}^{2+}}{56} + \frac{3\text{Fe}^{3+}}{56} + \frac{3\text{Al}}{27} + \frac{2\text{Mn}}{55} + 1000(10^{-\text{pH}})\right\} \quad (1.6)$$

"Where concentrations of metals are given in mg l^{-1} and 50 is the equivalent weight of $CaCO_3$, thus transforming the milliequivalent per litre of acidity into mg l^{-1} $CaCO_3$ equivalent" (Hedin *et al.*, 1994).

Hedin and co-workers (1994) showed that for waters measuring a pH of <4.5 using a standard H_2O_2 method, their calculated acidities were generally found to be within 10% of that measured figure. Using examples from three sites in the U.S., they went on to show that, the acidity created by minerals, forms an important part of the waters' overall acidity.

Besides varying acidity (proton and mineral), other environmental parameters affect the overall characteristics of the water, such as; dissolved oxygen which can be as low as <10% saturation of the ambient aerated water (Emerson and Moyer, 1997); dissolved organic carbon; metals (discussed in depth by Sullivan and Yelton (1988)) and salinity. These may in-turn affect the waters redox potential. Redox (where both oxidation and reduction occur in the same reaction) is influenced by the presence of sulphides and other reduced sulphur compounds in the water's sediment (Boulegue and Michard, 1979), although, it is primarily affected by the ratio of Fe^{2+} : Fe^{3+}. Therefore, the two halves of this redox reaction are as follows (Nordstrom *et al.*, 1979; Johnson *et al.*, 1993):

$$Fe^{2+} \rightarrow Fe^{3+} + e^{-} \qquad (1.7)$$

Here, for the oxidation part of the reaction, the rate is determined by availability of the Fe^{3+} ion. Although this reaction is slow in acidic conditions, it can be enhanced considerably with the presence of iron oxidising bacteria such as *Thiobacillus ferrooxidans*. The role of this organism (and others) will be discussed in more detail later. This reaction continues until all the pyrite is used or ochre formation occurs. The reduction part of the reaction is as follows. Note that the electrons in this reaction are derived from the oxidation of iron:

$$\tfrac{1}{2}O_2 + 2H^{+} + 2e^{-} \rightarrow H_2O \qquad (1.8)$$

To ensure appropriate aerobic conditions for the respiration of these bacteria, oxygen is a vital factor and need only be present in small quantities to drive this reaction. However, oxygen can be a limiting factor in this process (Hustwit *et al.*, 1992). Anaerobic iron oxidation has been reported by (Straub *et al.*, 1996) in some strains of denitrifying bacteria. These strains were originally isolated using "aromatic substrates and nitrate" and could oxidise, within a week, over half of the ferrous iron present, but they needed the addition of 1mM acetate.

It should be noted that the two parts of the above redox reaction do not necessarily have to be in equilibrium (Nordstrom *et al.*, 1979) and the measurement of redox potentials (Eh) can be used to evaluate these systems. Brookins (1988) showed diagrammatically the relationship between pH and Eh.

The majority of aquatic life, including microorganisms, are unable to withstand these acidic environments, although, some species of bacteria thrive in the conditions denoted as acid mine drainage (Mills and Mallory, 1987). These organisms are classed as acid-tolerant or acidophiles, each with varying tolerance. Some of which, for example the chemolithotroph *T. ferrooxidans*, known for its role in the genesis of acid mine drainage, have been isolated, selected and enumerated using solid media such as the 'overlay' technique (Johnson, 1995b; Amero *et al.*, 1994). Therefore, of the acidophiles, *Thiobacillus* is one of the most extensively studied groups (e.g. Hallberg and Lindström (1996); Schrenk *et al.* (1998); Kleinmann *et al.* (1981) and Pronk *et al.* (1992, 1991)) although not all are obligate acidophiles. As aforementioned, these bacteria are known to accelerate the oxidation of the sulphide minerals associated with acid mine drainage (Johnson, 1998), although recent research (Schrenk *et al.*, 1998) shows that *T. ferrooxidans* has a "limited role in acid generation". But, it is thought that the numbers of these bacteria (*Thiobacillus* spp. and *Leptospirillum ferrooxidans*) may be equal to or out numbered by indigenous acidophilic heterotrophic microorganisms (Johnson and Roberto, 1997b). These heterotrophs were first characterised in the 1980's from cultures, which were supposedly pure, of *T. ferrooxidans* (Wichlacz and Unz, 1986). They have been reported to form 'acid streamers' (macroscopic gelatinous growths, white or cream in colouration) in acidic mine waters (Dougan *et al.*, 1970; Johnson *et al.*, 1992) and in 1991, Johnson published a paper on their evidence in Cae Coch, an abandoned pyrite mine (North Wales) (Johnson and McGinness, 1991). It is thought, (Johnson and McGinness, 1991; Johnson, 1995a) that these acidophilic heterotrophic microorganisms, may catalyse either the reduction of ferric iron or the dissimilatory oxidation of ferrous iron. These iron oxidising acidophiles display characteristics, both morphological and behavioral, similar to those of the neutrophilic *Sphaerotilus-leptothrix* species of filamentous bacteria (Johnson *et al.*, 1992). Acidophilic heterotrophic bacteria and other acidophiles, are influenced in their distribution by their individual metal or metalloid (a non-metal displaying some of the characteristics of metals) tolerance. Chemolithotrophic iron-oxidising bacteria, especially *T. ferrooxidans*, have a greater tolerance to dissolved metals than their heterotrophic counterparts although, increased tolerance could possibly be brought about by sub-culturing the organisms in increasing metal concentrations (Johnson and Roberto, 1997a). Johnson and Rang (1993) also found evidence to suggest that sulphate reducing bacteria (SRB) are present in stream sediments exposed to acid mine drainage. These bacteria exist heterotrophically and are found at the oxic/anoxic interface in the highest numbers (Jørgensen and Bak, 1991). The most commonly known of these is *Desulfobacter* spp. although, many other novel strains are now being discovered (Sen and Johnson, 1999). Other organisms noted in these water bodies include rotifers, fungi, yeast and protozoa (Johnson and McGinness, 1991; Johnson and Rang, 1993).

The distribution of these organisms, as we have seen, is dependent upon many parameters, but as the oxidation of pyrite is an exothermic reaction, it follows that the concept of temperature affecting species distribution and diversity, must also be considered. Temperature in underground mines varies considerably with depth, but shows very little seasonal variation; with temperatures of $<10°C$ not being uncommon (Ahonen and Tuovinen, 1991). 'Shake flask' experiments were set up by Ahonen and Tuovinen (1991) recording different activation energies for different sulphide minerals and they found that at the higher incubation temperatures there was a considerable change in pH and redox potential. The decrease in pH was attributed to the increased sulphuric acid production via the biological oxidation of sulphide minerals. At the lower temperatures tested, redox potentials displayed either a lag period before increasing or they stayed at a lower level, indicating at least some retardation in biological activity. Higher temperatures, especially those as a consequence of exothermic reactions, occur in areas such as spoil heaps where the internal environment is insulated against external thermodynamic changes. According to Norris and Johnson (1996), in their detailed account of these extremophiles, only three main groups have been identified and characterised. They were found to be active in temperatures of approximately 40–60°C and are classed as moderate thermophiles. These include: "Gram negative, sulphur oxidising autotrophs" found in areas such as the acidic hot soils of Yellowstone National Park (Fliermans and Brock, 1972) and species such as *Thiobacillus caldus*, characterised in 1994 by Hallberg and Lindström (1994); "Gram positive, *Bacillus*-like facultative chemolithotrophs and heterotrophs" such as *Sulfobacillus* spp. found in acidic mine waters and spoil heaps derived from coal mining; and "two genera of archaea" found in coal spoil heaps and hot springs. These include species of *Thermoplasma* (Norris and Johnson, 1996). In particular, the facultative anaerobe *Thermoplasma acidophilum* has been shown to be able to utilise sulphur as an electron sink. Strains of (hyper) thermophilic bacteria have also be identified that can function effectively, degrading sulphide minerals at 85°C namely species of *Sulfolobus* (Norris and Johnson, 1996). In 1994, Amero *et al.* published their work on the new and specific method for detection and enumeration of thermophilic bacteria such as these (namely *Sulfolobus acidocaldarius* and *Thiobacillus caldus*) using immunobinding techniques.

All of the organisms, described above, which have been isolated and characterised from acid mine waters, display different inter- and intra-specific relationships and are described in some detail in Johnson and Roberto (1997a). Each organism has its own ecological niche and competes in a very complex and unique environment.

It should be noted that, not all mine drainage is acidic, for example, water from a disused deep coal mine at Ynysarwed, South Wales is of circa neutral pH (~6.3). Some mines do not have the acid generating minerals, whilst others contain sufficient neutralising compounds such as carbonates to override the affect of sulphide mineral oxidation. In these near neutral pH conditions, iron-oxidising bacteria have been isolated and characterised from areas such as East Lansing, Michigan (USA) (Emerson and Moyer, 1997).

1.1.2 Traditional Methods of Abatement

Many strategies have been put forward, and in some cases implemented, to try and contain the threat posed by acid mine drainage (e.g. Dougan and Apel (1983); Brierly (1990)). Some were more problematic than others, requiring collection of water, possible diversion of watercourses and as in all cases, vast amounts of financial investment. The most favourable solutions involve trying to prevent, solve or confine the problem at source i.e. within the mine itself or the associated spoil heaps. But, according to Younger (1997), the most financially sound method of remediation would be a passive one such as the use of artificially constructed wetlands. Before undertaking such a strategy, the long-term effects, both physical and chemical, on the environment by the continual release of acid mine waters should be thoroughly researched. This should possibly include data from water quality assessments (WQA's) and full environmental impact assessments (EIA's). Strategies for the toxicity testing of acid mine drainage waters and their utilisation in setting water quality standards, using techniques such as 'Microtox (R)', a toxicity testing tool which uses the luminescent bacterium *Vibrio fischeri* as an indicator of a change (decrease) in respiration, were outlined by Gray and O'Neil (1997).

Characterisation of these mine sites using the information ascertained above, enables the hydrology of the mine to be modelled leading to possible predictions as to the long-term effects (Clarke, 1995). In most cases, maps of the intricate passages would have been useful in aiding this modelling process, but unfortunately, experience has shown that many of the maps are unreliable.

Younger (1997) suggests, that there are two components to acidity generation over time which need to be taken into account. These are: "Vestigial" – this refers to the post abandonment "geochemical trauma" exerted on the mine voids, as they fill with water for the first time. This takes into solution, some of the products of pyrite oxidation, such as ferrous and ferric hydroxysulphate salts (Wichlacz and Unz, 1986) which are intermediates of this oxidative process; and "juvenile" – this refers to the continual pyrite oxidation (until exhaustion), within the restrictions of the natural fluctuations of the water table, based in and around the disused mine. It should be noted (Clarke, 1995) that, the initial release of water from a flooded disused mine, is usually the most acidic and saturated with dissolved substances, in particular metals. This is due to the fact that the initial flushing contains the majority of products, which have accumulated in the mine pre and post abandonment. Gray (1997) tries to go further, by categorising the acid mine drainage sites, to aid the development of specifically tailored water quality, management and remediation strategies. However, he states that this is difficult, due to the fact that acid mine drainage is a "multi-factor pollutant" i.e. it affects directly or indirectly, via complicated pathways, the aquatic system as a whole. The immediate solution must be to try and contain the problem preventing further contamination of the water-courses. This can be done by employing a wide range of "barrier methods" (Clarke, 1995), which are designed to limit mineral (pyrite) oxidation and thus AMD genesis, by their isolation from water and oxygen. The oxidation of pyrite by these two variables has been previously outlined and is well documented elsewhere (e.g. Hustwit *et al.* (1992)). Sometimes, simple methods such as the compaction of the surrounding soil provide sufficient protection, whereas in other situations, more elaborate techniques must be deployed.

Chemical and Practical Methods

Spoil heaps associated with the mining process can be slightly easier to protect from the oxidative processes and elements than the emergent mine waters. Plastic/synthetic liners of 0.5–1.0mm thickness have been placed effectively over the spoils, ideally covering them completely. If an area is left exposed to the elements i.e. partially uncovered due to a tear for example, water can penetrate deep into the spoil thus limiting the affect of the liner and possibly rendering it useless. A protective layer of soil may be placed on top of the liner to prevent tearing, leaking and degradation of the plastic by ultraviolet light. The soil layer and subsequent colonisation by vegetation can also encourage the stabilisation of the spoil. The liners efficiency is also increased, by the fact that the internal water-table is lowered, reducing the rate of mineral oxidation. Clarke (1995) states that, although this method is effective, it is both expensive and labour intensive, thus restricting its use. Other capping/sealing materials employed to prevent rainwater percolation include asphalt, tar, concrete and cement. In America (Scheetz *et al.*, 1995), a more permanent method of abating the effects of acid mine waters, has been trialed, involving the use of a grouting technique. Using magnetometry, the areas with potential "acid-producing minerals" are sought out and injected under pressure, through boreholes, with a cement and fly ash mix/slurry. This engulfs and stabilises the area rendering it inert and impermeable. The success and long-term consequences of this method are still being researched, some details of which are outlined in Clarke (1995) and Scheetz *et al.* (1995).

Using column leaching experiments, Stewart *et al.* (1997), investigated the affect of combining alkaline fly ash with the acid forming refuse associated with coal mining. The effectiveness of this method is due to the reduction in pyrite oxidation. This is brought about by the presence of the fly ash retarding gas and water flow through the column. It is also thought that the fly ash may be adsorbing some of the free ferric iron, resulting from the oxidative process, preventing it from oxidising the pyrite itself. This co-disposal consequently solves two environmental problems at once.

Submergence and underwater storage of sulphide minerals (especially mine tailings) once again can be used to prevent their contact with oxygen, as the diffusion rates of oxygen through water are considerably slower than through other media. Sometimes, these minerals are covered with a layer of sediment to prevent their resuspension into the water column through wave or wind action. But a more effective way is to use a blanket of organic matter. Not only does this act as a barrier, but it also consumes oxygen through its own degradation, providing an ideal substrate for sulphate reducing bacteria (SRB). The use of SRB in the remediation process will be discussed in more detail later. In the past, it has been common practice to flood and seal disused mines especially those occurring below the water table (Clarke, 1995). This causes periodical problems of mineral exposure to oxidative processes, if and when the water table drops or undergoes lateral movement, acid mine drainage may result. Clarke (1995) suggests that initially this water will be extremely acidic, but after subsequent flushings this will be reduced.

As we have seen, there are many methods to aid the prevention of watercourse contamination by AMD, but inevitably, contamination does occur, requiring some sort of treatment to lessen the effects. Many methods are so called "active" ones due to the fact that they require continual/long-term treatment or the addition of chemicals. One of these methods includes the addition of small quantities of additives to encourage the precipitation and settling of compounds. The use of flocculating agents, which may be either of inorganic or organic materials, encourage the aggregation of precipitates leading to their recovery in large settling ponds. A pilot scheme was set up in Yorkshire to test the combined effect of using 24g of sodium hydroxide and 200g of flocculent. This resulted in the separation of only 20g of dry ochre, which was recovered through raking. The disadvantages of this system were found to be, that flocculation was most effective at near neutral pHs and required continual injection of pH conditioning agents, as well as flocculent, to the inflow (Clarke, 1995). Clarke (1995) also describes the use of coagulants such as aluminium sulphate, which forms aluminium flocs, which in turn attract other metals for precipitation. This method requires pH adjustment, due to the fact that the formation of aluminium flocs is dependent upon the reaction of aluminium sulphate with alkaline waters. To eliminate/reduce the need for this pH adjustment, precipitation can be enhanced by increasing the dissolved oxygen concentrations. This can be achieved by increasing the aeration of the system or, by the addition of oxidising compounds such as peroxide or hypochlorite (Clarke, 1995). Phosphate has also been trailed in solution with peroxide, in the form of hydrogen peroxide (Clarke, 1995). The mode of action is as follows: the pyrite surfaces are oxidised by the peroxide, producing Fe^{3+}, which in turn precipitates out as iron phosphate. The following reaction shows the oxidation of pyrite by hydrogen peroxide, in the presence of phosphate (Clarke, 1995):

$$FeS_2 + 7\tfrac{1}{2}H_2O_2 + H_2PO_4^- \rightarrow FePO_4 + 2SO_4^{2-} + 3H^+ + 7H_2O \qquad (1.9)$$

The iron phosphate forms a passive coating upon the pyrite surface preventing further pyrite oxidation (Zhang and Evangelou, 1996), but it does require some sort of buffering to enable it to work effectively. Although this method is still only experimental, the results are promising. The additional bonus of this method is that only small quantities of peroxide and phosphate are needed thus keeping the running costs to a minimum (Clarke, 1995).

Today, the most common method used for reducing the effect of the acidic pHs characteristic of most mine drainage water systems, is via the addition of alkaline chemicals or compounds. Some mine drainage systems, due to the surrounding geology, have their own built in neutralising capacities, in the form of carbonate minerals. This counterbalances the acidity produced as a result of pyrite oxidation. Clarke (1995) calls this "acid-base accounting" and it can be used to determine the potential of the local geology to produce AMD. To date, lime, sodium hydroxide, sodium carbonate and calcium carbonate (limestone), have been used as cost-effective agents of water neutralisation on a large scale. The use of lime to reduce water acidity, is not a new process. In 1991 the Environment Agency and Hyder (E.A. and Hyder, 1991) used lime dosing on Upper Tywi, South Wales, to combat the acidification which had arisen from conifer afforestation. The acidic pH was killing the susceptible salmonid stocks and as a result, in 1996, two purpose built limers, were installed on the Rivers Tywi and Camddwr, the first of their kind in the UK. They could measure the water acidity and supply the correct dose of lime to increase the pH from 5.1 to ca 6. Similar water treatment strategies were also implemented in the Loch Dee catchment (Tervet and Harriman, 1988). Furthermore, limestone and lime, can also be used as an additional buffering zone between spoil heaps and the overlying soil, and mixtures of limestone and coal waste have also been used as an acidity buffer. Other mixes, to increase the ability of limestone to neutralise AMD, include that of limestone and other calcareous material. A five-year column study was set up to evaluate the effect of these mixtures and it was concluded that columns containing limestone were the most effective at delaying the release of acid (Clarke, 1995).

Those faced with the abatement of AMD welcome new ideas which are cost effective and require little or no maintenance. One of the solutions, which combines both of these, is the use of anoxic limestone channels/drains (ALD). Although, this is classed as a passive treatment rather than an active one, like those discussed above, it uses limestone in the treatment of AMD (Nairn *et al.*, 1992). It involves a principle called "limestone armouring", which as the name suggests, on the passage of oxidising ferrous iron over the limestone it becomes coated or protected in ferric hydroxides, reducing the limestone's neutralising capacity. Results from laboratory experiments showed that the "armoured" limestone compared to its unarmoured counterpart, "was only 2 to 45% less effective" (Ziemkiewicz *et al.*, 1997) at neutralising a solution of hydrochloric acid (dependent on solution pH), but was "90% as effective" (Ziemkiewicz *et al.*, 1997) at neutralising AMD. Field studies followed, using constructed channels, lined with pieces/chips of specific rock types. The results showed that, the limestone, albeit armoured, was more effective at reducing the acidity of AMD, than sandstone (Ziemkiewicz *et al.*, 1997). This system was developed primarily as a method of raising the pH of AMD waters prior to their passage through (constructed) wetlands. If the water were passed through an anoxic pond to reduce the dissolved oxygen level, prior to its passage through the ALD, then less iron would oxidise creating a more efficient system. However, problems do arise when waters contain high concentrations of ferric iron or aluminium. This in the long term, due to hydroxide build up, reduces the ALD's permeability rendering it useless in a relatively short time. One way to solve this problem is to prevent the biological oxidation of the sulphide minerals through the use of bactericides such as sodium lauryl sulphate and sodium benzoate (Dougan and Apel, 1983). These substances work via a mechanism of destroying the cell wall integrity, allowing the influx of acid into the near neutral interior. If high enough concentrations are used, the cell may be killed (Dougan and Apel, 1983; Dougan, 1987).

Biological Methods

In the past, bioremediation, or the harnessing of biological systems as a solution to aquatic environmental problems has been associated with the 'clean up' of organic pollutants (Head, 1998; Kelley *et al.*, 1993). Only since the 1980's, has the use of this technique, as a remediation process for the problems posed by AMD, been gathering momentum. As described earlier, the biological oxidation and dissolution of sulphide minerals cause the problem of AMD. New techniques involve microbially reversing those processes, which lead to AMD, giving rise to the removal of metals from solution and the raising of pH in these waters. It should be noted that whilst some microbial processes, like those described earlier, give rise to a net increase in acidity, others produce alkalinity, for example by sulphate reduction, a process which will be discussed further, later in this chapter.

Photosynthesising acid tolerant microorganisms have been reported in some aquatic environments affected by AMD. Under 'normal' circumstances, there is no change in pH, unless, as in aquatic systems, the CO_2 is removed as carbonate or bicarbonate from solution by photosynthetic organisms. This causes a localised pH increase during the hours of daylight. If photosynthesis is considered in conjunction with nitrate assimilation, the removal of nitrate, phosphate and hydrogen ions are seen to increase alkalinity. The equation for this reaction is as follows (Wendt-Potthoff and Neu, 1998):

$$106CO_2 + 16NO_3^- + HPO_4^{2-} + 122H_2O + 18H^+ \rightarrow (C_{106}H_{263}O_{110}N_{16}P) + 138O_2 \qquad (1.10)$$

Not all aquatic environments affected by AMD can support photosynthesising microorganisms, many experience little or no light and as a consequence become anoxic. Nitrate and ammonia, are amongst the most commonly used compounds as electron acceptors, in anaerobic respiration (Madigan *et al.*, 1997). Nitrate can be reduced (denitrification) to products such as nitrous oxide and nitrogen gas, which can be lost to the atmosphere. Therefore, once again, a net gain in alkalinity will be observed if, as in this process, hydrogen ions and nitrate are removed (Wendt-Potthoff and Neu, 1998). It should be noted that organisms such as purple bacteria have been observed anaerobically (at pH 7) using ferrous iron as an electron donor for photosynthesis (Ehrenreich and Widdel, 1994).

The relationships between specific species of bacteria have been discussed in detail elsewhere (e.g. Johnson and Roberto (1997b)). These relationships, especially those of competition and inhibition, have a possible use in the abatement of AMD. In 1988, Shuttleworth and Unz (1988) published a paper on the findings of a mathematical model that calculated the growth inhibition of *T. ferrooxidans* when grown on a substrate specific for the heterotroph *Acidiphilium* spp.. Although, no firm conclusions were made, laboratory trials were undertaken to test the hypothesis generated e.g. the mathematical model, so as to see if biological AMD genesis could be inhibited. Shearer and co-workers (Shearer *et al.*, 1970) showed that strains of Caulobacters, isolated from neutral waters, could inhibit acid production from sulphur- and iron- oxidising bacteria. Although, only one strain of antibiotic-producing bacteria (*Streptomyces aureofaciens*) was shown in laboratory experiments to reduce net acidity and be capable of withstanding AMD environments.

The microbial catalysation of Fe (III) and Mn (IV) reduction is extremely well documented elsewhere (e.g. Unz and Dietz (1986); Pronk *et al.* (1992)) and originally thought to be solely an abiotic process (Wendt-Potthoff and Neu, 1998). In fact, during anaerobic respiration, bacteria can use these two metals as electron acceptors (Wendt-Potthoff and Neu, 1998). In suboxic microbial ecosystems, ferric iron oxides are extremely abundant and can be reduced to ferrous compounds by many species of bacteria such as *Thiobacillus* spp.. This reaction can be coupled to one of oxidation involving many organic and inorganic electron donors. For example, if the carbon source were that of organic acids, the reactions for iron and manganese respectively, would be as follows (Wendt-Potthoff and Neu, 1998), producing alkalinity as the metal ions are removed:

$$FeOOH + CH_3CHOHCOO^- + H_2O \rightarrow Fe^{2+} + CH_3COO^- + CO_2 + 1.5H_2 + 2OH^- \tag{1.11}$$

$$MnO_2 + CH_3CHOHCOO^- + H_2O \rightarrow Mn^{2+} + CH_3COO^- + CO_2 + H_2 + 2OH^- \tag{1.12}$$

Thiobacillus ferrooxidans under anaerobic conditions has been shown to be able to use ferric iron as an electron acceptor instead of oxygen, when oxidising elemental sulphur (Pronk *et al.*, 1992). Consequently, anaerobic conditions may not guarantee the immobilisation of metals and inhibition of sulphide mineral oxidation (Das *et al.*, 1992).

The significance of iron reduction in the metal cycling process has already been discussed in terms of bacteria such as the aforementioned *Thiobacillus ferrooxidans* and documented by Johnson *et al.* (1993), but the importance of SRB should be noted. In some marine environments, iron sulphides are formed through the reduction of iron oxyhydroxides by the H_2S produced by the SRB (Coleman *et al.*, 1993). The formation of these mono-sulphides has been shown to be evident in anoxic (sulphidic) mine tailings (Fortin and Beveridge, 1997). Therefore, the use of sulphate reducing bacteria (SRB), such as *Desulfovibrio* spp., have more recently been suggested, as a cost effective method of treating mine drainage waters (Webb *et al.*, 1998; Banks *et al.*, 1997; Christensen *et al.*, 1996) – a process by which net alkalinity is produced. Under anaerobic conditions, they use sulphate, as a terminal acceptor resulting in the production of H_2S (hydrogen sulphide), which is excreted. In addition, metals form insoluble sulphides. The reactions are summarised as follows (Wendt-Potthoff and Neu, 1998; Webb *et al.*, 1998):

$$2CH_2O + SO_4^{2-} \rightarrow 2HCO_3^{2-} + H_2S \tag{1.13}$$

$$M^{2+} + S^{2-} \rightarrow MS_{ppt} \tag{1.14}$$

Where M^{2+} represents Fe^{2+} or other divalent metal ions. These highly reactive iron sulphides can adsorb or coprecipitate with other dissolved metals such as copper and zinc. Their ability to control the solubility of these metals and others such as cadmium and lead could lead to the possibility of their use in remediation techniques, although, in the presence of other intermediate sulphur species e.g. thiosulphate, the eventual formation of pyrite is observed. It should be noted that if the bacteria utilise organic acids, then the reaction will be as follows (Wendt-Potthoff and Neu, 1998):

$$SO_4^{2-} + CH_3CHOHCOO^- + 2H_2 \rightarrow H_2S_{(g)} + CO_2 + CH_3COO^- + 2OH^- + H_2O \tag{1.15}$$

Clarke (1995) suggests that there may be one disadvantage with this method of remediation, in that a substrate of organic carbon must be provided. Dvorak *et al.* (1992) suggested that to overcome this problem, the use of mushroom compost or sawdust, both with or without limestone addition (Wendt-Potthoff and Neu, 1998) have provided some success. Substrates of straw have also been suggested (Béchard *et al.*, 1994) for aiding microbial treatment of AMD and were included in bioreactors along with a "mixed aerobic-anaerobic microbial treatment". It was found that the use of cellulosic substrates in this form were only effective if a supplement of sucrose was added to the AMD. In contrast, a microbial treatment with a substrate of alfalfa and without a sucrose addition, was thought to be a low maintenance answer (Béchard *et al.*, 1994). If SRB and other bacteria can utilise these waste products in this way, then there is an added advantage of this remediation technique. For example, Benner *et al.* (1997) suggested the use of organic matter in a "porous wall" to be placed into aquifers affected by AMD. Such a wall was successfully implanted into the Nickel Rim mine site near Sudbury, Ontario in August 1995. Nine months after installation, it was found that sulphate reduction and metal sulphide precipitation was occurring. As a consequence, the acidity emerging at the surface was much reduced. It is this increased acidity that brings about the solution of many heavy metals such as cadmium and iron, which are evident in some AMD effluents (Mason, 1995; Unz and Dietz, 1986). It should also be noted that the aerobic and facultative heterotrophic species present in the surface regions of a biofilm, have been reported to create the appropriate physiochemical and nutrient conditions required by SRB for growth (Hamilton, 1994).

The interactions between metal and microbe are extremely well documented elsewhere (e.g. Ferris *et al.* (1989a). As aforementioned, metals can be immobilised, mobilised or transformed by reactions such as oxidation and reduction; methylation and demethylation; complexing and binding, precipitation and accumulation. It is therefore, of additional benefit if the bioremediation technique can cope with this additional stress. White and Gadd (1998) have found SRB biofilms that can undertake the immobilisation of metals. When subjected to cadmium (Cd), pure and mixed cultures of SRB showed no inhibition of growth and it was found that they were immobilising and precipitating vast quantities in the form of CdS. The importance of these biofilms in the immobilisation of metals was also recognised by Ferris and co-workers (Ferris *et al.*, 1989b), who used non-SRB microbial films. Christensen *et al.* (1996) set up bench scale experiments in cylinders with sand and stone substrata. On the addition of AMD, followed by incubation, a "black sludge" appeared and an accumulation of acetate was noted. In addition, they found that the majority of microbial activity was taking place in the stony layer rather than the "free water phase", suggesting that a surface provided a more favourable environment. It was concluded that SRB were contributing to metal removal and this technique would provide a low cost and low maintenance answer to the problem posed by AMD, providing that the sludge handling cost could be minimised. In Alabama and Colorado, metal removal from mine drainage waters was encouraged by the presence of mixed microbial mats, which due to photosynthesis, didn't require any additions such as nutrients, once again providing a low cost alternative (Phillips and Bender, 1998). On the other hand, the majority of bioremediation processes involving sulphate reduction require some form of bioreactor. A process described by Rowley *et al.* (1994) called a 'biosulfide system' does just that. It consists of two independent components, one biological and the other chemical (Clarke, 1995) and the latter precedes the first, allowing almost metal free water to enter the biological system. The biological system, containing pH and metal sensitive SRB, produces hydrogen sulphide (and alkalinity) which comes into contact with the water upon its entering into the chemical system. For optimal effect, sulphate concentration and pH must be regulated.

Combined Strategies

Passive treatments are already in operation throughout Britain such as on the River Pelenna, South Wales and Wheal Jane, Cornwall, SW England. Treatment at the Wheal Jane site involved initially, immediate and temporary strategies that were implemented to try and reduce the effects. These included lime-dosing, pumping of mine water from underground and flocculation to promote precipitation and consequential settlementation. Since then long-term abatement strategies have been proposed for the site (Lamb *et al.*, 1998) and during 1993 and 1994, a large pilot treatment plant, in the form of wetlands, was constructed downstream of the site. These included both aerobic and anaerobic systems to allow the precipitation of metal oxides/hydroxides and metal sulphides respectively but, as so many different metals were involved, three sub variants were devised, which were as follows (Lamb *et al.*, 1998; Johnson, 1998):

1. A 'lime-dosed' system – initially lime is added to bring about a pH increase before entering constructed aerobic wetlands or cells. A buried anaerobic,

organic rich (cow manure and sawdust mix) cell follows overlying a plastic liner. Here anaerobic microbial activity is promoted, not just by SRB, to remove toxic metals associated with chalcopyrite. The water then passes through a 'rock filter' to remove manganese and allow heterotrophic degradation of organic compounds thus reducing the biological oxygen demand.

2. An ALD system – as the name suggests an anoxic limestone drain is incorporated into the system prior to flow through the anaerobic cells, to encourage partial water neutralisation. The rest of the technique is as above except, for a pre-treatment of an anoxic pond once again containing organic material. This once again removes dissolved oxygen preventing deposition of iron compounds in the ALD.

3. A 'lime-free' system – is the same as the two treatments above except there is no alkali addition before the water flows through the aerobic cells.

Preliminary results from the three treatment variables are encouraging with the ALD system (2.) being the most efficient at removing metals, although it is thought that the ability for metal removal, especially iron, in the aerobic cells will increase with maturity. (Lamb *et al.*, 1998). Lamb *et al.* (1998) suggests that it is too early to evaluate the complete efficiency of the system, although passive treatment of Wheal Jane water seems viable. Although, to cope with the vast quantities of water from the mine, a larger site would possibly be needed. Whether that amount of land would be available is another problem in itself.

1.2 Wetlands for the Treatment of (Acid) Mine Drainage

Wetlands, both constructed and natural, have been used in the past to treat other polluted waters such as domestic agricultural and industrial waste-water (Knight, 1997; Corbitt and Bowen, 1994; Davies and Cottingham, 1994; Dunbabin and Bowmer, 1992; Bavor *et al.*, 1995; Cooper and Hobson, 1989). The use of wetlands for the treatment of (acid) mine drainage was possibly first noted when observing natural, volunteer wetlands in America located near 'acid seeps' (Mitsch and Gosselink, 1993). They have long been recognised as natural sinks for metals and the ability of plants to take up and store metals precedes industrial pollution and is therefore, not a new phenomenon. Engleman Jr. and McDiffett (1996) studied the effect of aluminum and iron accumulation on bryophytes found in Pennsylvanian streams affected by AMD. They noted that the accumulation of each of these metals was related to pH with the most acidic sites (pH3.5) containing the highest concentrations of iron. The use of wetlands, as opposed to chemical or biological techniques, is passive in that once the system has been constructed it requires little or no maintenance. Wetlands may, therefore, provide an inexpensive solution to the problems posed by AMD. Occasionally, this abatement strategy is seen in tandem with that of the anoxic limestone drains (ALD) which were referred to earlier (Brodie *et al.*, 1993; Nairn *et al.*, 1992).

Historically, natural wetlands covered approximately a quarter of the British Isles and until relatively recently, their importance both ecologically and as a treatment method for problematic waters has gone unnoticed (Rackham, 1995). These complex ecosystems can support a wide diversity of life including microorganisms. It is these microorganisms, through processes such as reduction of iron and sulphate and denitrification, that produce the alkali that make wetland systems so effective in reducing mine drainage acidity. It follows, therefore, that the abatement efficiency of these areas is wholly governed by the rate at which alkali can be generated. It has also been suggested that the addition of sawdust may increase the rate of sulphate reduction, which will in turn, reduce the acidity of the waters (Kalin *et al.*, 1991).

Alkali generation is also brought about by the resident moderately acid-tolerant plants that inhabit both natural and constructed wetlands. Wetland vegetation is an integral part of the wetland ecosystem. Brix (1994, 1997) reported on what he perceived to be the 'role' and 'function' of wetland macrophytes beside being aesthetically pleasing and a habitat for wildlife. The aerial plant material is thought to reduce the incidents of wetland freezing and it also reduces the wind velocity which in turn limits the re-suspension of particulate matter. But, more importantly, this aerial vegetation stores nutrients which are only released from the wetland on plant decay. The role of the sub-aqua vegetation is very different and possibly the most important, providing micro-niches for the microflora, stabilizing the sediment and acting as a biofilter, thus facilitating the treatment of certain problematic waters. Macrophytic treatment systems can decrease the severity of many contaminants such as trace metals, pathogens, trace organics, suspended solids, phosphorus, nitrogen and the biological oxygen demand (Perkins and Hunter, 2000; Reddy and D'Angelo, 1997; Kowalik *et al.*, 1995).

The type of vegetation found in natural wetlands and used in constructed wetlands is largely dependent upon climate and geographical location. The dominant vegetational type in U.K. constructed wetlands is *Phragmites* spp., but according to Kadlec (1995), the most commonly used plants are *Typha* spp. (cattails) and *Scirpus* spp. (bullrushes), although, natural *Sphagnum* spp. based wetlands have been used to treat problematic waters such as acid mine drainage (Hedin, 1997). The majority of these treatment wetlands have been specially constructed in non-indigenous wetland areas, for the specific purpose of treating problem waters. As a consequence, much research, investigates these constructed systems (e.g. Corbitt and Bowen (1994) and Kadlec (1995)). From an environmental prospective, these constructed ecosystems create new habitats for the benefit of wildlife and thus rescuing some species from the brink of extinction (Brenner, 1995; McCleary and Kepler, 1994). There are many types of constructed wetland and it is incumbent upon scientists to work alongside the wetland managers to create a system that is tailored to a given remediation programme. This may involve using specific substrates or flora to achieve the water quality targets expected and of course in designing such a system the geographical location and availability of local resources will be a major consideration because they will dictate the type of system deployed. Therefore, according to Witthar (1993), there are three categories of wetland treatment systems:

- free-water surface systems (FWS) – low velocity water flows over the substrate and through the vegetation, in shallow basins or channels;
- subsurface flow systems (SFS) – Similar to the FWS system but instead the water flows through the substrate. Though this is reported to be harder to maintain it is said to require less land (Witthar, 1993);
- aquatic plant systems – again similar to FWS but instead of over-substrate flow, the water is found in deeper ponds with submergent or floating vegetation. Therefore, this system requires a higher level of maintenance. Kadlec (1995) splits this group further into floating leaved aquatics (FAS) and submerged aquatic beds (SAB).

Wetland soil/substrate are important, in terms of chemical transformations, primary storage, cycling and vegetational support, along with providing micro-habitats and surfaces for microbial activity. Indeed, the substrate type used when constructing a wetland will greatly influence the wetlands' ability to act as an effective treatment system. Therefore, when constructing a wetland the following considerations should be made as to the soil texture (eg. sandy or clay); organic content (organic soils tend to have a higher cation exchange capacity than mineral soils); overland and through flow of the system – linked to this is the fact that if the water flows straight over the surface, there is a chance that it may remain untreated; finally the depth of substrate.

Many constructed wetlands are planted in gravel which facilitates the percolation of water to the rhizosphere where the microbial activity is high (Gersberg *et al.*, 1986). Witthar (1993) suggests that the best substrate/soil for FWS systems is spent mushroom compost or peat, though many soils have been tested in a variety of systems. Indeed some such as mushroom compost provide organic carbon for sulphate reducing bacteria, as previously discussed. In naturally occurring ecosystems, low molecular weight dissolved organic carbon (DOC) compounds are not readily available to microorganisms and thus larger ones must be broken down enzymatically into smaller molecular weight molecules, such as lactic acid, alcohols and CO_2, before they can be utilized (Chrost, 1992). Anaerobic bacteria degrade large molecular weight carbon into smaller molecular weight molecules such as lactic acid, alcohols and CO_2 thereby increasing their availability to other microbes. Methanogenesis can also occur when this CO_2 is used by methanogens as a terminal acceptor in the production of methane, which is released into the environment as 'swamp or marsh gas' (Mitsch and Gosselink, 1993). CO_2 fluxes and methane production are affected by temperature and moisture conditions (Silvola *et al.*, 1996). Indeed, when methane is found in low concentrations, those of sulphate are generally high (Valiela, 1984), as found in mine drainage and generally if sulphate is abundant, sulphate reducing bacteria will out-compete methanogens. In anaerobic soils, sulphate reducing bacteria oxidise the low molecular weight organic compounds, to produce hydrogen sulphide and bicarbonate (Hedin, 1997). But, providing an energy source for the microflora is not the only role of dissolved organic carbon in wetland ecosytems. DOC can form complexes with metals such as iron, along with some hydrophobic organics (Pinney *et al.*, 2000), which has remediation implications.

Transition metals such as iron and manganese, are found in wetlands in their more soluble reduced forms making them more readily available to the micro-fauna. Iron in its reduced form produces the effect known as gleying but it can be oxidised microbially in the presence of oxygen to the insoluble ferric form, described by Atlas and Bartha (1981) as 'bog iron' and known to be used by the early iron and steel industry.

There are two types of constructed wetland systems used in the treatment of (acid) mine drainage. The first is aerobic, promoting ferrous iron oxidation. Here emergent plant species such as *Typha* and *Phragmites* are planted into soil, although occasionally, directly onto mine tailings or other acidic spoil (Dunbabin and Bowmer, 1992). *Sphagnum* moss, which does occur naturally, has been used, but due to its toxic accumulation of iron and susceptibility to other water chemistry changes, it is usually not used (Witthar, 1993). The second type of constructed wetland creates anaerobic conditions with a capacity for reduction. These conditions are brought about with the addition of organic matter such as compost, cow manure and spent mushroom compost. In addition, organic matter is supplied by dead and decaying vegetation. To help maintain the anaerobic state, floating plants are sometimes employed to ensure that winds and storms do not cause significant aeration. The effect of storms on constructed wetlands and predominately their discharges, is reviewed by Taylor *et al.* (1993). It was found that concentrations of total iron, manganese and suspended solids, as well as pH, increased. In addition, rainfall intensity was also important, as was the flush capacity/mechanism. But these are not the only factors influencing the wetland ecosystem. There are five interrelated factors, some of which have already been described and they include aerobic status, temperature, redox potential, pH and hydrology. Redox potential, which can be measured by inserting a platinum electrode into the soil, describes the soils ability to oxidise or reduce substances. Therefore, when waterlogged organic soils are oxidised by iron or oxygen for example, a cascade of reductive processes can take place (after oxygen, nitrate is the first to be reduced then manganese, iron, sulphate and carbon dioxide), thus reducing the soils redox potential and allowing cycling processes to occur (Faulkner and Richardson, 1989). As with all the other processes described, redox potential is affected by pH (and vice versa), and soil type and its overlying waters affect pH. In addition, the wetlands' hydrology, in particular its water balance/budget, is an important factor which should be considered when discussing the biogeochemistry of a wetland. The evaporative, flooding and droughting mechanisms not only affect the chemical balance but also the micro and macro, flora and fauna.

The importance of microbes in these wetland areas in promoting alkali genesis was described earlier. Vile and Wieder (1993) compared the alkalinity produced by ferric iron reduction to that of sulphate reduction in constructed wetlands. It was concluded that sulphate reduction and iron oxyhydroxide accumulation were possibly more important during the initial stages of AMD by constructed wetlands. It was also noted that these oxyhydroxides could accumulate over time. Therefore, in the long-term the re-mobilisation of such precipitated metals should be considered because their accumulation leads to changes in the water chemistry, which in turn affects those bacteria which drive the process. Constructed wetlands provide ecological niches for many rare species and are inexpensive in terms of labour cost. These wetlands tend not to form vast quantities of sludge characterised by many other abatement programs.

1.2.1 Advantages as a Method for Abatement of (Acid) Mine Drainage

The use of wetland systems to treat water is not new and as 'clean' water is needed world-wide to sustain life and population densities are increasing, science is required to develop systems that are low cost and eco-friendly. According to Wood (1995) wetlands are gaining international interest and application as a treatment mechanism. He states that due to several high status specialist conferences approximately one thousand wetland systems were implemented during the period 1986–1994. Indeed, in developing countries their importance as a water purification system has been discussed, but unfortunately, due to the lack of aid, these wetland systems have not been executed (Denny, 1997).

Waters affected by AMD require much treatment before they are considered acceptable for human consumption or a non-threat to other environmental parameters: for example, animals, plants and other ecosystems. Singh and Chowdhury

(1999) investigated the effect of mine discharge on the use of the Indian riverine habitat, by elephants and other mammals, finding, not surprisingly, that over the study period, the animals were frequenting the regulated waters which had fewer suspended solids and were less turbid than unregulated waters. The use of these strategies therefore, increases habitat diversity and if land is reclaimed from the mining processes and companies, an environmental success is made out of an environmental disaster (Brenner, 1995).

Treatment of problematic waters using wetlands is a passive process as opposed to the active chemical ones previously described. The wetlands used, can either be natural or those constructed specifically for the purpose of treating AMD. It should be noted, that not all natural wetlands are capable of tolerating AMD and Wenerick *et al.* (1989) undertook an investigation into three types of wetland plants; *Typha latifolia*, *Sphagnum recurvum* and *Pohila nutans*, finding *Typha* spp. to be the most tolerant. Though natural wetlands are used in the treatment of AMD limitations for their use exist (Wildeman *et al.*, 1991). According to Kent (1994) this is due to the possible accumulation of humic acid thus lowering the wetlands ability to neutralise acid mine drainage. He also suggests, that the movement of water through the substrate hinders the potential for anaerobic processes to occur, as more water tends to flow across the surface. Therefore, constructed wetlands seem to be the most viable option for the treatment of mine drainage.

Wetlands constructed to ameliorate the problems posed by acid mine drainage are cost effective, aesthetically pleasing and once established, low maintenance. In an era of being 'environmentally friendly', these constructed systems have a purpose that is two fold, habitat generation and amelioration. In the treatment of acid mine drainage, wetlands can reduce acidity, metal concentrations, pathogenic organisms and trace organics (Witthar, 1993), the mechanisms by which have already been discussed.

1.3 Thesis Objectives

The main aims of this thesis are to investigate iron oxidation in mine drainage waters and attempt to elucidate the biogeochemical processes occurring in wetlands treating mine drainage waters. Therefore, the objectives of this study are as follows:

1. To determine the factor(s) that affect the rate of iron oxidation in (acid) mine drainage waters.
2. To determine the relative importance of indigenous plants (and their associated benthic microflora) in the bioremediation of (acid) mine drainage waters

3. To investigate the use of artificial (non-native) substrates in wetlands constructed to treat (acid) mine drainage waters.

4. To investigate the mechanisms by which mine drainage remediation occurs within constructed wetlands.

5. To make recommendations for the treatment of these waters.

To enable the aforementioned objectives to be addressed, the thesis is compiled as follows;

- Laboratory-based shake flask experiments were undertaken to investigate the effect of temperature, oxygen, nutrients and microbial biomass on two British mine drainage waters (chapter3).

- Bench-scale microcosms were set up to investigate varying soil type on the remediation of mine drainage waters. As a consequence a soil(s) was proposed for use in treatment wetlands and its longevity investigated (chapter 4). The universality of the proposed soil(s) was determined using waters from throughout the British Isles (chapter 6).

- Microcosms were also set up to investigate the effect of planting with indigenous flora from a natural wetland receiving acid mine drainage, located in north-east corner of Anglesey (chapter 4).

- Investigations were carried out to elucidate the biochemical processes occurring within these microcosms leading to facilitation or hindrance of mine water remediation (chapters 5 and 7).

- Finally, these resultant data were summarised and conclusions made on the viability of wetlands for the treatment of mine drainage water, leading to the proposition of a treatment system (chapter 8).

Chapter 2

Methods for Field Sampling and Laboratory Analysis

To prevent repetition, the methods and techniques detailed in this chapter are those that were common to all parts of the research undertaken. It should be noted that the research contained in this thesis is in two parts, that which investigates the microbial aspects of (acid) mine drainage, and secondly, the remediation of (acid) mine drainage using wetland systems.

Note, 'ANALAR' Grade chemicals are used throughout this research.

	Ynysarwed	Wheal Jane	Mona
pH	6.3	3.4	2.9
[Fe^{2+}] (mg l^{-1})	300	251	280
[SO_4^{2-}] (mg l^{-1})	2000	797	2250
[Cu^{2+}] (mg l^{-1})	ND	0.34	44.0
[Zn^{2+}] (mg l^{-1})	ND	48.0	60.0
[Cd^{2+}] (mg l^{-1})	ND	0.32	0.4

Table 2.1: Comparison of major physio-chemical characteristics of Ynysarwed, Wheal Jane and Mona mine drainage waters. ND=Not Detected.

2.1 Field Sampling and Analysis

2.1.1 Study Sites

There are three main study sites mentioned in this research and their locations are shown in figure 2.1. They are Ynysarwed (South Wales), Wheal Jane (Cornwall) and Mona (Mynydd Parys, Anglesey, North Wales). Despite their variance in geographical location, these discharging waters contain similar concentrations of ferrous iron (ca. 300 mg l^{-1}), but the pHs of Wheal Jane (~3.4) and Mona (~2.9) differ considerably from the more alkali Ynysarwed (pH ~6.3). A comparison of these physio-chemical characteristics of these waters is shown in table 2.1. It should also be noted, that these drainage waters, contain quantities of dissolved heavy metals such as cadmium, zinc and copper.

The Village of Ynysarwed (SN 819 018) which is located north of Neath, South Wales, is where the discharge of a disused coal mine has been flowing, via a

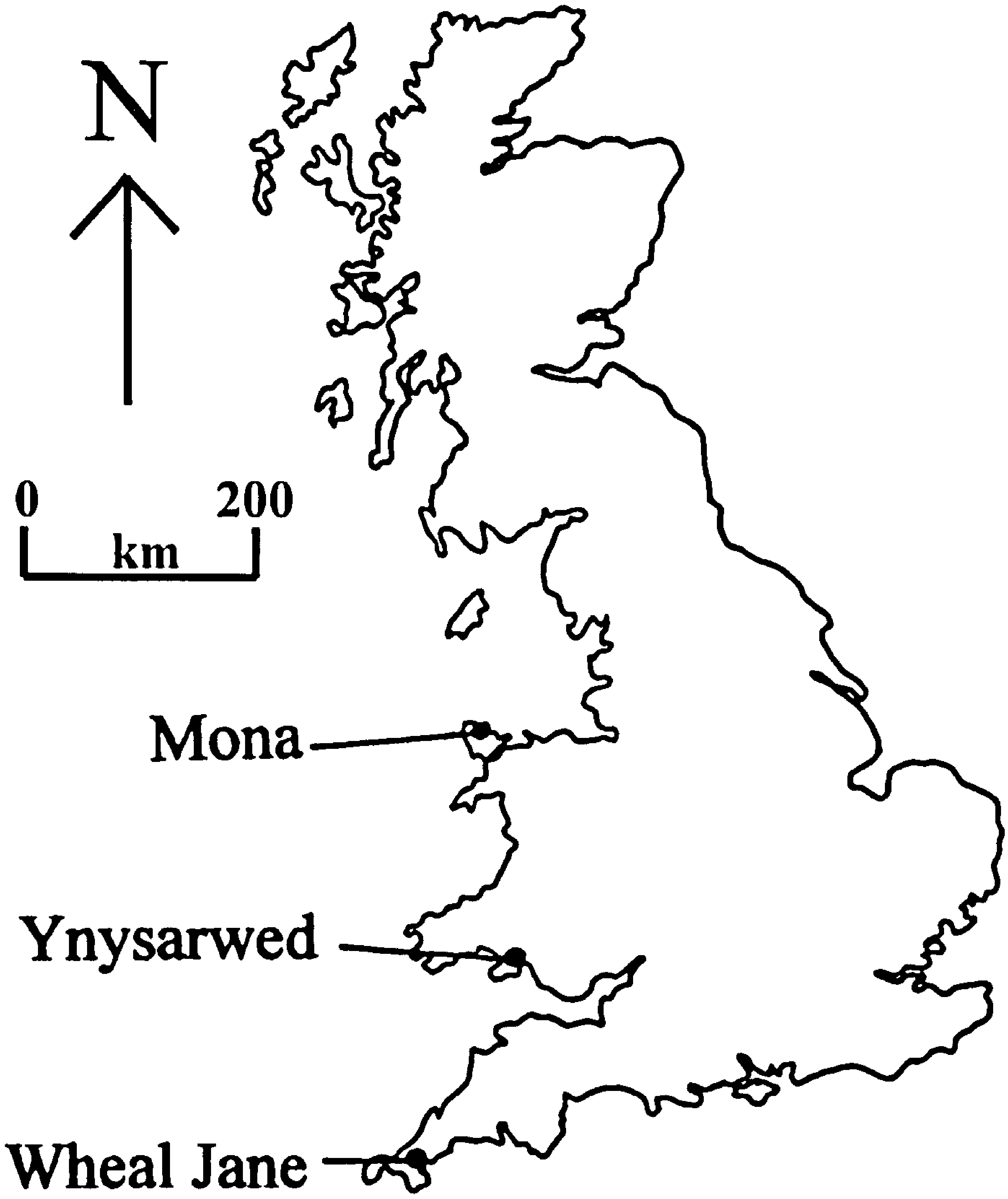


Figure 2.1: Map of Great Britain, showing the location of the mine drainage sampling sites.

ORIGINAL IN COLOUR

Figure 2.2: Ynysarwed mine water discharge adit.

partially culverted discharge channel, into the Neath Canal since 1993 (Younger, 1997). As a consequence, an ochre plume can be observed in the canal stretching for ~5km from the discharge point. The sample sites, for the first part of the research are as follows: Site 1, at the discharge point (Figure 2.2); Site 2, the confluence between the canal and the discharge source; Site 3, one kilometre downstream from the confluence and Site 4, further downstream at Tonna Lock. For the second part of the research all samples were taken from the adit/point-of-discharge at site 1.

The recently closed (1991) tin mine called Wheal Jane (SW 775 427; figure 2.3),

ORIGINAL IN COLOUR

Figure 2.3: Wheal Jane treatment works.

is located in the Cornish Valley of the River Carnon, SW England. This high profile site was brought to the public attention in 1992 when the Nangiles adit plug failed, releasing acidic ferruginous waters into the into the River Carnon and subsequently a large 'ochre' plume could be seen in the Fal Estuary. The National Rivers Authority (now the Environment Agency) immediately implemented short-term treatment of this discharge water, although now, more long-term abatement strategies have been proposed (Lamb *et al.*, 1998). Water for the present study was removed on site prior to any form of on-site treatment.

The derelict mine workings of Mynydd Parys or Parys Mountain, are located in the north-east corner of Anglesey, North Wales (SH 444 905). Historically, this area was exploited for copper ore in the 18th and 19th centuries (Rowlands, 1966). Mining ceased in this area over one hundred years ago, although the reclamation of copper from solution continued by precipitation in large ponds, a feature in this area. The waters for this part of the research were collected from Mona Adit (figure 2.4), which is part of the Mynydd Parys complex. For the second part of this research, indigenous plant samples of *Eriophorum augustifolium* were also taken from a natural wetland (figure 2.5) receiving acid mine drainage from Dyffryn Adda, which also forms part of the Mynydd Parys complex (SH 444 905 figure 2.6). This adit discharges ferruginous waters (pH 2.4) into a stream which drains eventually into the Afon Goch (north) river.

2.1.2 Field Sampling Techniques

Water samples, to be used in the experiments, were collected at each of the sites using 20 litre high-density polyethylene water carriers. These were filled to capacity to minimise iron oxidation by atmospheric oxygen, through agitation (shaking) whilst in transit, and used immediately on returning to the laboratory. It should be noted that these fresh mine water samples were used on the day of collection for

ORIGINAL IN COLOUR

Figure 2.4: Mona adit, post collapse. After this photograph was taken the adit was cleared and a support system was put in place. Note filamentous growths/streamers.

ORIGINAL IN COLOUR

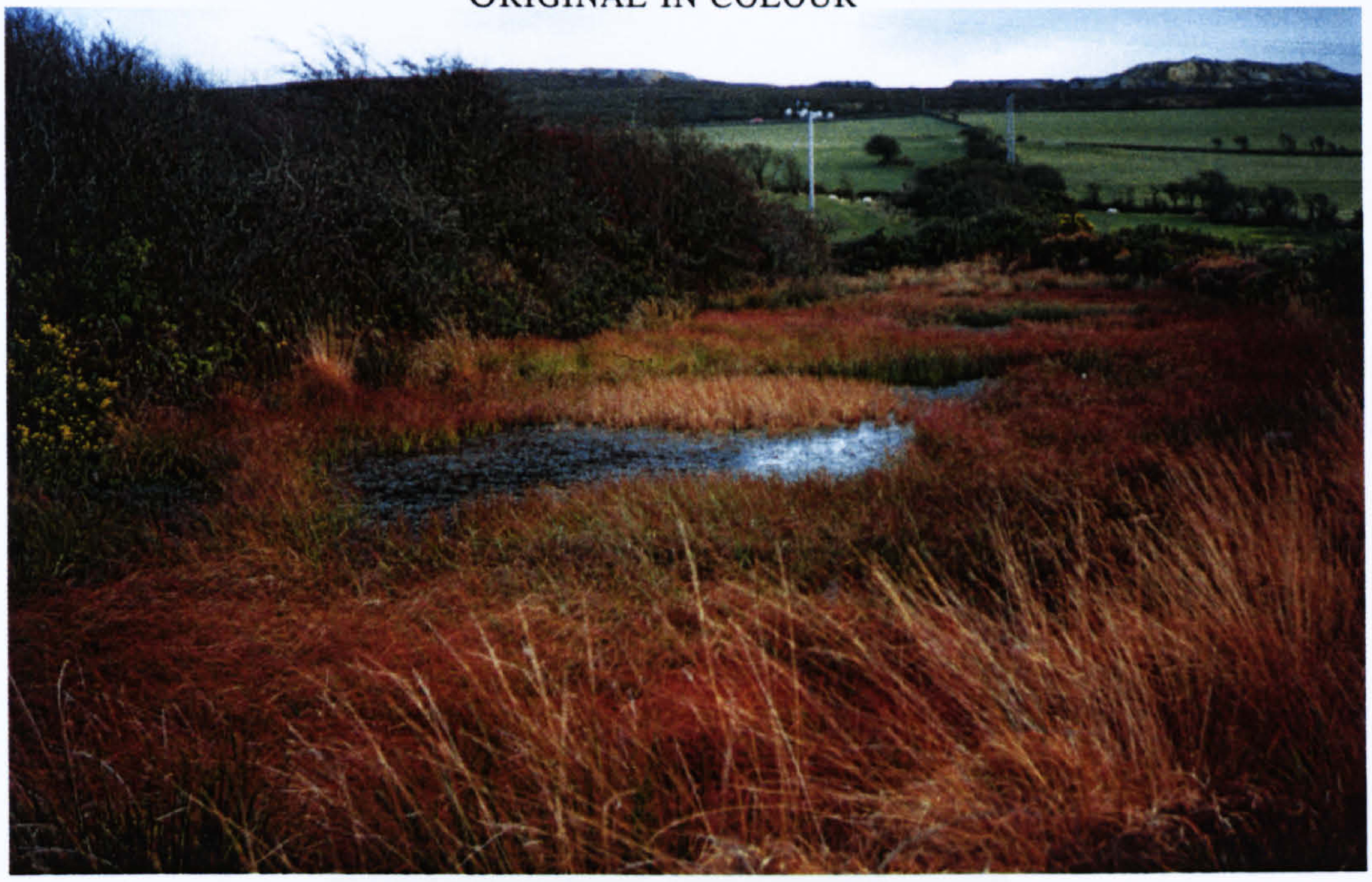


Figure 2.5: Natural wetland receiving acid mine drainage from Dyffryn Adda.

each experiment. In addition, 20ml samples were taken in sterile bottles. These smaller samples were filtered using membranes of pore size 0.2μm or left unfiltered and left un-acidified or acidified with 0.1M nitric acid. The acidification and filtering limited any biological or chemical transformations that may have occurred in the sample. These samples were refrigerated at 4°C until analysis could be undertaken. Samples from each of the sites were also filtered. This filtration technique ensures that the water is preserved from microbial activity so that accurate metal analysis could be undertaken in the laboratory.

At each site, *in situ* water analyses was undertaken to determine the relevant envi-

ORIGINAL IN COLOUR

Figure 2.6: Dyffryn Adda mine drainage discharge point.

ronmental variables. These were:

I. Dissolved oxygen, using a Whatman DO400 dissolved oxygen meter.

II. Redox potential, pH, conductivity and temperature, using Whatman water testers for the first part of the research then a Hannah HI 8424 pH meter for the second.

III. Iron, sulphate and nitrate concentrations using Merkoquant test strips. Although these are not as accurate as laboratory methods, they give an immediate approximate indication of levels.

Sediment samples were also taken for the first part of the research using sterile containers.

2.2 Laboratory Sampling

2.2.1 Aseptic Techniques

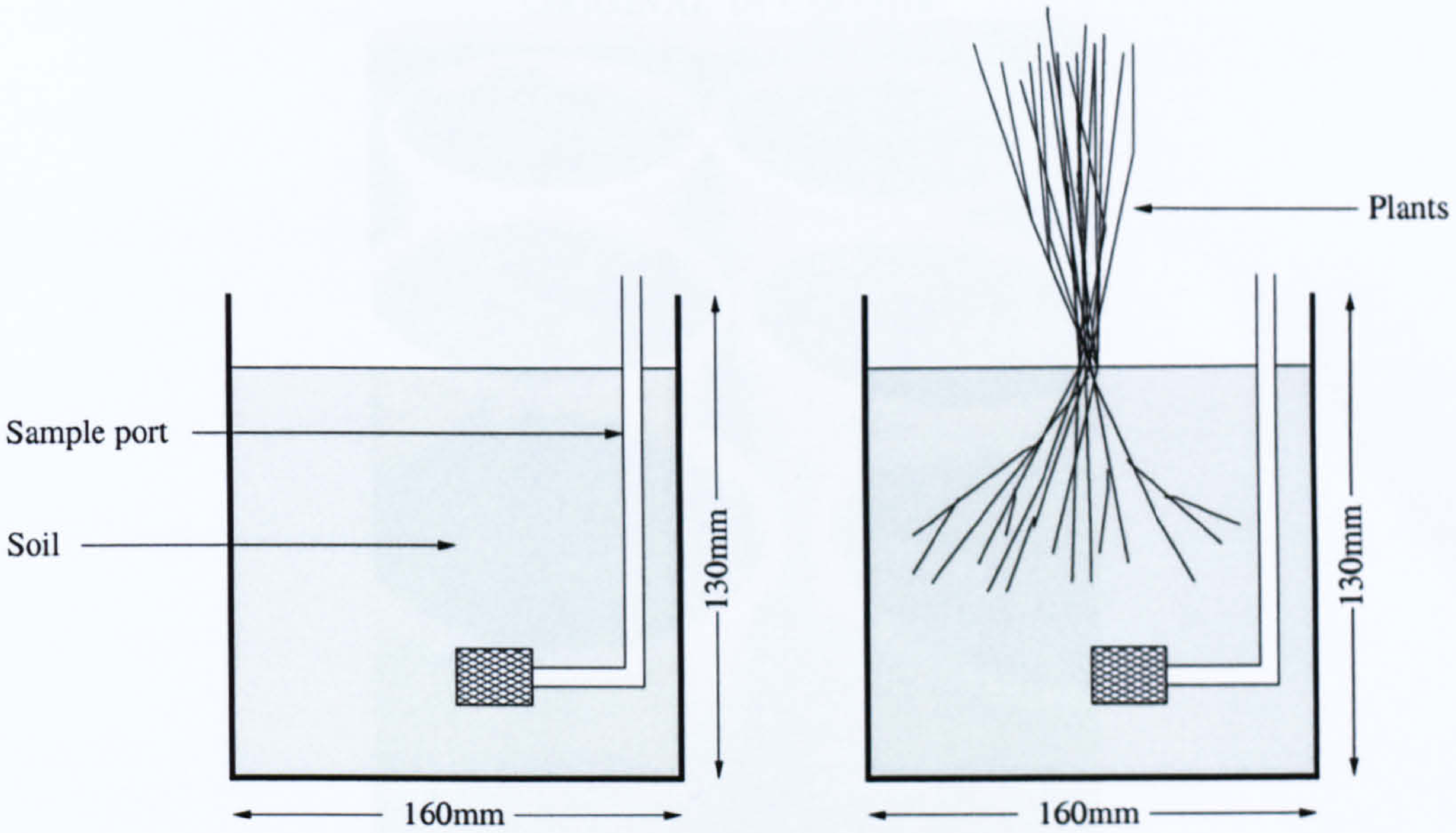
Sterilisation of apparatus and samples for the microbial part of this research was undertaken by one of three methods:

1. Metal objects such as forceps and inoculating loops were heated in a Bunsen flame until glowed red and glass rods were flame sterilised using alcohol.
2. Glassware including glass pipettes were sterilised in an oven at 160°C for a minimum of two hours.
3. An autoclave was used as a method of decontamination of plates, media and equipment after use, and also as a method of sterilisation of plastic pipette tips and media (not heat sensitive media). Aqueous samples were removed from flasks using the aseptic technique widely used in laboratories, where a sterile glass pipette or plastic sampling tip is inserted into a flask having flamed the neck of the flask prior to insertion.

2.2.2 Microcosm Set Up and Sampling Techniques

For the second part of the research, laboratory based microcosms were set up in a climate-controlled room at the University of Wales, Bangor. The temperature of which was set 10-12°C. These microcosms were constructed using 2.5 litre buckets (130mm deep, 160mm diameter) purchased from AW Gregory and Company Limited, London. Microcosms were set up as shown schematically in figure 2.7 and as in figures 2.9 and 2.8. Dry soil was placed into the buckets to a depth of 110mm. The soil was then saturated (bubbles ceased to emerge implying no air pockets) with mine drainage water, approximately 1.5 litres, leaving 4–5mm standing water on the surface. Planted microcosms were constructed in the same way with a plant addition. Plants were planted at a depth of 50mm from the bottom of the bucket, ensuring 20mm clearance from the sample port. At the end of each experiment the buckets were acid washed to remove any ferric deposits.

To facilitate water sample extraction, a sampling port was constructed using part of a syringe and flexible tubing, with a glass wool filter to prevent large particles clogging the port. This port was placed into the microcosms at a depth of 20–30mm from the bottom of the bucket. New sample ports were used for each experiment to prevent cross contamination between experiments. At regular intervals, water samples were removed from these microcosms using the sampling ports and filtered (0.2μm cellulose acetate filters 'Whatman' Catalogue Number: 6880-2502), then subsequently stored at 4°C until analysis could be undertaken.



Unplanted Microcosm Planted Microcosm

Figure 2.7: Schematic diagram illustrating microcosm set up. Not to scale.

ORIGINAL IN COLOUR

Figure 2.8: An example of planted microcosm set up, treating mine drainage water.

ORIGINAL IN COLOUR

Figure 2.9: An example of unplanted microcosm treating mine drainage water.

2.3 Laboratory Based Analysis

2.3.1 Metal Analysis

Metal analysis was undertaken using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES) to determine the concentrations of iron, copper, magnesium, aluminium, calcium, zinc, manganese, arsenic, cobalt, cadmium and lead. Appropriate standards were made using the corresponding standard solutions. Detection limits varied from one element to another and were as follows; iron 4.7ppb; manganese 6.2ppb; cadmium 4.5ppb; copper 12.9ppb; zinc 6.2ppb;

cobalt 6.8ppb and aluminum 5.5ppb.

Determination of Ferrous Iron Content

Ferrous iron content was determined in the water samples colormetrically using the 'Ferrozine' assay, adapted from Lovley and Phillips (1987). Standards of concentrations of 0–1mM Fe(II) were made using 1mM ammonium ferrous sulphate solution and acidified water (pH 2). Optical density was determined using a Cecil 292 spectrophotometer and latterly a CAMSPEC spectrophotometer.

The Ferrozine reagent was made by dissolving Hepes (Sigma H-3375) and ferrozine ((3-(2-Pyridyl)-5,6-bis(4-phenyl-sulpfonic acid)-1,2,4-Triazine) (Sigma P-9762)) in 1 litre ultra pure water (MilliQ) and adjusting to pH 7 using potassium hydroxide. This reagent was then stable if stored in the dark for up to one month.

Ferrozine reagent (0.95ml) was added to a 0.05ml aliquot of sample and mixed thoroughly. The absorbance was measured at 562nm against a distilled water blank. It should be noted that the same cuvette was used for each sample to minimise error.

It should be noted that some samples required centrifugation prior to undertaking this assay, to remove any particulate matter, in particularly ferric compounds.

Total Iron and Aluminium

This was determined using atomic absorption spectrometry (AAS). Iron and aluminium were measured using a nitrous oxide/acetylene flame at wavelengths of 248.3 and 396.2nm respectively. Suitable standards were made from Iron and Aluminium standard solutions courtesy of the Environmental Chemistry Laboratory.

2.3.2 Sulphate

The turbidimetric method was undertaken according to Rand *et al.* (1975) for the determination of sulphate. Precipitates of barium sulphate arise from the reaction between the sulphate ion and barium chloride. The turbitity produced is read at 420nm using a Cecil 292 spectrophotometer.

A 0.2ml of sample was made up to 10ml using MilliQ water, in a small beaker. To this, 5.0ml conditioning reagent (300ml MilliQ water + 30ml conc. HCl + 100ml 95% ethyl or isopropyl alcohol + 75g NaCl + 50ml glycerol, mixed thoroughly) was added and whilst constantly stirring, “a spoonful” (Rand *et al.*, 1975) of barium Chloride ($BaCl_2$) crystals (5ml spoon of 20 to 30 mesh crystals). Stirring at a constant speed was maintained for 1 minute. 1ml aliquot of this mixture was put into a cuvette and the turbidity was measured at 420nm using a spectrophotometer. Readings were taken every 30 seconds over a period of 4 minutes to obtain the maximum turbidity.

Standards of 0 – 3.0 mg l^{-1} were made using a solution of anhydrous sodium sulphate and a calibration curve produced. The amount of sulphate (mg l^{-1}) present was calculated according to the calculation provided by Rand *et al.* (1975).

In the second part of the research (chapters 4 to 7) sulphate was measured using ion chromatography (DIONEX 120), which is detailed later in this chapter (section 2.4).

2.3.3 Thiosulphate, Trithionate and Tetrathionate

The colorimetric method was undertaken as outlined by Kelly *et al.* (1969). Optical density was determined at 460nm using a Cecil 292 spectrophotometer.

Method for thiosulphate ($S_2O_3^{2-}$):

The sample for analysis (containing up to 8μmoles of thionate) was added to 4ml of NaH_2PO_4-NaOH buffer pH 7.4. MilliQ water was then added until a total volume of 10ml was obtained. This mixture was cooled to 5°C and 5ml of 0.1M potassium cyanide was added. A temperature of 5°C was maintained for 10 minutes. 1.5ml of 0.1M copper sulphate was then added, thus lowering the pH to 7.35 and the 'mixture' was maintained at 5°C, for a further 10-15 minutes. Next, 3ml of 1.5M ferric nitrate in 4N $HClO_4$ was added and agitated continuously whilst warming to room temperature. The volume was then increased to 25ml and a 1ml aliquot removed for analysis in a spectrophotometer. The optical density was read at 460nm.

Method for tetrathionate ($S_4O_6^{2-}$):

The same procedure applies as above except the copper sulphate addition was omitted and the solution was maintained at 5°C for 20 minutes instead of 10minutes prior to the ferric nitrate addition.

Method for trithionate ($S_3O_6^{2-}$):

This procedure is the same as that for thiosulphate, but after the solution was heated in boiling water bath for 45 minutes and then cooled prior to copper sulphate addition.

It should be noted that the same cuvette was used for each sample to minimise error.

2.4 Anion and Cation Analysis

Both anion (Fluoride, chloride, nitrate, phosphate and sulphate) and cation (lithium, ammonium, sodium, potassium and calcium) analysis were undertaken using ion chromatography – DIONEX 120 with an IonPac AG4A-SC 4mm (P/N 043174) and an IonPac CS12 4mm (P/N 044001) columns respectively. 31mM sulphuric acid was used as an eluent for cation analysis, whilst 1.8mM sodium carbonate:1.7mM sodium hydrogen carbonate was used for anion determinations. A flow rate of 1ml min^{-1} was used throughout. Standards were made using the DIONEX five-anion (Product Number 37157) and six-cation (Product Number 40187) standard solutions. 0.6ml of sample to be analysed was injected into the machine.

2.5 Enzyme Analysis

The widely used (Gee *et al.*, 1999) fluorogenic substrates methylumbelliferyl (MUF) β-D glucoside and MUF-sulphate were used in the present study to investigate the activity of the extracellular enzymes β-D-glucosidase and sulphatase in accordance with the procedure outlined by Freeman *et al.* (1995). In triplicate, 0.5g of the sample to be analysed was homogenised with 3.5ml of each of the substrates and incubated for 60 minutes at 12°C, with frequent mixing. After which a 1.5ml aliquot was pipetted into an eppendorf tube and centrifuged at 10000rpm for 5mins. 0.5ml of the supernatant is then added to 2.5ml of MilliQ water and the fluorescence measured at emission wavelength 450nm, excitation energy 330nm and at slit widths of 2.5 using a Perkin Elmer LS 50. For each of the samples tested calibration curves were made using 0–50μM of MUF free acid solution in a soil sample to correct for interference from phenolics and the adsorption of the product to the soil matrix (Freeman *et al.*, 1995). It should be noted that prior to the analysis, all substrates were pre-dissolved in 1.5ml methylcellosolve (Hoppe, 1983) and were freshly prepared for the assay.

2.6 Determination of Polyphenolic Substances

Determination of polyphenolic substances was undertaken using Folin-Ciocalteau phenol reagent and the method outlined by Box (1983). A saturated solution of sodium carbonate (200 g/l) was prepared freshly prior to the analysis, along with phenol standards of 0.1–1.0 ppm. 0.15ml of alkali and 50ml of Folin-Ciocalteau phenol reagent was added to 1ml of the sample to be analysed and shaken well. It was then left for 1 hour for the colour to develop. The absorbance was measured at 750nm using a spectrophotometer using a blank containing MilliQ water instead of sample water. A calibration curve was constructed from the standards to allow the sample concentrations to be calculated. It should be noted that the same cuvette was used for each sample to minimise error.

2.7 Carbon Analysis

Analysis of total carbon (TC), inorganic carbon (IC) and subsequently total organic carbon (TOC) was measured using a Shimadzu TOC analyser (TOC-500). 40ppm standards were made using potassium hydrogen phthalate (TC) and sodium hydrogen carbonate (IC).

2.8 Molecular Weight Analysis

Determination of the molecular weights of dissolved organic carbon, was undertaken in the laboratory, using high performance liquid chromatography (HPLC - Cecil 1100) at a detection level of 254.0nm, with an injection volume of 150μl. A Tris-HCl buffer was used (pH 7.5). The column was heated to 35°C to increase column efficiency and reduce variance between chromatograms with fluctuating laboratory temperature (Laboratories, 1994).

2.9 Plating and Liquid Culture

2.9.1 Solid Media

To determine the indigenous types of microbial populations and their population densities, the water samples from each of the sites were plated on a variety of appropriate solid media at acidic and neutral pHs. These included iron and sulphur overlays, WAYE (Washed Agarose Yeast Extract), ferrous iron/thiosulphate plates using both washed and unwashed agarose and the recently developed ferrous iron/thiosulphate overlays. This facilitated the selection for acidophilic bacteria, neutrophilic bacteria, iron oxidisers and heterotrophs etc. The protocols for these plates have been detailed elsewhere (e.g. Johnson (1995b)), though the development of the new iron/thiosulphate plates (pH 6.5) was designed specifically for this research by myself and Dr. Johnson. This involved adding 0.99g sodium thiosulphate ($Na_2S_2O_3$) to the media prior to it being autoclaved and the washing of the agarose (referred to Fe/thio hereafter). The colonies were then classed according to their morphology for example, those with ferric deposits (iron oxidising bacteria) and those which were not (heterotrophic bacteria).

Plates were incubated at 30°C unless otherwise stated and aerobic, microaerobic and anerobic conditions were simulated. Microaerobic and anaerobic conditions were created using Oxiod 'CamyGen' and 'AnaeroGen' sachets and indicators in 'Oxoid (AG25) AnaeroJars'.

2.9.2 Liquid Media and Liquid Culture

Liquid medium was used for isolation of possible novel stains and their subsequent culture. This was initiated from a single colony found on a plate and transferred into a universal bottle containing the appropriate media. Following complete oxidation, it was transferred to a conical flask, containing the corresponding media. All were incubated at 30°C until complete oxidation was observed. Liquid media for the maintenance of the novel isolates was ferrous iron/ thiosulphate, pH4.

The use of porous recycled glass balls (Poravor) in the culture of isolates was being investigated prior to change in research direction. These balls offer a large surface area over which a biofilm can develop.

Acridine Orange direct counts were used to enumerate and calculate the plating efficiency of acidophilic bacteria. This was undertaken using the protocol outlined by Hobbie *et al.* (1977). Samples were filtered through a black polycarbonate membrane filter and fixed with 4% gluteraldehyde for 10 minutes. They were then stained with 0.01% acridine orange for 6 minutes before rinsing with 5ml of MilliQ water and being allowed to air dry. The membrane filter was then mounted on a glass slide with immersion oil and viewed under a fluorescence microscope at ×1000 magnification. The bacterial counts were estimated for a minimum of 20 randomly chosen fields of view and a minimum of 200 cells. The total number of bacterial cells per ml of culture could therefore, be calculated as follows:

$$n = \frac{xAd}{va}$$

where: x - average count per area

A - effective filtration area of membrane

d - dilution factor

a - graticule area

v - volume of sample used

n - number of bacterial cells per unit volume

Note all of the reagents must be filtered through 0.2μm prior to use.

2.9.3 Microscopy

Phase contrast microscopes were used to examine bacterial cells from liquid culture and plate colonies enabling their morphology, density and spore presence/absence to be noted.

More detailed examination of cell morphology and size was undertaken using a scanning electron microscope (SEM). Bacterial cultures were prepared as follows for scanning electron microscopy. The volume of culture used is dependent upon the cell density, which can be determined prior to this procedure using a Thoma counting chamber. For a cell density of 10^7 ml^{-1}, for example, 1.0ml of culture is required.

A Swinnex unit with a 0.2μm Nuclepore (12mm diameter) membrane was set up and the assembled unit was then flushed using a syringe containing 2ml 0.1M H_2SO_4. 5ml of basal salts (Appendix B) was placed into the syringe, whilst connected to the unit, along with the microbial sample, which was then pushed through the membrane. The membrane was then flushed with the following sequence – 1ml 0.1M H_2SO_4, 3ml basal salts and 3ml of MilliQ water in that order. The samples were fixed using 4% glutaraldehyde and then dehydrated using the following sequence of ethanol solutions - 30%, 60%, 90% and 100% (× 2). For each of the fixation and dehydration steps, 3ml of solution was placed in the syringe. 1 ml was then pushed through the membrane, leaving the remaining 2ml as a reservoir whilst the process was held for 10–15 minutes. After this time, the remaining solution was pushed through the membrane. Following the final flush, the membrane was removed and critical–point drying was undertaken. The sample was then mounted on a SEM stub, coated with gold using a sputter coater and viewed.

2.10 Statistical Analysis

In the majority of cases, the data presented in this thesis was produced through the sampling of particular observables over the time-course of a given experiment. Statistical analysis of this data was necessary to qualitatively address the answers to the following:

- Between a given set of experiments, do the particular experimental variables significantly affect the population distributions of an observable over the duration of the experiment?
- Given the time-resolved data, can the observed value(s) taken on a particular day be completely attributed to the experimental variable, over that of random fluctuations?

To address the first question, a one-way analysis of variance (ANOVA) was undertaken to ascertain whether the population distributions of the data sets were significantly different. In response to the second, it was necessary to adopt a multi-factorial approach, as the fluctuations in the value of the observable between sampling intervals needed to be attributed to fluctuations from day to day, in addition to experimental factors, rather than random error. Therefore, two or three way factorial ANOVAs were employed, with day attributed to one of the factors in order that this criterion is fulfilled. In order to identify which experimental variables lead to significantly different observations, *post hoc* (least significant difference) analysis was undertaken. The results of this test are reported according to convention, where the results are arranged in ascending order of mean and then lines are drawn under groups that are not significantly different (Dytham, 1990).

To calculate the significance of percentage change in the observables, a t-test was undertaken using Instat version 2. This enables the significance difference in the means to be calculated, taking into account natural variability within the data.

Statistical analysis was conducted on the data contained in this thesis, using SPSS UK version 10, Instat version 2, Kinnear and Gray (2001) and Dytham (1990). Statistical tables found in Fowler and Cohen (1990) were used to determine the significance of some of the tests.

Chapter 3

Factors Affecting the Rate of Iron Oxidation In Ynysarwed and Wheal Jane Mine Drainage Waters

In chapter one, the environmental problems associated with the metalliferous discharges from disused mines were discussed along with the mechanisms by which iron oxidation takes place, be they biotic or abiotic. It is thought that the oxidation of ferrous iron is the rate limiting step in the treatment of acid mine drainage (Kirby *et al.*, 1999) and proposed remediation strategies should involve controlling this oxidative process (Singer and Stumm, 1970; Maree *et al.*, 1998). Therefore, it was pertinent to investigate those factors, which affect the rate of iron oxidation. For this purpose waters were collected from Ynysarwed (S. Wales) and Wheal Jane (S. England). Conical flasks were set up containing the aforementioned acid mine drainage waters thus allowing the following parameters to be investigated. These were aerobic status, temperature, inorganic nutrient availability and bacterial numbers.

Some of the findings detailed in this chapter have been presented in Dennison *et al.* (2001) a copy of which can be found in appendix A.

3.1 Iron Oxidation

3.1.1 Materials and Methods

Mine drainage water was collected and treated prior to the analysis detailed in chapter 2 section 2.1.2. An initial investigation was undertaken to determine the physico-chemical variables of Ynysarwed and Wheal Jane waters. A shake flask experiment (shaken at 100rpm), using sterile one hundred millilitre conical flasks, containing water samples from the Ynysarwed adit and Wheal Jane, was set up in such a manner as to allow a regular small aliquot of sample to be removed for ferrous iron analysis using the ferrozine assay (see section 2.3.1) and pH determination. Flasks were set up to enable the effect of the following biotic and abiotic factors to be investigated:

- Temperature – flasks were incubated at 10°C, 20°C, 30°C and 37°C.
- Oxygen – flasks were set up using appropriate volumes of water to create conditions denoted as aerobic and "microaerobic". To test the effect of anaerobic conditions, serum bottles were used. These bottles were also filled with an appropriate amount of mine drainage water to create conditions denoted as anaerobic and were purged with nitrogen gas after samples were removed for analysis, thus maintaining anaerobic conditions.
- Nutrients – 0.5ml of nutrient supplement was added in the form of basal salts made to departmental formulation (see appendix B).
- Bacteria – 1ml of indigenous Cae Coch bacterial suspension was added to each of the flasks as a source of acidophilic iron oxidising bacteria. Cae Coch (SH 775 653) is an extensively studied disused sulphur mine located in the Conwy Valley, North Wales (Johnson *et al.*, 1992). One millilitre filtered Cae Coch water additions (0.2μm) were made to the Ynysarwed and Wheal Jane waters as a control. This practice removes the bacteria present in the water, whilst ensuring that there are no discrepancies in water

chemistry e.g. dilution or pH.

Appropriate controls i.e. filtered and unamended samples, were also set up in each case, to generate data for comparison with the experimental data. All flasks were incubated at 20°C unless otherwise stated.

At the end of each experimental period, water samples were serially diluted and plated onto appropriate solid media as outlined in section 2.9 and incubated aerobically. A water sample from the start of the experiment was also plated in the same manner for comparison.

3.1.2 Results

Table 3.1 summarises the major physico-chemical and biotic variables of the Ynysarwed and Wheal Jane discharge waters. It can be seen from this table that the discharging water for Wheal Jane (pH 3.4) is lower than that of Ynysarwed (pH 6.3) and is more comparable in its acidity to Ynysarwed Site 4 (Tonna Lock, pH 3.5). The concentration of dissolved iron was found to be similar at Wheal Jane to that at Ynysarwed. This latter location also exhibited a decrease in concentration of iron, sulphate and manganese with distance from discharge, although there was a slight increase in the concentration of aluminium. It should also be noted that despite all precautions been taken, during the transportation of Ynysarwed water, 50% of the water oxidised. Water obtained from Wheal Jane was found to be more stable during transportation and storage (at 4°C) for under two weeks. The analytical results also show that the sulphate concentrations in the Wheal Jane water were more comparable with the sites further down stream from the Ynysarwed discharge point than at the adit. It is important to note that although there was a distinct smell of H_2S (hydrogen sulphide), at Ynysarwed adit, no sulphide was detected during water analysis. Table 3.1 also shows that Wheal Jane and Ynysarwed Site 4 have higher numbers of iron-oxidising neutrophilic bacteria and acidophilic heterotrophic bacteria then any of the other sites sampled. In the case of Ynysarwed, these bacterial numbers increase with distance from discharge.

The results of an iron oxidation experiment using the water collected from the adit at Ynysarwed (site 1) containing low numbers of indigenous microbes (table 3.1), when amended with acidophilic iron-oxidising bacteria from Cae Coch (Cae Coch AMD) and after the addition of inorganic nutrients, are shown in figure 3.1. On each sampling occasion (day), the unamended mine drainage water, which was functioning as a control, showed ferrous iron concentrations which did not vary significantly from the twelve-day sample mean (125.14 ± 4.53). Therefore, no net iron oxidation is observed in the unamended mine water over the

	Ynys Site 1	Ynys Site 2	Ynys Site 3	Ynys Site 4	Wheal Jane
pH	6.3	6.3	6.1	3.5	3.4
Temp. (°C)	14.1	14.5	15.35	15.8	18.0
Eh (mV)	+213	–	–	+428	+462
Conductivity (μS cm^{-1})	1926	1423	1122	149	ND
DO (mg l^{-1})	4.97	7.53	6.11	5.97	6.88
[Fe^{2+}] (mg l^{-1})	300	101	84	16	251.4
[SO_4^{2-}] (mg l^{-1})	2000	1620	960	560	796.8
[Al^{3+}] (mg l^{-1})	23	23	ND	27	ND
[Mn^{2+}] (mg l^{-1})	8	7	ND	4	ND
[$S_4O_6^{2-}$] (μg l^{-1})	17.68	ND	ND	ND	ND
[$S_2O_3^{2-}$] (μg l^{-1})	UD	ND	ND	ND	ND
Fe^{2+} oxidising acidophilic bacteria (/ml)	<10	<10	<10	<10	3.10×10^4
Fe^{2+} oxidising neutrophilic bacteria (/ml)	<10	<10	<10	640	2.95×10^4
Acidophilic heterotrophs (/ml)	<10	<10	10	140	217
Neutrophilic heterotrophs (/ml)	<10	1.26×10^4	3.9×10^3	1.0×10^4	3.4×10^3
Fungi (/ml)	<10	<10	<10	<10	<10

Table 3.1: Analytical data summary of major physico-chemical and biotic variables of the Ynysarwed (Ynys) and Wheal Jane waters obtained during site visits between September 1997 and October 1998. Key: ND – Not Determined; UD – Undetectable. Due to the change of tutor, these samples were not analysed until much later. As a consequence, some of the samples had degraded and were unable to be analysed, whilst of those that were analysed – some of the analytical data was not made available to me.

experimental period. This was also observed over the first two to four days in amended mine water experiments. However, these experiments then exhibited a net decrease in ferrous iron concentration over the remainder of the experimental period. The most marked decrease in ferrous iron concentration was observed following the addition of inorganic nutrients to Ynysarwed mine water, resulting in almost complete ferrous iron removal (<0.05 mg l^{-1}) within 7 days. In flasks containing filtered and unfiltered Cae Coch additions, 8 days was required to yield a similar, low, ferrous iron concentration. A two way/repeated measures ANOVA proved that there was no significant difference between the ferrous iron concentration in those experiments amended with filtered or unfiltered Cae Coch water. Once a low concentration (<0.05 mg l^{-1}) is achieved in all amended experimental flasks, it is maintained over the remainder of the experimental period. It is therefore evident that iron oxidation is occurring in these experiments, with the fastest rate being observed following the addition of inorganic nutrients.

In a parallel experiment using Wheal Jane mine water which naturally contains high microbial numbers (table 3.1), the unamended water did not show stability about a constant value and consequently the variation about the sample mean (90.78 ± 97.03) is much greater than that observed at Ynysarwed. Over the twelve-day experimental period (figure 3.2), a net decrease in ferrous iron concentration was observed, reaching similar low concentrations to those observed in amended Ynysarwed mine water by the twelfth day. Thus, gradual iron oxidation was observed in unamended Wheal Jane mine water over the duration of the experiment. This phenomena was enhanced in the amended mine drainage water, in a similar fashion to that observed in Ynysarwed mine waters, with the most marked decrease apparent in mine water amended with inorganic nutrients. Complete iron oxidation was observed within three days, which was much faster than that in the corresponding experiment using Ynysarwed mine water. In comparison, the addition of filtered or unfiltered Cae Coch mine drainage water resulted in complete ferrous iron removal within 8 days. Once again, a two-way/repeated measures ANOVA on these two experiments, showed that there was no significant difference between the ferrous iron concentrations. Similarly, once low ferrous iron concentrations are achieved, as at Ynysarwed, these are maintained for the remainder of the experiment.

Over the twenty-day experimental period to investigate the effect of temperature, iron oxidation in Ynysarwed water was shown graphically to be most rapid at 30°C (figure 3.3). However, statistical analysis showed that there was no significant difference in the mean iron concentration on day 14 (complete iron oxidation was observed at 30°C) between flasks incubated at 30°C and 37°C. In the interests of clarity, the filtered samples were omitted from this figure as temperature seemed to have very little effect on the rate of iron oxidation (fluctuations about a

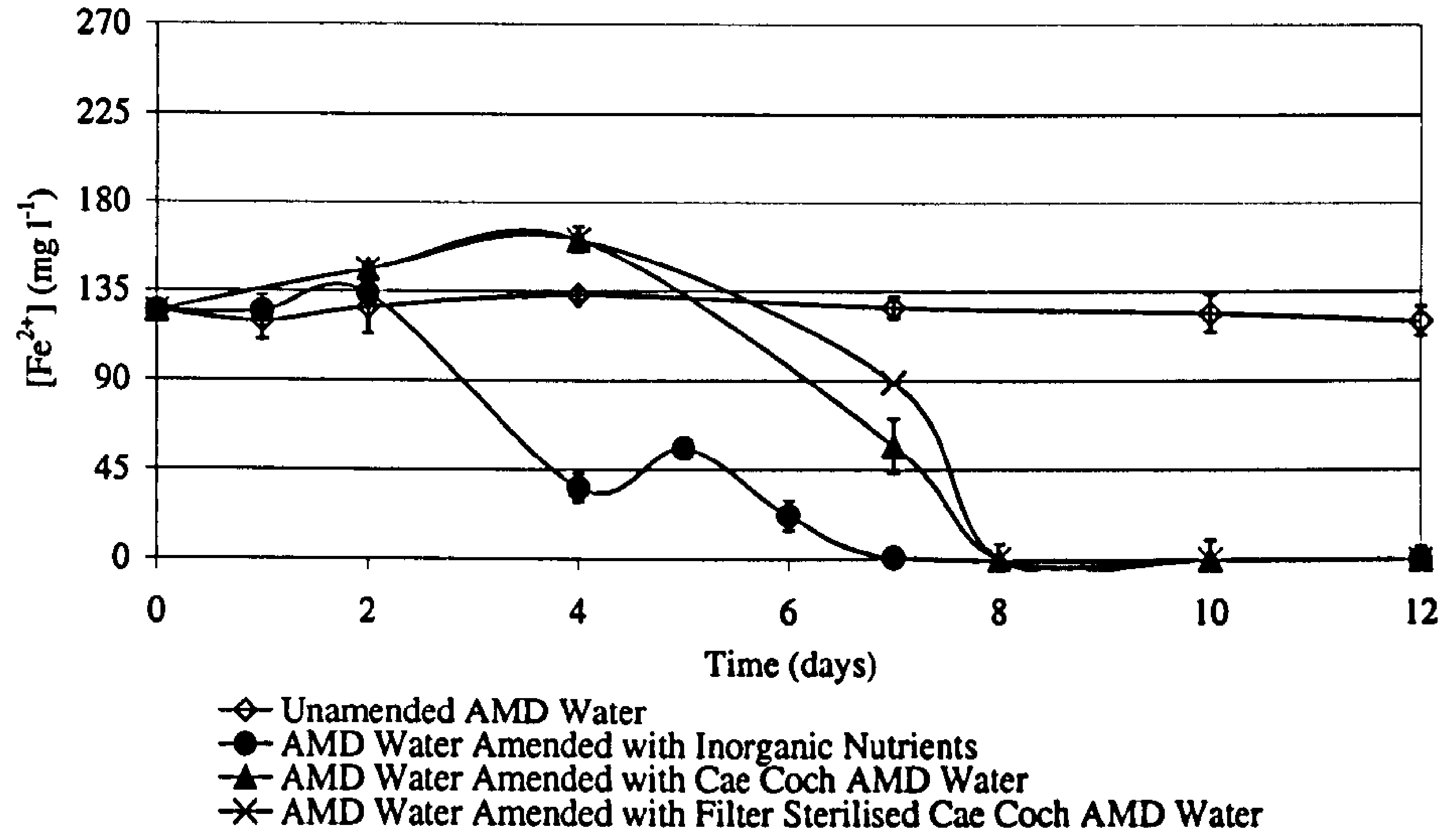


Figure 3.1: Iron oxidation in Ynysarwed after the addition of aqueous inorganic nutrients and Cae Coch indigenous bacterial suspension. Mean±s.e., n=3.

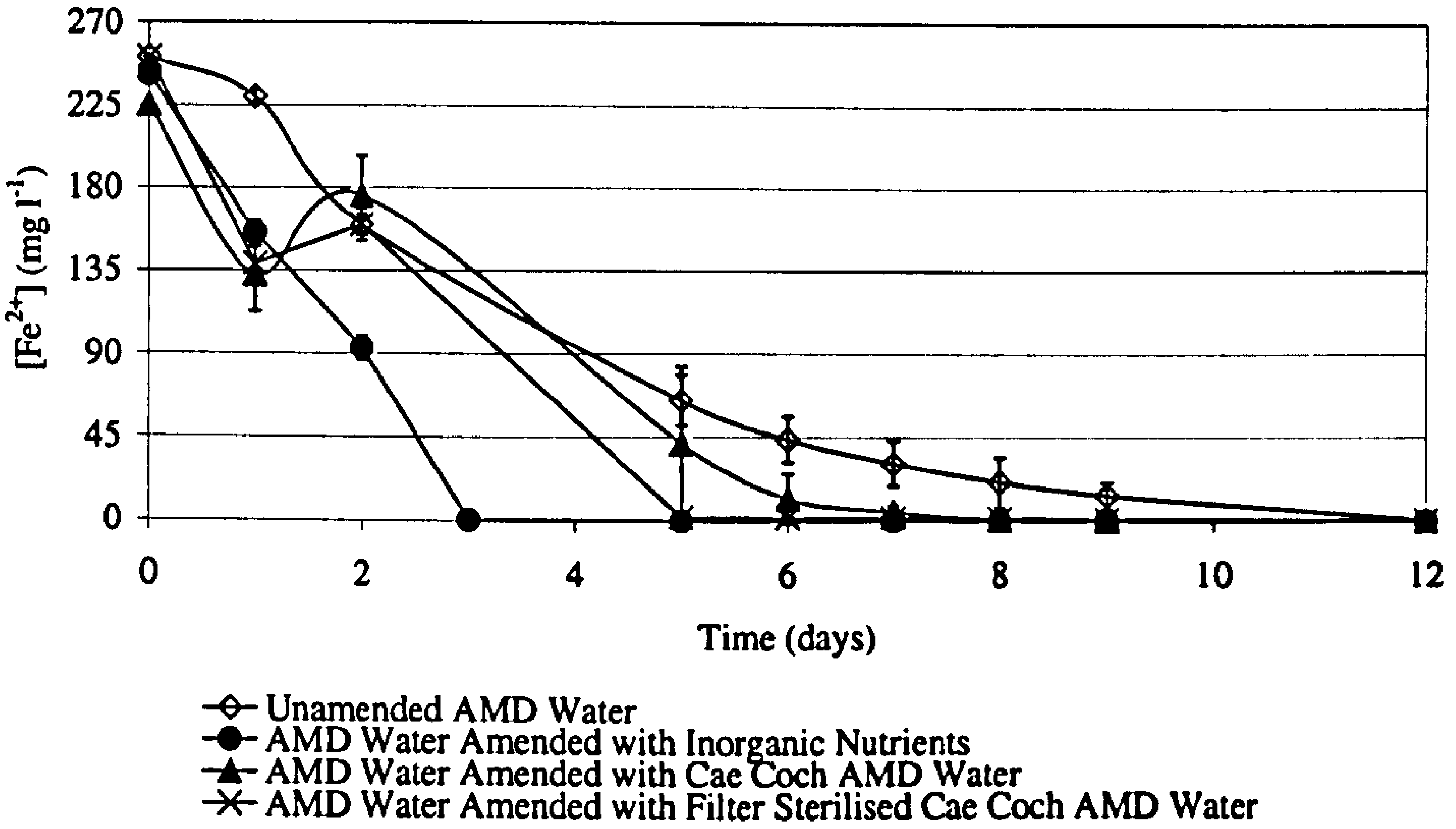


Figure 3.2: Iron oxidation in Wheal Jane water after the addition of aqueous inorganic nutrients and Cae Coch indigenous bacterial suspension. Mean±s.e., n=3.

Treatment	Mean Rate of Iron Oxidation (mg l^{-1} hr^{-1})	
	Ynysarwed	Wheal Jane
10°C	0.24 ± 0.01	0.73 ± 0.02
20°C	0.46 ± 0.26	1.46 ± 0.17
30°C	0.82 ± 0.26	2.83 ± 0.11
37°C	0.63 ± 0.21	4.96 ± 0.38

Table 3.2: Comparison of iron oxidation rates, expressed as mg l^{-1} hr^{-1}, for Ynysarwed and Wheal Jane waters with respect to temperature (mean ± s.d., n=3). Rate was determined from the gradient of the line of best fit for iron concentration, with respect to time.

mean of 124.27 ± 2.1), with no significant difference being observed between the filtered samples over the experimental period using a repeated measures ANOVA. At 10°C, which was used to mimic the natural environmental temperature, approximately half of the total iron was oxidised during the experimental period. Continuation of the 10°C experiment showed that full oxidation did not occur until 54 days. However, in the Wheal Jane water (figure 3.4) iron oxidation was more rapid at 10°C and full oxidation had occurred after 22 days. It can be seen from figure 3.4 that the optimal temperature for the most rapid rate of iron oxidation in Wheal Jane water, was 30°C. Here, full oxidation was reached at 5 days compared to the 14 days exhibited in the Ynysarwed water. Once again, the mean iron concentration at this temperature was not found to be significantly different from that obtained at 37°C. Analysis of the reaction rates shown in table 3.2 shows that 37°C has a faster rate of iron oxidation in Wheal Jane water.

The iron oxidation rates for Ynysarwed and Wheal Jane waters with respect to

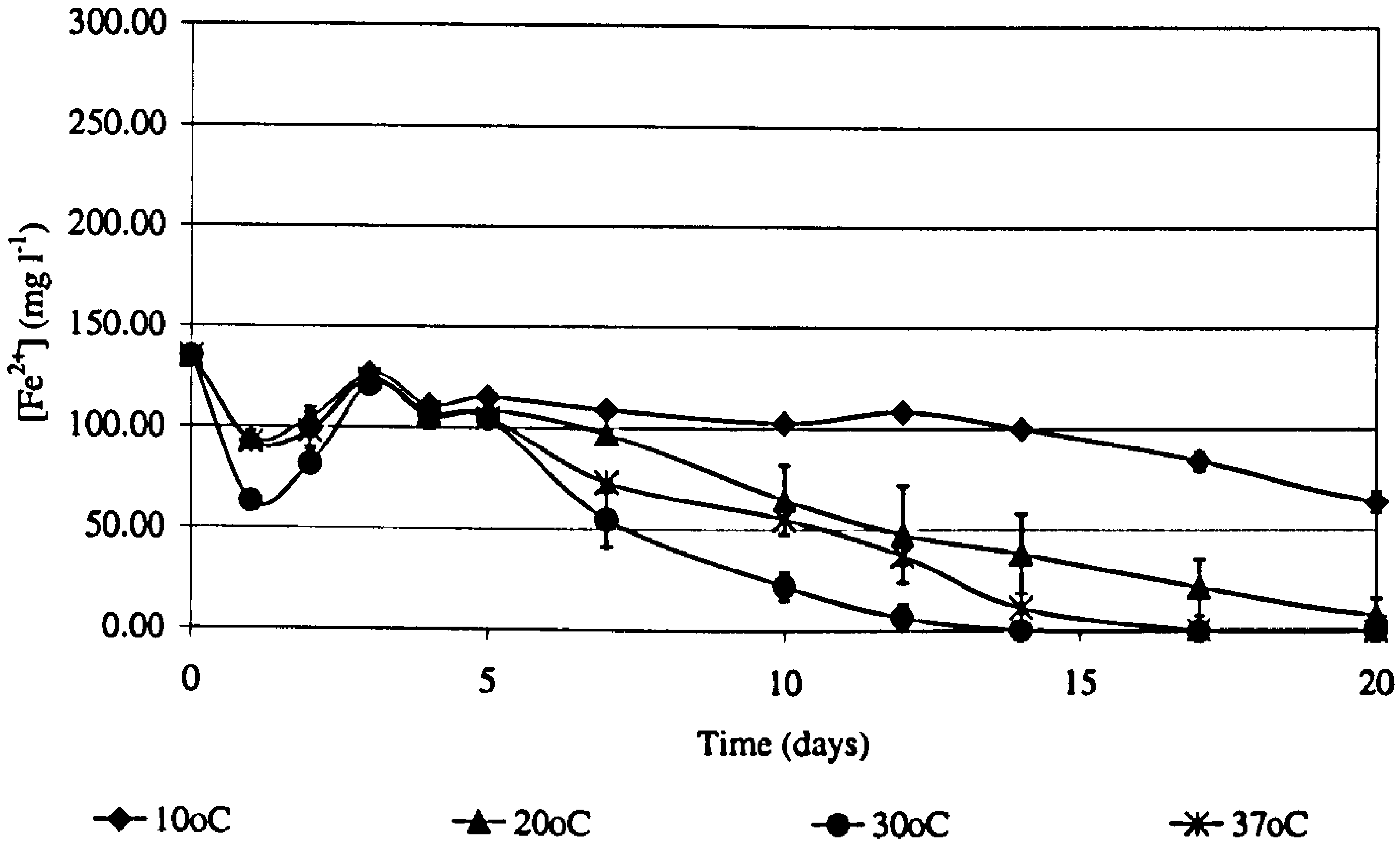


Figure 3.3: The effect of temperature on iron oxidation in Ynysarwed water. Note: filtered samples have been omitted in interest of clarity (see text). Mean±s.e., n=3.

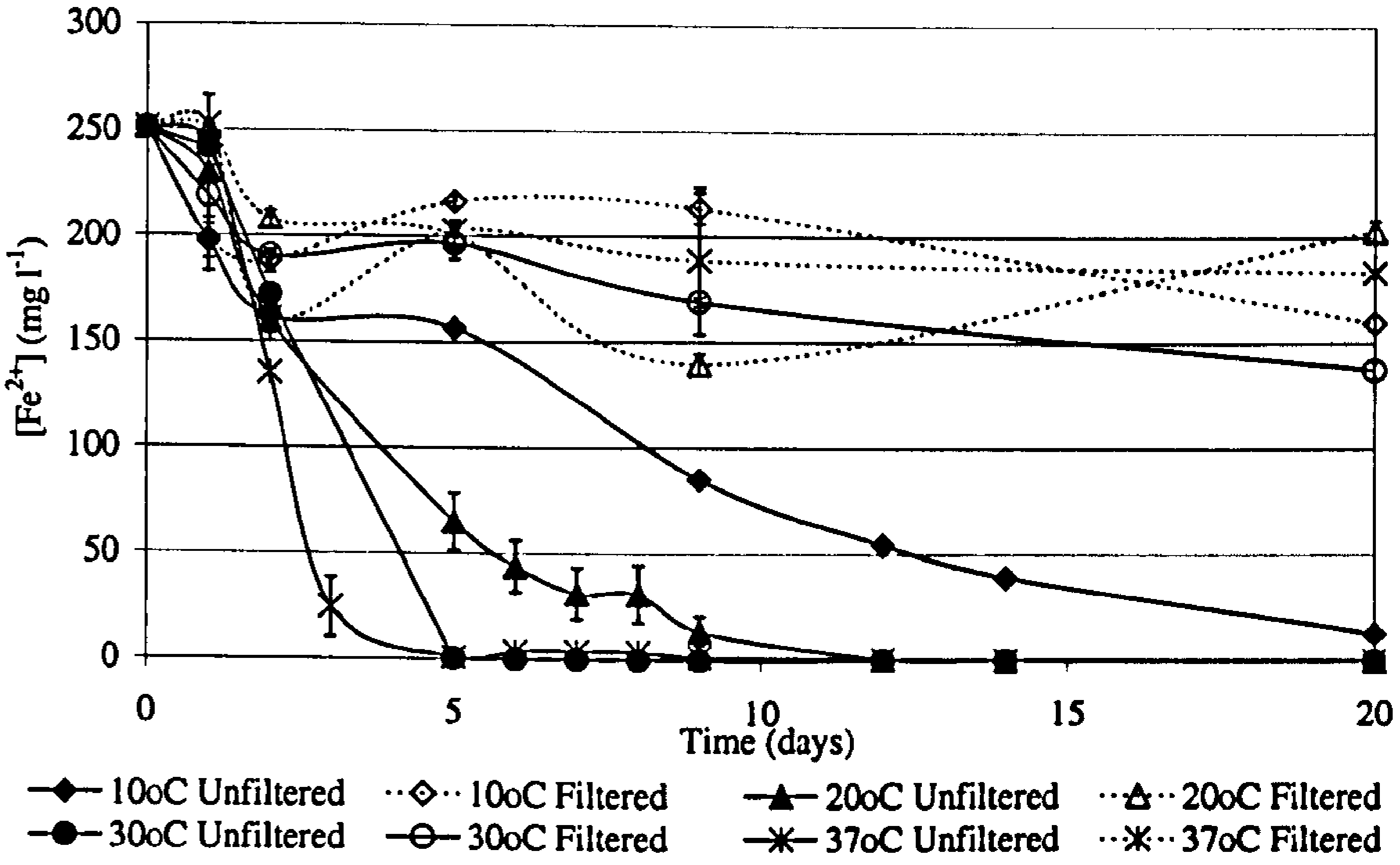


Figure 3.4: The effect of temperature on iron oxidation in Wheal Jane water. Mean±s.e., n=3.

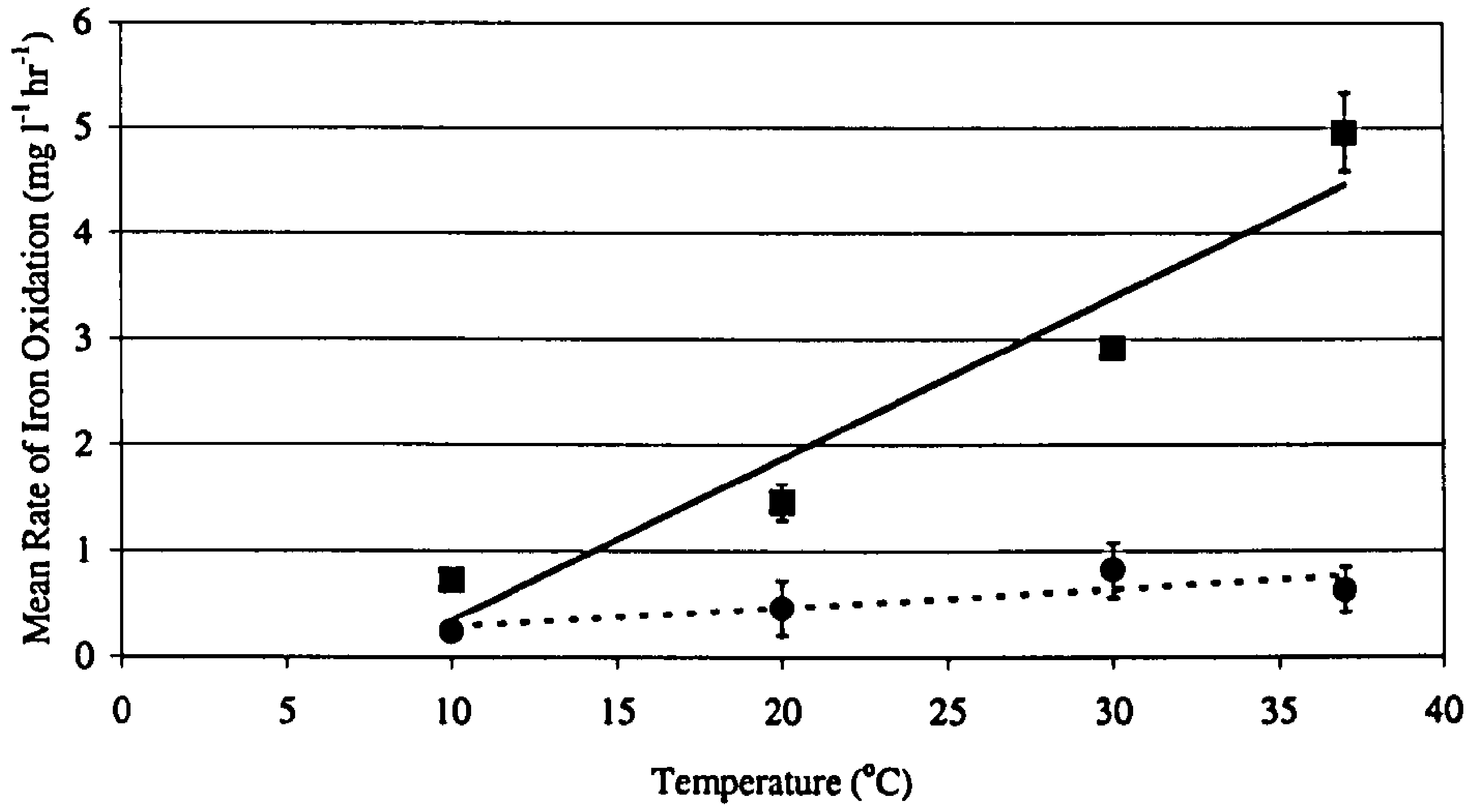


Figure 3.5: The relationship between iron oxidation rate and temperature exhibited by waters obtained from Ynysarwed (circles, broken line of regression)and Wheal Jane (squares, solid line of regression). Mean±s.e., n= 3.

temperature, are compared in figure 3.5. It can be seen from this figure that the relationship between temperature and reaction rate for Ynysarwed and Wheal Jane waters is linear with the coefficient of determination (R^2) values R^2=0.7132 and R^2=0.9244 respectively, which indicate that 29% and 7.56% of the variation in reaction rates is not accounted for by temperature. If the product moment correlation coefficient (r) is analysed, it is found that these values of 0.8445 and 0.9615 respectively exceed the tabulated value at 11df of 0.684 at P=0.01 and are therefore, highly significant.

Temperature	Temperature Coefficients	
	Ynysarwed	Wheal Jane
10 - 20°C	1.9	2.0
20 - 30°C	1.8	1.9
30+°C	1.0	2.0

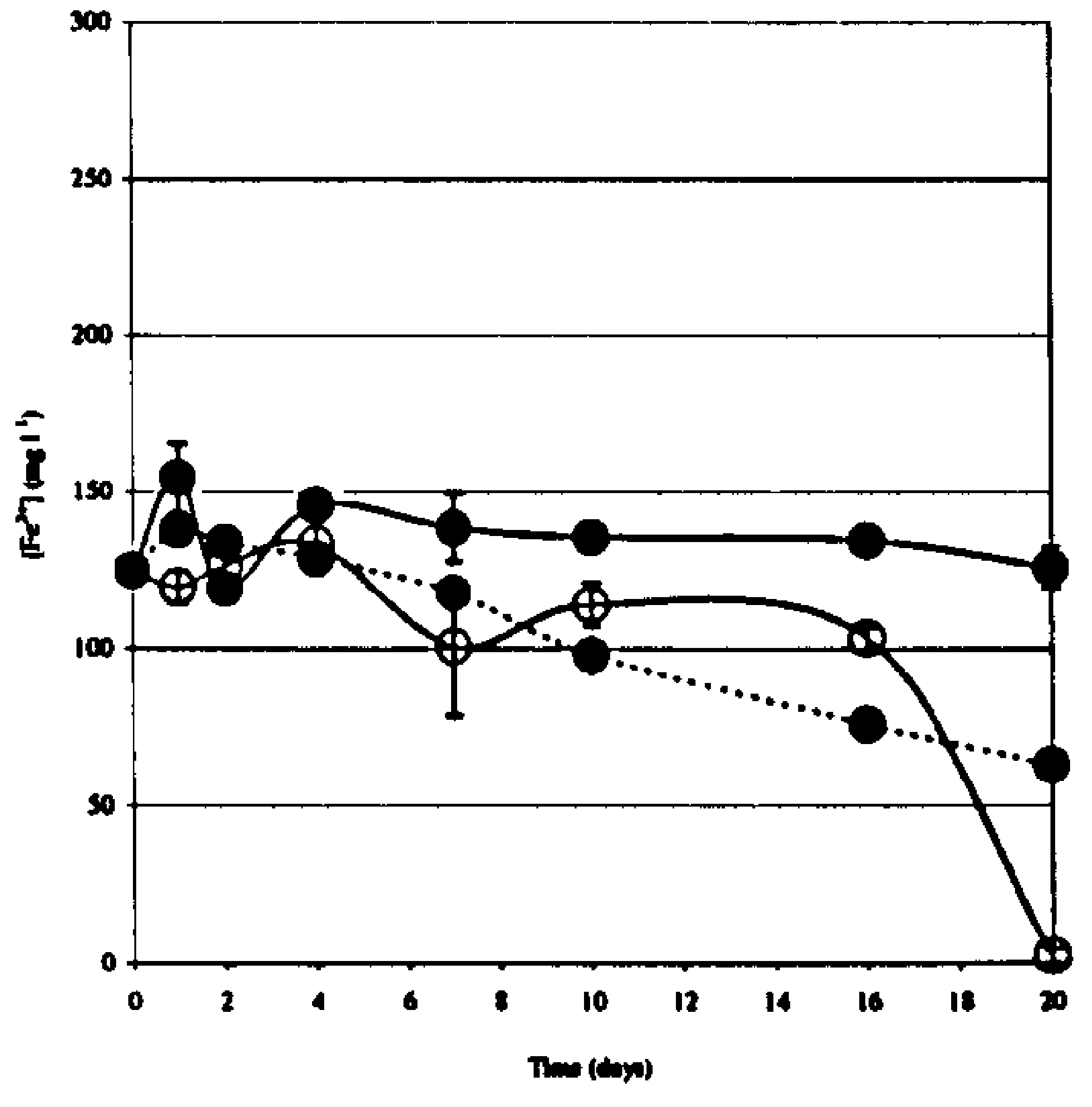
Table 3.3: Comparison of temperature coefficients (Q_{10}), for Ynysarwed and Wheal Jane waters.

The temperature coefficients (Q_{10}) i.e. the effect of a 10°C rise in temperature on the reaction rate, can be calculated for these waters, the results of which are shown in table 3.3.

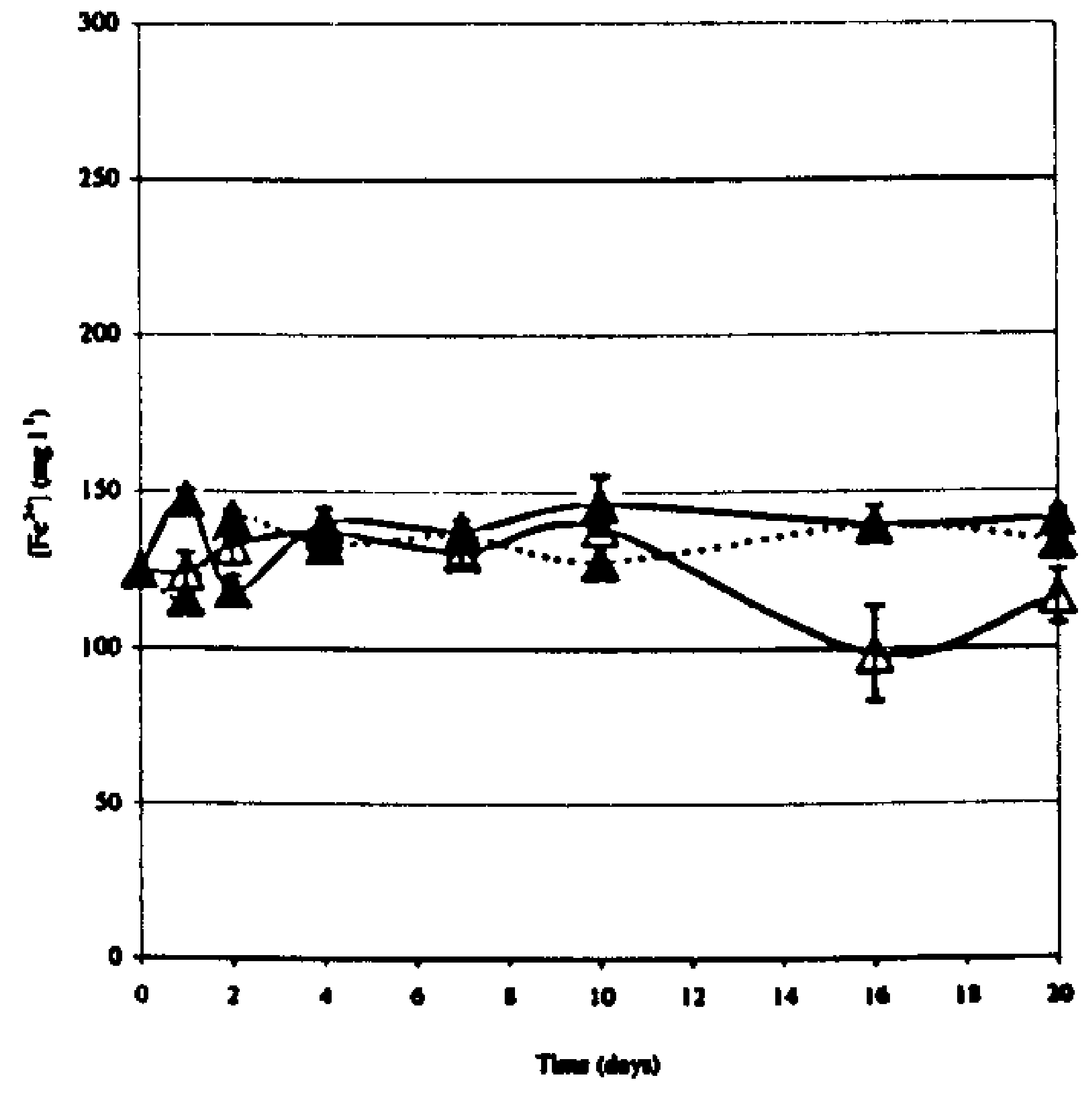
The temperature coefficients (Q_{10}) between 10 and 30°C are shown (table 3.3) to be approximately 2 in both mine drainage samples, with those for Wheal Jane being slightly higher than those calculated for Ynysarwed.

The effect of aerobic status on iron oxidation in Ynysarwed and Wheal Jane mine waters was investigated and found that when the mine waters were filtered, very little iron oxidation occurred over the twenty-day experimental period (figures 3.6(b) and 3.7(b)). Analysis of variance showed there to be no significant difference between the iron concentrations for each of the treatments over time. In unfiltered water samples (figures 3.6(a) and 3.7(a)), very little iron oxidation occurred over the twenty-day experimental period in anaerobic conditions, but both water sources required aerobic conditions, particularly in Ynysarwed which demonstrated the fastest rate. In Wheal Jane waters, graphically (figure 3.7), microaerobic conditions were required for the faster rate of iron oxidation, although, statistical analysis showed that there was no significant difference in the mean iron concentration observed on day 12 (complete oxidation was observed in flasks incubated under microaerobic conditions) in Wheal Jane water samples incubated under aerobic and microaerobic conditions, a phenomena which was not observed in Ynysarwed water, where analysis (t-test) showed there to be an extremely significant difference between the two treatments on day 20 (t=12.066, 4df, P=0.003).

Microbial population densities at the end of the experimental period are detailed in figures 3.8–3.10. Microbial numbers, in this instance, denote colony forming units per millilitre (CFU ml^{-1}). Figure 3.8 shows the results of the microbial enumeration following the addition to Ynysarwed water of inorganic nutrients; addition of Cae Coch AMD; addition of filtered Cae Coch AMD and 20°C aerobic (unamended). These data should be compared with that of Ynysarwed site 1 (the discharge point, <10 microorganisms ml^{-1}), found in Table 3.1. It can be seen that at the end of incubation, the unamended water (20°C) showed significant numbers of neutrophilic bacteria, including some of iron oxidising bacteria and fungi. However, following the addition of inorganic nutrients, microbial numbers and biodiversity increased to include acidophilic bacteria and fungi, although no iron oxidisers were detected.

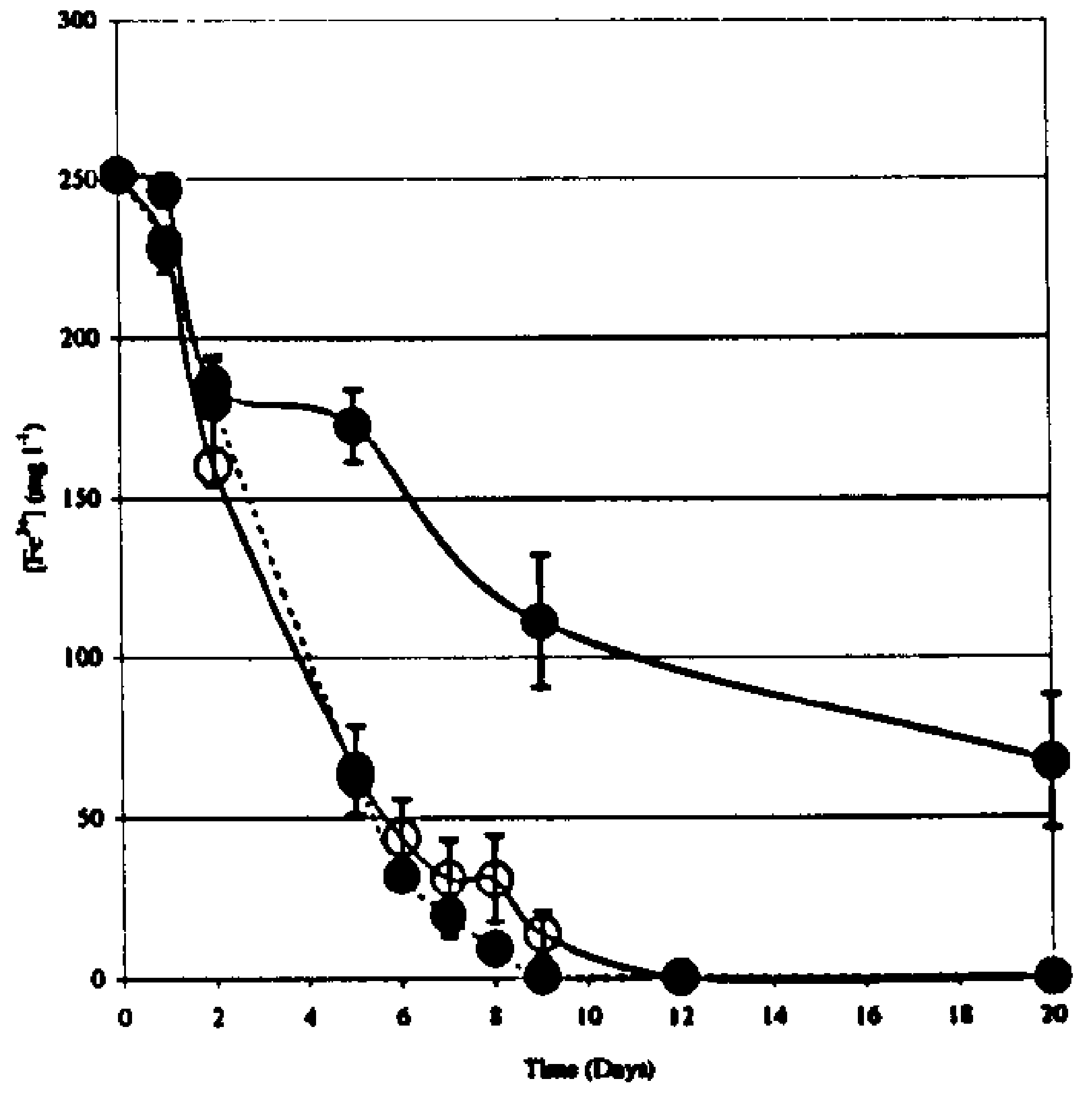


(a) Unfiltered Ynysarwed Mine Drainage Water

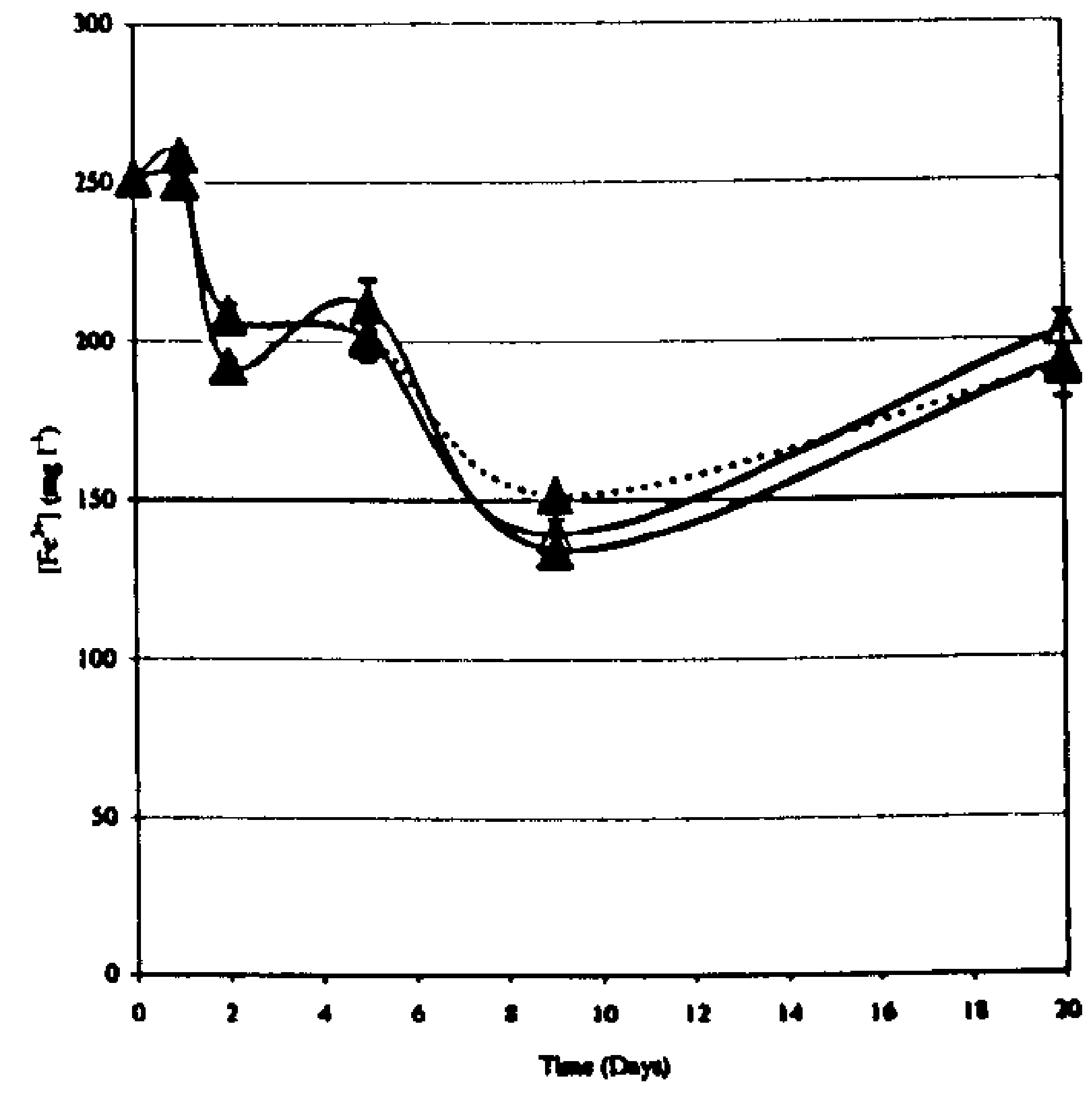


(b) Filtered Ynysarwed Mine Drainage Water

Figure 3.6: The effect of aerobic status on iron oxidation in Ynysarwed water (mean±s.e., n=3). Where, the filled symbols represent anaerobic conditions, unfilled symbols represent aerobic conditions and those symbols with a dotted line, denote microaerobic conditions.



(a) Unfiltered Wheal Jane Mine Drainage Water



(b) Filtered Wheal Jane Mine Drainage Water

Figure 3.7: The effect of aerobic status on iron oxidation in water obtained from Wheal Jane (mean±s.e., n=3). Where, the filled symbols represent anaerobic conditions, unfilled symbols represent aerobic conditions and those symbols with a dotted line, denote microaerobic conditions.

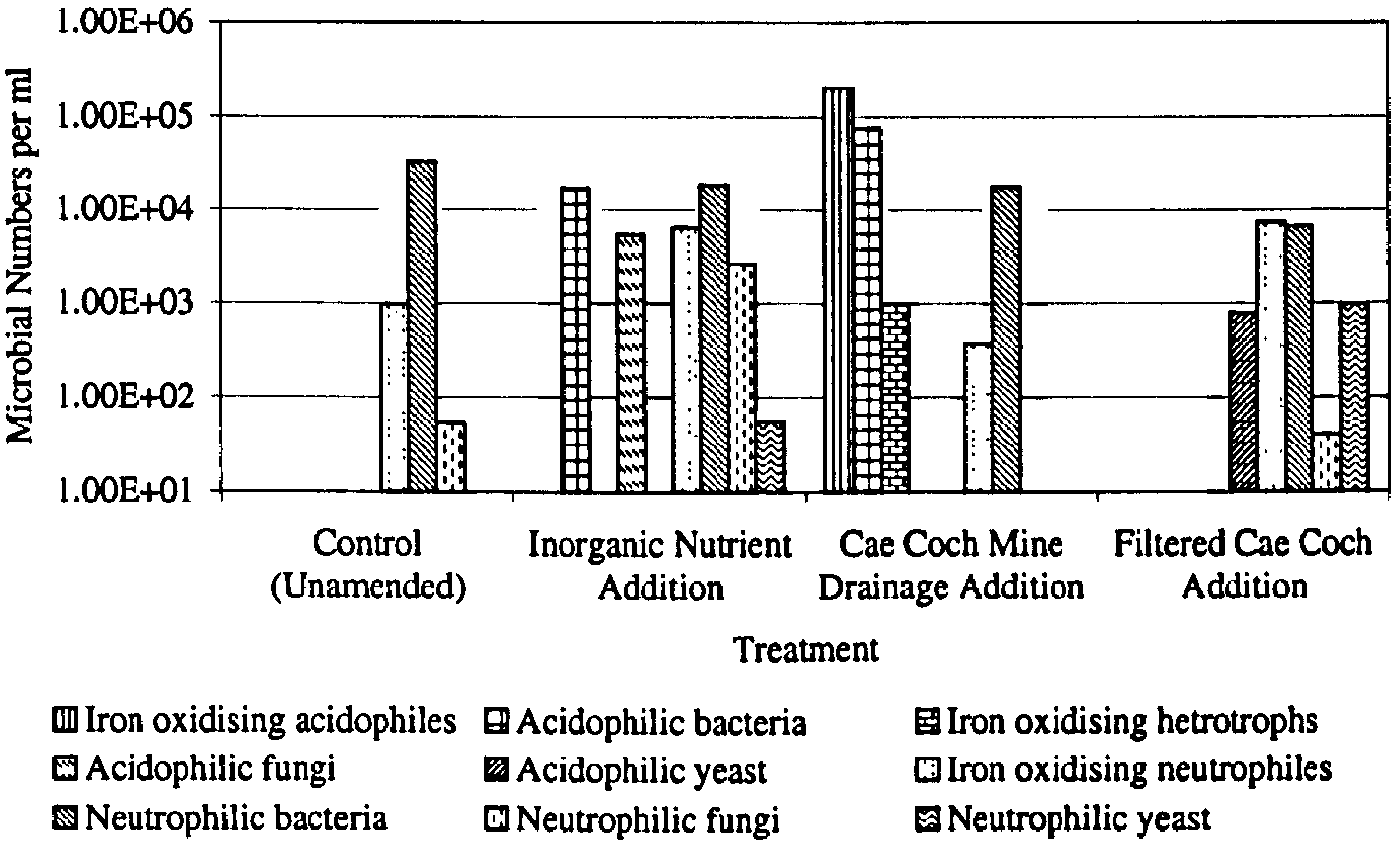


Figure 3.8: The effect on microbial numbers of inorganic nutrients, Cae Coch mine drainage and filtered Cae Coch mine drainage following their addition to Ynysarwed water. The solid media was inoculated at the end of the experimental period.

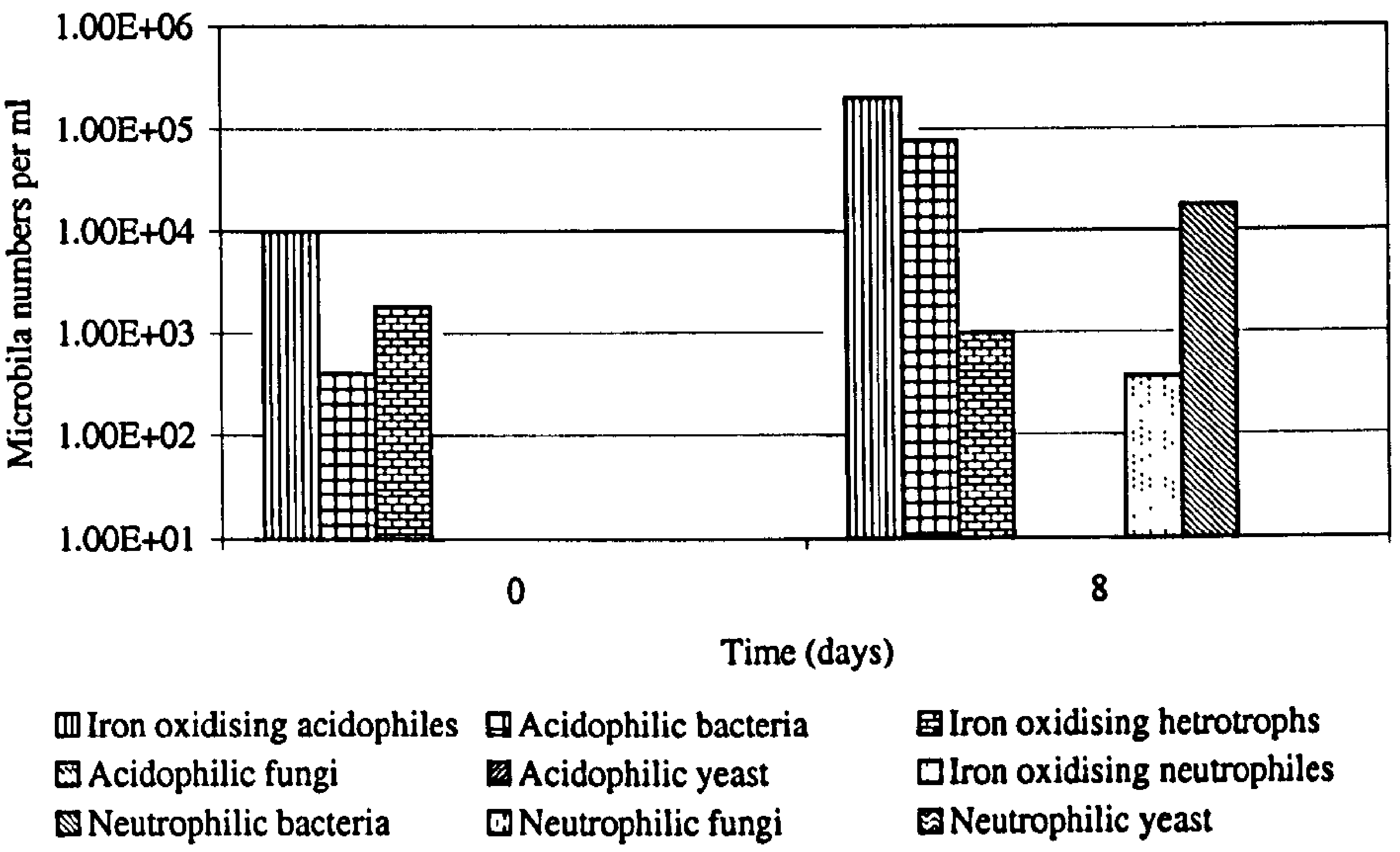


Figure 3.9: Bacterial numbers in Ynysarwed water at the start of incubation (day 0) and at the end of incubation (day 8), following the addition of Cae Coch mine drainage water.

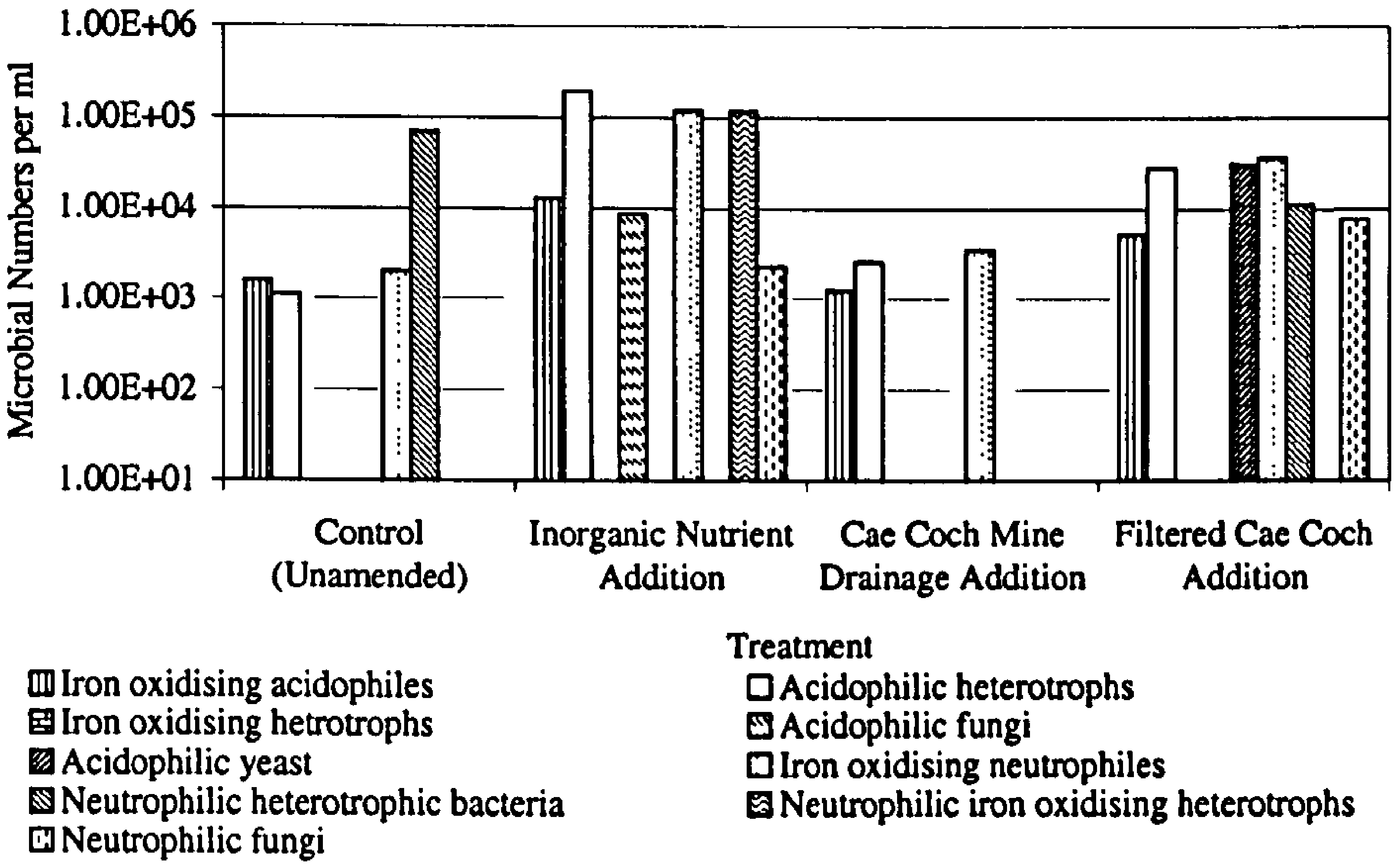


Figure 3.10: The effect on microbial numbers of the addition of inorganic nutrients, Cae Coch mine drainage and filtered Cae Coch mine drainage to water obtained from Wheal Jane. The solid media was inoculated at the end of the incubation period.

Figure 3.9 shows that Cae Coch acid mine drainage contained a variety of acidophilic bacteria, including iron oxidisers. Innoculation of mine waters obtained from Ynysarwed with this water, yielded an increased number of microorganisms.

The effect of the addition of inorganic nutrients; addition of Cae Coch AMD; addition of filtered Cae Coch AMD and 20°C aerobic (unamended) to the post-incubatory microbial numbers in water obtained from Wheal Jane, can be seen in figure 3.10. Once again, these numbers should be compared to those found in table 3.1 showing the untreated water. On comparison with Ynysarwed (figure 3.8) Wheal Jane water appears to be able to support a greater number of microorganisms and again, biodiversity is affected by the addition of inorganic nutrients.

Detailed examination of the solid media inoculated with unamended and amended Ynysarwed water samples at the end of incubation, showed two (possibly 3) distinct, ostensibly novel isolates (figures 3.11 3.12 and 3.13) were found. They were growing optimally under moderately acidic (~pH 3.8) conditions. These organisms had dark red/brown deposits, presumably ferric iron, at the centre of the colonies, though their general colonial morphology was observed to be different. One had a cream gelatinous disposition (christened Jelly), approximately 1-5mm in diameter and the other, likened more to a sun depicted by an artist. Each colony had the red/brown centre. Under close examination using phase contrast microscopy and later S.E.M., they were found to be rod-shaped. It should be

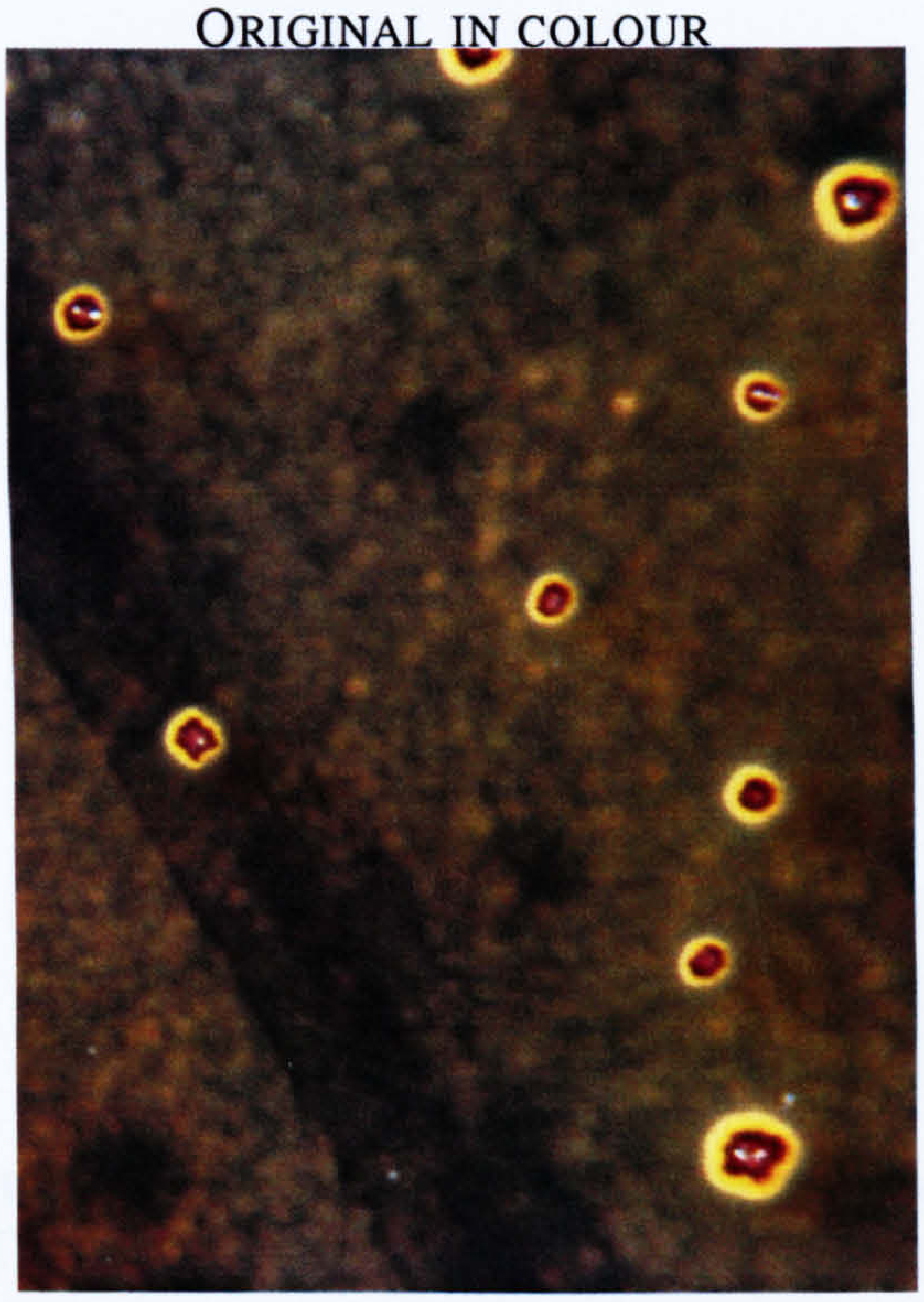


Figure 3.11: Showing the colonial morphology (x65) of isolate christened 'Sun' as observed on Fe/Thio plate.

noted that no *Thiobacillus ferroxidans* or *Leptospirillum ferrooxidans* like bacteria were isolated from Ynysarwed water. These bacteria (*Thiobacillus ferroxidans* and *Leptospirillum ferrooxidans*) were present at Wheal Jane, though, once again, the moderate acidophiles were numerically dominant. The ostensibly novel isolates were purified by isolating a single colony and maintained in liquid culture until further research could be undertaken. Some of the liquid cultures included PORAVOR (recycled glass) balls which acted as a surface for these isolates.

Further research was undertaken by Dr. Johnson and co-workers (see Dennison

ORIGINAL IN COLOUR

Figure 3.12: Showing the colonial morphology (x25) of isolate christened 'Jelly' as observed on Fe/Thio plate.

ORIGINAL IN COLOUR

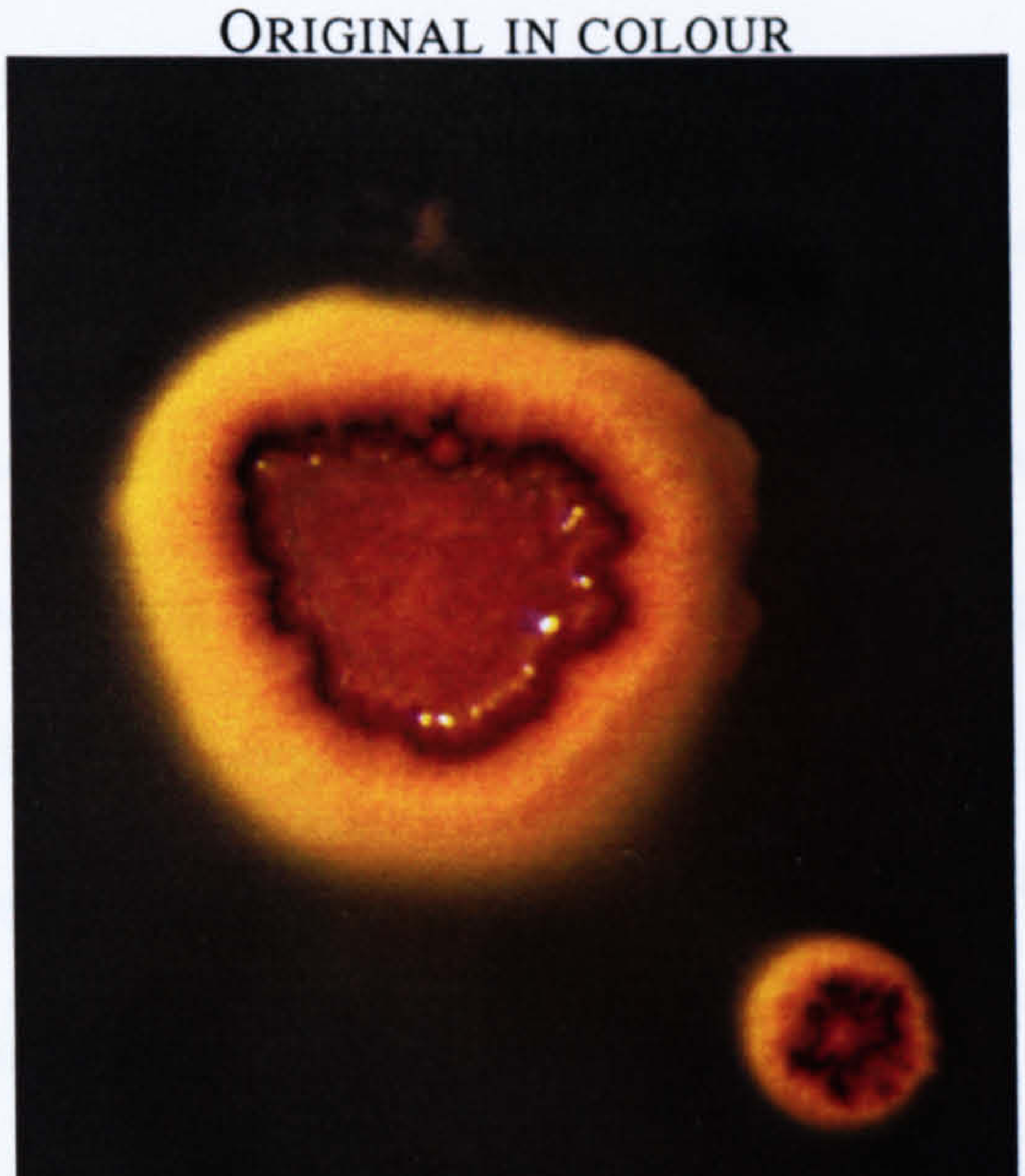


Figure 3.13: Both new isolates (x25), as isolated on Fe/thio plates.

Treatment	Mean Rate of Iron Oxidation (mg l^{-1} hr^{-1})	
	Ynysarwed	Wheal Jane
Anaerobic	0.08 ± 0.01	0.76 ± 0.13
Microaerobic	0.10 ± 0.01	1.52 ± 0.02
Aerobic/20°C	0.46 ± 0.26	1.46 ± 0.17
10°C	0.24 ± 0.01	0.73 ± 0.02
30°C	0.82 ± 0.26	2.83 ± 0.11
37°C	0.63 ± 0.21	4.96 ± 0.38
Inorganic Nutrient Addition	0.82 ± 0.12	3.51 ± 0.11
Cae Coch Addition	0.36 ± 0.06	2.75 ± 0.28
Filtered Cae Coch Addition	0.29 ± 0.05	1.53 ± 0.20

Table 3.4: Comparison of iron oxidation rates, expressed as mg l^{-1} hr^{-1}, for Ynysarwed and Wheal Jane waters under specific biotic and abiotic conditions. Note, inorganic nutrients were added in the form of basal salts (mean ± s.d., n=3).

et al. (2001)), who found that these organisms were, as we originally expected, autotrophic thiosulphate oxidising bacteria. 16S rRNA analysis was then undertaken on the isolates finding that they represented *β-Proteobacteria* – a group previously undescribed. Their closest relative being *Thiomonas thermosulfata.*

Table 3.4 summarises the rates of iron oxidation in Ynysarwed and Wheal Jane waters given in terms of mg l^{-1} of iron hr^{-1}. From this comparative table, it can be seen that the addition of inorganic nutrients enhances the rate of iron oxidation in both mine drainage waters, with only a temperature of 37°C having a greater impact on iron oxidation rate in Wheal Jane water.

3.1.3 Discussion

The physiochemical and biological characteristics of Ynysarwed and Wheal Jane water bodies were investigated. Preliminary analysis of the native water samples showed that these waters contain similar concentrations of ferrous iron despite their differing pHs (Ynysarwed pH 6.3 and Wheal Jane pH 3.4). Oxidation of this ferrous iron as a mechanism for the remediation of mine waters (Kirby *et al.*, 1999) is reliant upon the presence of the microflora, in particular iron-oxidisers. Indeed, Strömberg and Banwart (1995) suggested that by promoting the growth of iron-oxidising bacteria, the rate of iron oxidation can be manipulated. The mechanisms by which microbial oxidation occurs is outlined in chapter 1, section 1.1.1 (see equation 1.3). Microbial analysis of Ynysarwed water (table 3.1) showed that the total number of organisms was very low, compared to those found at Wheal Jane. Closer examination of the microflora at these sites showed that Ynysarwed had no *T. ferrooxidans*- and *L. ferrooxidans*- like bacteria, although three novel types were isolated and found to represent the undescribed (to date) *β-Proteobacteria*, their closest known relative being *Thiomonas thermosulfata*. When Ynysarwed was innoculated with Cae Coch mine water (3.1) the bacterial numbers increased indicating that Ynysarwed was capable of supporting these bacteria even though they were not native to Ynysarwed water. In contrast, Wheal Jane had a larger number of iron oxidising bacteria in particular the aforementioned *T. ferro.* and *L. ferro.* types. These findings were reinforced by direct counts of microbes using acridine orange. Therefore, iron oxidation proceeded at a comparatively faster rate (13.1% faster, P=0.0001 therefore, significant) in water samples obtained from Wheal Jane than those obtained from Ynysarwed. Indeed, the addition of indigenous bacteria from Cae Coch also increased the rate of iron oxidation, as expected. Therefore, the rate limiting factor in these waters has been identified as biological, but a parallel investigation involving the addition of inorganic nutrients, showed that the indigenous microorganisms were in-turn limited by the availability of inorganic nutrients (figures 3.8 and 3.10). Microorganisms require a variety of macronutrients such as carbon, nitrogen, potassium, magnesium and phosphorus etc for their successful development and growth. These constituents are added to culture media in varying ratios depending upon the organisms requirements. Therefore, it was necessary to investigate which constituent of the inorganic nutrient mixture, known in the laboratory as basal salts, was responsible for this increase in rate of iron oxidation. The results of this experiment are displayed in the next section (section 3.2).

Aerobic status was also investigated in this research in terms of its effects on iron oxidation. Flasks and plates were set up to create conditions denoted as aerobic, 'microaerobic' and anaerobic. The results show that in both water samples the fastest rate of oxidation (table 3.4) occurred in aerobic samples suggesting that these microorganisms require aerobic conditions although, no significant difference in iron concentration was observed between Wheal Jane water samples incubated under aerobic and microaerobic conditions. It is widely known that *T. ferro.*, found abundantly in Wheal Jane mine water, is an extremely adaptable organism which is capable of growing in aerobic and anaerobic conditions (Nemati *et al.*, 1998). Although this organism displays the characteristics of a typical aerobe, it can grow anaerobically using sulphur as a substrate (Das *et al.*, 1992; Pronk *et al.*, 1992).

In naturally occurring ecosystems, microbial activity may be affected by other environmental parameters such as temperature. Indeed, Walton and Johnson (1992) found that in waters obtained from Mynydd Parys (Anglesey) the main limiting factor was not nutrients, as found in these experiments, but rather temperature. Current investigations as to the remediation of these waters are usually undertaken at ambient environmental temperature or temperatures best-fitted to the culturing and in-vitro maintenance of these organisms, although, different microorganisms have different characteristic cardinal temperatures and organisms such as *T. ferro.* will only be found at detectable levels when the temperature is within its particular range (Edwards *et al.*, 1999). Therefore, in experiments such as these, a range of temperatures are used. The results of experiments outlined previously in this chapter have found that, as expected, those temperatures used for the culturing of these microorganisms (30°C) exhibited the most rapid rate of iron oxidation (figures 3.3 and 3.4). By comparison, samples at ambient environmental water temperature (10°C) were much slower. Walton and Johnson (1992) also observed a much slower rate of iron oxidation in water samples incubated at 10°C compared to 30°C, concluding that the microorganisms present were mesophilic, as found to be the case in these experiments. As with all organisms, the ability to adapt to varying environmental conditions is important as the environmental temperature may vary considerably from that which is exhibited within underground mines and spoil heaps for example, which may reach lows of <10°C with little seasonal variation (Ahonen and Tuovinen, 1991). Seasonal variations in microbial numbers have been described by numerous research groups such as Edwards *et al.* (1999) and McGinness and Johnson (1993). Temperature affect experiments may provide insights into the implications in terms of climate change, especially as some parts of the world affected by mine drainage may see an increase in ambient temperature. Indeed, the Intergovernmental Panel on Climate Change (Houghton *et al.*, 2001) predicted that we would see a increase of temperature by 5.9°C before the end of this century.

3.2 Effect of Inorganic Nutrient Addition to Ynysarwed and Wheal Jane Waters on Iron Oxidation

3.2.1 Materials and Methods

Conical flasks were set up, in triplicate, containing 50ml of Ynysarwed or Wheal Jane mine drainage water. To each, 0.5ml of either the composite nutrient solution (Appendix B) or its individual constituents (ammonium-N, potassium, magnesium and phosphate), made to the same concentration and pH (2.5) as found in the composite solution, was added to the mine drainage water. A control of unamended mine water was also set up and all flasks were incubated at 20°C until full oxidation had occurred.

Samples were taken using sterile technique, on a daily basis/twice daily for ferrous iron analysis.

3.2.2 Results

A comparison of the effect of adding the composite nutrient solution and of its separate constituents, on rates of iron oxidation in Ynysarwed water are presented in figure 3.14. As before, some iron oxidation was observed in uninoculated water. However, the addition of either the composite inorganic nutrients or of phosphate, considerably enhanced the rate of iron oxidation, reaching total iron oxidation by five days. Statistical analysis, using one-way ANOVA showed that there was a significant difference between the ferrous iron concentration observed in each of the experiments ($F_{5,120}=6.157$, $P<0.001$). Further analysis using post-hoc (least significant difference (LSD)) tests showed that at $P=0.05$, there was no significant difference between the ferrous iron concentration in experiments containing phosphate and combined inorganic nutrient additions, nor was a significant difference observed between the remaining experiments. However, a significant difference was observed between phosphate and combined compared to the remaining experiments ($P=0.05$). This is summarised below according to convention where the results are arranged in ascending order of mean and then lines are drawn under groups that are not significantly different (Dytham, 1990).

$$\underline{\text{Pho.}\quad\text{Combined}}\quad\underline{\text{Mag.}\quad\text{Pot.}\quad\text{No}\quad\text{Amm.}}$$

Where, Pho. = phosphate, combined = combined inorganic nutrients, Mag. = magnesium, Pot. = potassium, No = No inorganic nutrients, Amm. = ammonium.

This rapid rate of iron oxidation in flasks containing an additive of phosphate was also observed in the Wheal Jane water (figure 3.15), reaching full oxidation after four days. Although, in this instance identical statistical analysis to that presented for the Ynysarwed data, failed to show any significant differences between any of the experimental data sets. This is due to the fact that iron oxidation is occurring to a greater extent in Wheal Jane water than the corresponding Ynysarwed experiment. As visible from figure 3.15, all other treatments were found to be significantly different (using a t-test) from one another with 4df and $P<0.1$ on day 5 when complete iron oxidation occurred in flasks amended with phosphate, except those flasks containing ammonium-N and magnesium additions, and unamended flasks and those containing an additive of potassium. The rates of reaction are summarised in table 3.5 and a faster rate of iron oxidation, in terms of mg l^{-1} hr^{-1}, can be seen in both Ynysarwed and Wheal Jane mine water, following the addition of phosphate compared to the combined inorganic nutrients.

At 10°C, a temperature akin to the mean environmental temperature of British water, the addition of inorganic nutrients, in particular phosphate, increased oxidation rates. In terms of Ynysarwed, it increased from 0.18 ± 0.02 mg l^{-1} hr^{-1} to 0.59 ± 0.01 mg l^{-1} hr^{-1} following the addition of nutrients.

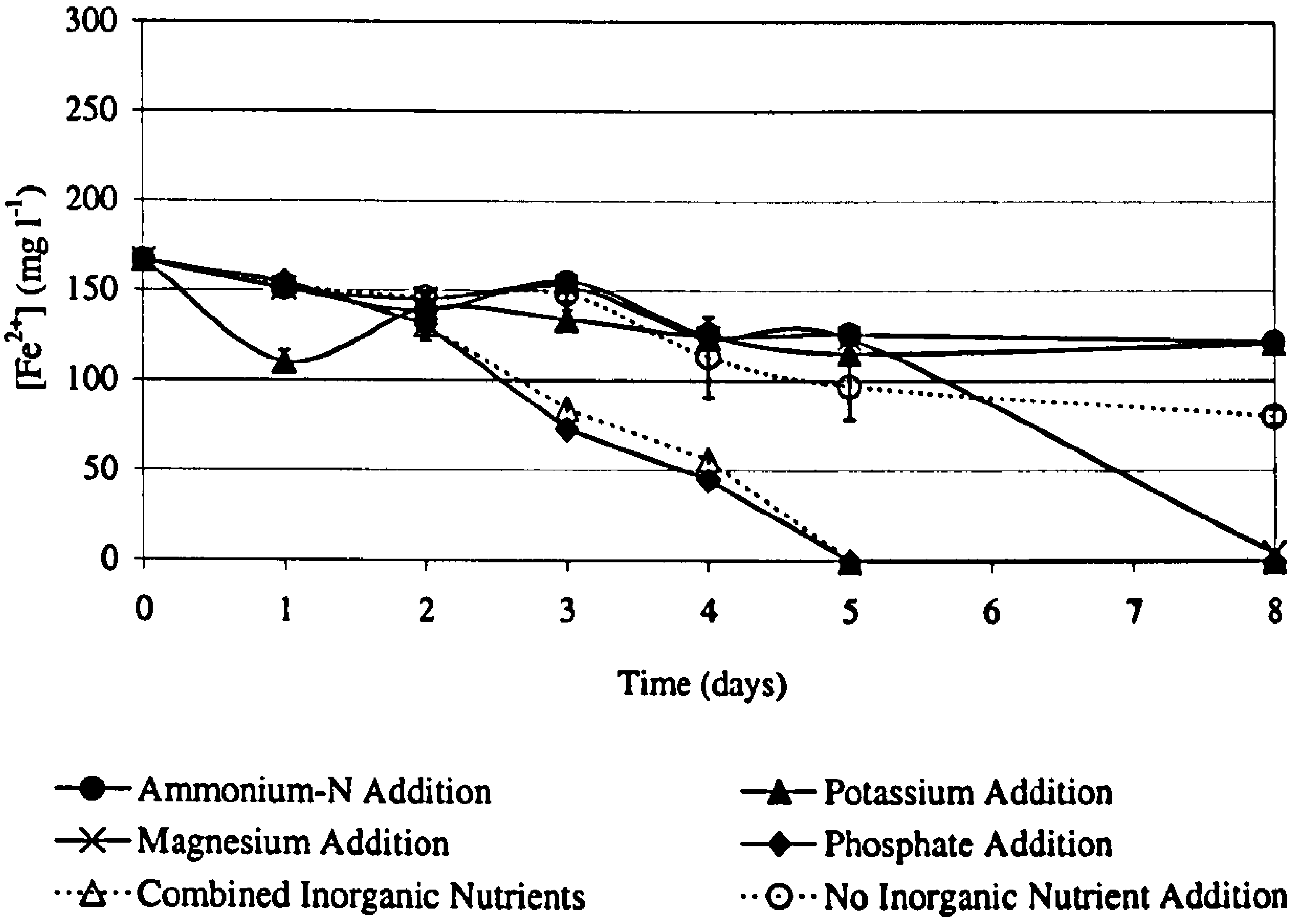


Figure 3.14: The effect of combined inorganic nutrients (basal salts) and its individual constituents, on iron oxidation in waters obtained from Ynysarwed. Mean±s.e., n=3.

Treatment	Mean Rate of Iron Oxidation (mg l^{-1} hr^{-1})	
	Ynysarwed	Wheal Jane
+Ammonium-N	0.40 ± 0.05	1.76 ± 0.02
+Potassium	0.47 ± 0.05	1.18 ± 0.22
+Magnesium	0.39 ± 0.10	1.26 ± 0.05
+Phosphate	1.73 ± 0.23	4.08 ± 0.74
+Combined Inorganic Nutrients	1.29 ± 0.04	2.86 ± 0.16
No Nutrient Additions	0.70 ± 0.40	1.71 ± 0.11

Table 3.5: The effect of inorganic nutrient addition to Wheal Jane and Ynysarwed waters (mean ± s.d., n=3).

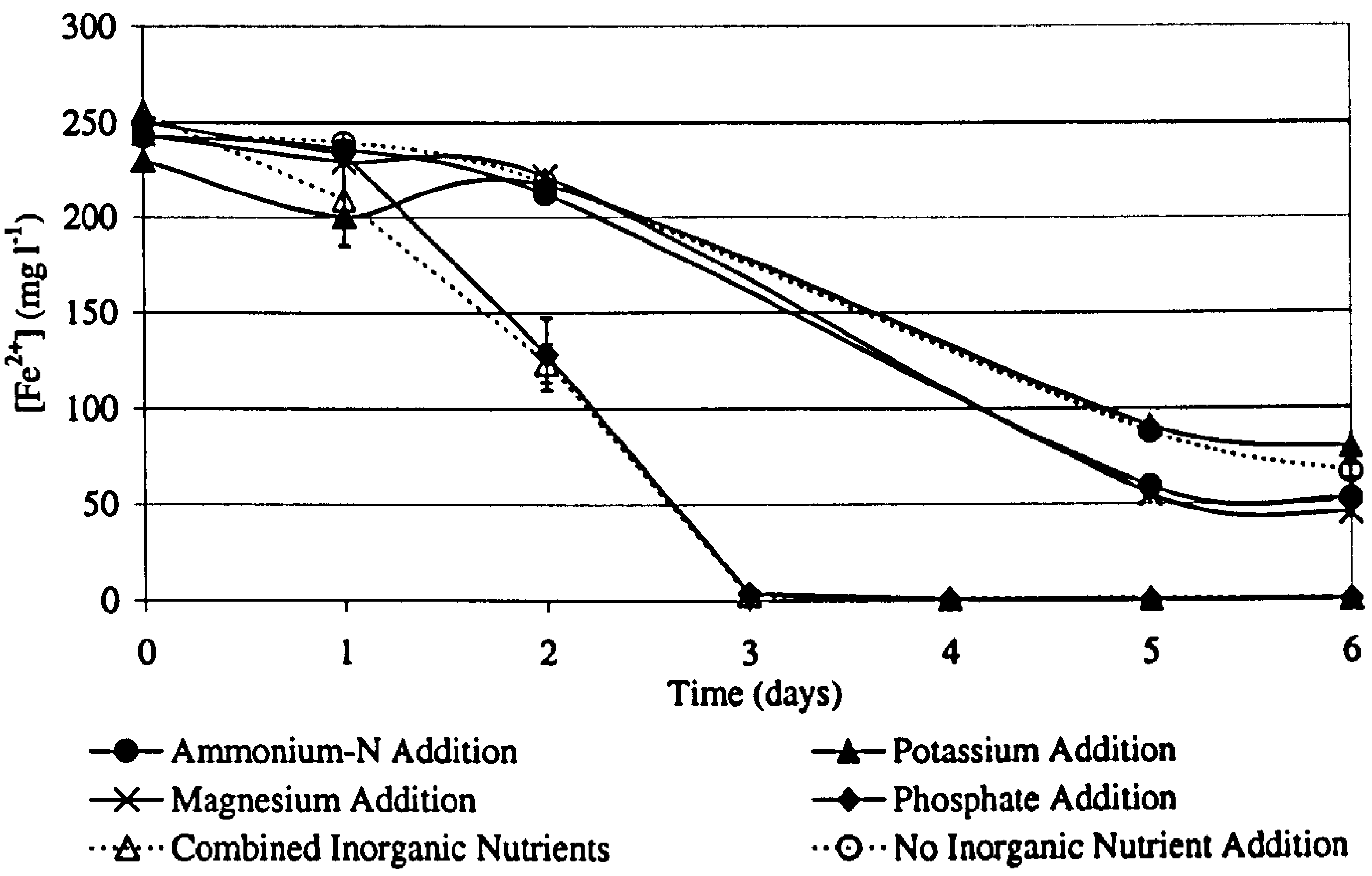


Figure 3.15: The effect of combined inorganic nutrients (basal salts) and its individual constituents, on iron oxidation in water obtained from Wheal Jane. Mean±s.e., n=3.

3.2.3 Discussion

In the previous discussion (section 3.1.3) it was stated that certain macronutrients were required by microbes for their growth and development. This was supported by experimental evidence that the addition of inorganic nutrients to Ynysarwed and Wheal Jane mine waters, in the form of basal salts (figures 3.1 and 3.2), showed a marked increase in the rate of iron oxidation and microbial biomass (figures 3.8 and 3.10). The similarity between the rates of iron oxidation (table 3.5) in the composite inorganic nutrient solution and the phosphate amended samples would suggest that phosphate is the limiting factor in these waters. Therefore, it can be speculated that the growth of the indigenous iron-oxidising bacteria are limited by phosphate availability.

Phosphate is the common form by which phosphorus is found in the environment. This macronutrient is primarily used for the synthesis of phospholipids and nucleic acids within cells (Madigan *et al.*, 1997). According to Geiger *et al.* (1999), phosphate stress is frequently encountered by some organisms and bacteria such as *Escherichia coli* and *Bacillus subtilis* have evolved their own mechanisms to allow them to function in a phosphate limiting environment. There are two mechanisms by which Geiger *et al.* (1999) report this to occur. The first, as exhibited by the aforementioned organisms, involves the activation of some of the genes involved in phosphate assimilation and transport, the second is by the formation of "phosphorus-free membrane lipids" (Geiger *et al.*, 1999). Further research is required to ascertain if such mechanisms are exhibited by organisms subjected to the additional stresses posed by (acid) mine drainage.

As a consequence of the research detailed within this section, it was essential to investigate the critical concentration of phosphate required to enhance the oxidation rate.

3.3 Effect of Phosphate Addition to Ynysarwed and Wheal Jane Waters on Iron Oxidation Rate

3.3.1 Materials and Methods

Conical flasks were set up containing 50ml of Ynysarwed or Wheal Jane mine drainage water. Phosphate was added in the form of NaH_2PO_4 (0.04M sterile stock solution) to the flask in the following volumes of the stock solution and made up to 55ml with distilled water: 0.5ml (0.004M), 1.0ml (0.008M), 5.0ml (0.04M), 0.05ml (0.0004M) and 0.005ml (0.00004M). Filtered and unamended controls were also set up. Aliquots of water were taken using sterile technique on a regular basis for ferrous iron analysis.

3.3.2 Results

The effect of the addition of different volumes of phosphate solution to Ynysarwed and Wheal Jane waters respectively are shown in figures 3.16 and 3.17, and the reaction rates for this experiment are presented in table 3.6. In the Ynysarwed samples (figure 3.16), all volumes show an initial rapid oxidation of iron before a brief reductive phase between days 2 and 3, except those flasks containing filtered mine drainage water. Oxidation resumes leading to full oxidation in flasks containing 0.5ml (0.004M) of phosphate solution after 7 days, a week before the control samples. Whilst in the Wheal Jane water samples (figure 3.17) iron oxidation is initially slow, but increasing after day 1. Analysis of the data found in figure 3.17 shows that iron oxidation occurs rapidly in those flask with an addition of 0.5ml (0.004M) and 1.0ml (0.008M) of phosphate solution, leading to full oxidation after 5 days. However, when iron oxidation rates are compared for these two experiments (table 3.6), it can be seen that iron oxidation is most rapid in those samples containing 1.0ml (0.008M)of phosphate solution.

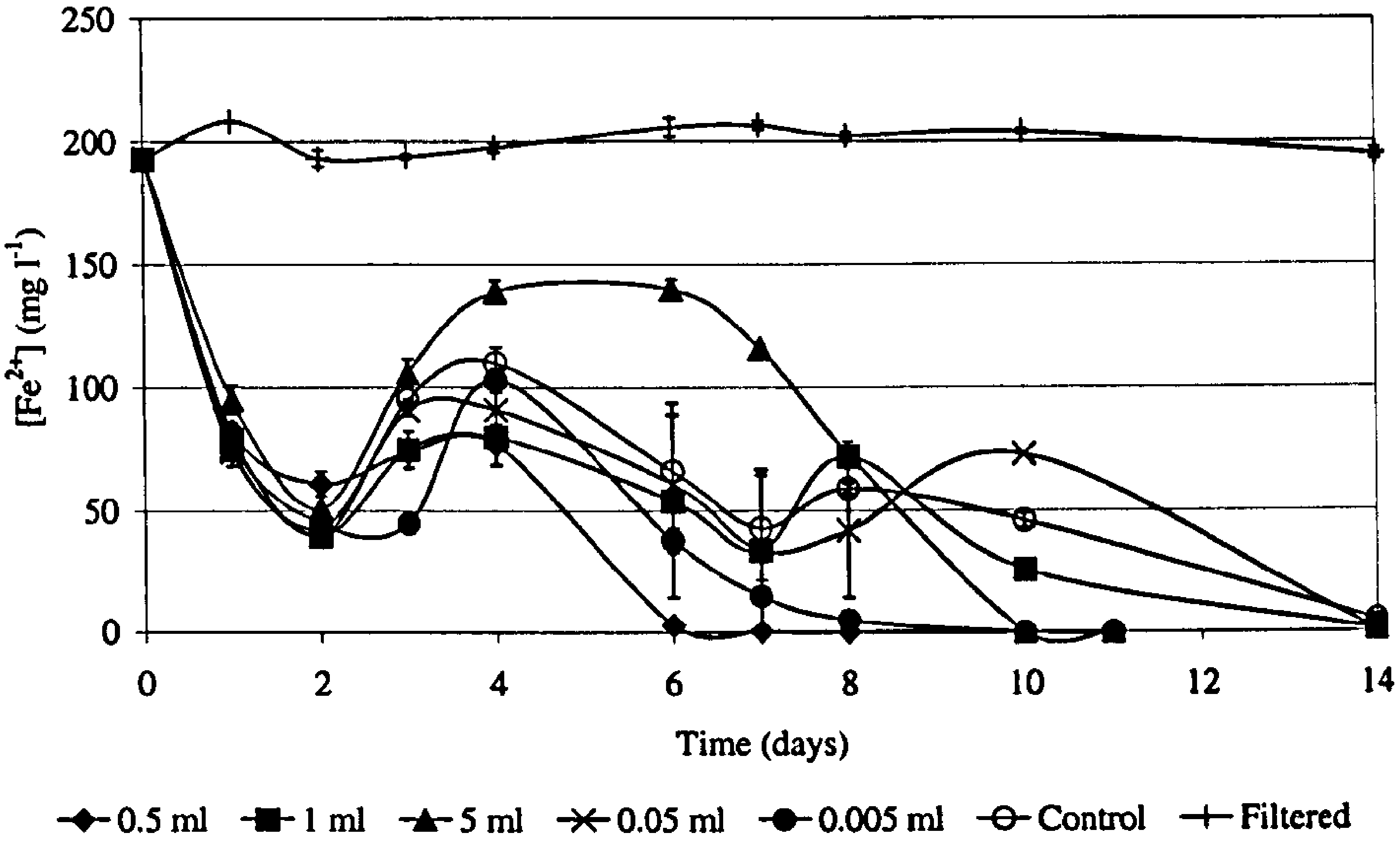


Figure 3.16: The effect of adding different volumes of phosphate solution to Ynysarwed drainage water on iron oxidation. Mean±s.e., n=3.

Volume of Phosphate Solution (ml)	Mean Rate of Iron Oxidation (mg l^{-1} hr^{-1})	
	Ynysarwed	Wheal Jane
0.0	0.88 ± 0.21	1.23 ± 0.02
0.005	1.09 ± 0.03	1.68 ± 0.23
0.05	0.97 ± 0.24	1.53 ± 0.21
0.5	1.95 ± 0.06	1.99 ± 0.12
1.0	1.47 ± 0.15	2.07 ± 0.01
5.0	0.53 ± 0.02	1.39 ± 0.13
0.5 + Filtered (A)MD	0.01 ± 0.02	0.02 ± 0.03

Table 3.6: The effect of phosphate addition to Wheal Jane and Ynysarwed waters, on the rate of iron oxidation (mean ± s.d., n=3).

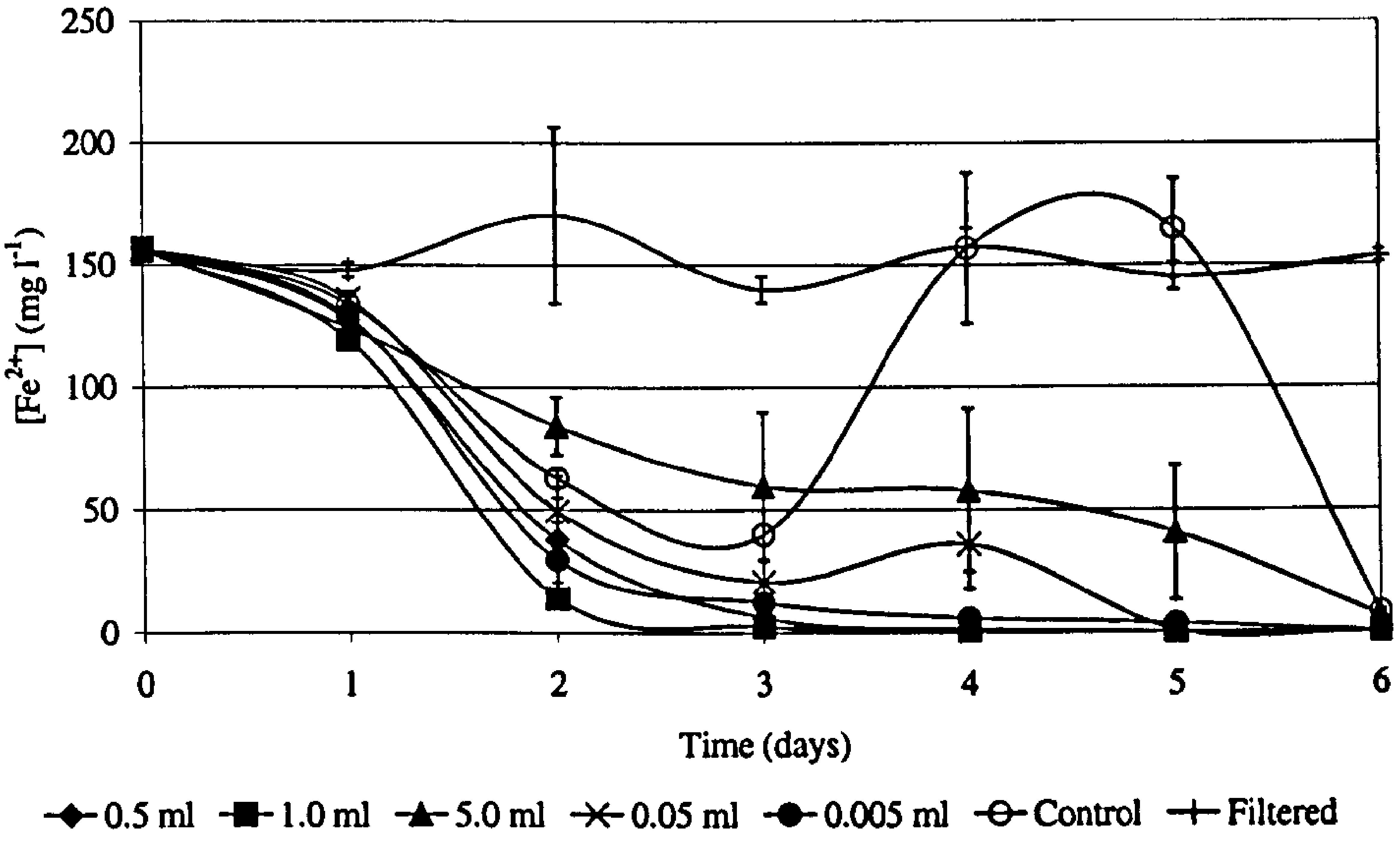


Figure 3.17: The effect of adding different volumes of phosphate solution to Wheal Jane drainage water on iron oxidation. Mean±s.e., n=3.

3.3.3 Discussion

The results from this experiment have shown that the critical phosphate concentration required to enhance the rate of iron oxidation in Ynysarwed and Wheal Jane waters is in the region of 0.004M and 0.008M respectively. To date there is no literature available for the comparison of this figure, nor to suggest that the microbial population in other mine drainage waters are also limited by the concentration of phosphate. Further research is needed into this area as a tool in the control of acid mine drainage.

3.4 The Mechanism By Which Iron Oxidation Occurs In Ynysarwed Mine Drainage Water

Throughout the experiments undertaken on Ynysarwed drainage water, a phenomenon was observed that was not found to be present in Wheal Jane water. This phenomena was seen to be more exaggerated with the addition of inorganic nutrients (e.g. figure 3.16).

3.4.1 Materials and Methods

A shake flask experiment (shaken at 100rpm) was set up using sterile 100ml conical flasks containing 50ml of Ynysarwed mine drainage water. An inorganic nutrient was added in the form of 0.5ml of phosphate solution (see section 3.3) and the total volume in the conical flasks made up to 55ml using distilled water. Small aliquots of amended mine water were taken regularly from the flasks over the 90 hour experimental period, using sterile technique, for ferrous iron analysis.

3.4.2 Results

As can be seen from figure 3.18, iron oxidation in Ynysarwed water occurs initially very rapidly, where approximately 75% of the ferrous iron was converted to ferric form within the first 17 hours. Thereafter, iron oxidation occurs at a much slower rate and does not increase when the water is actively aerated. Thus, it is possible that iron oxidation in Ynysarwed water is a biphasic phenomena rather than monophasic as displayed by Wheal Jane water.

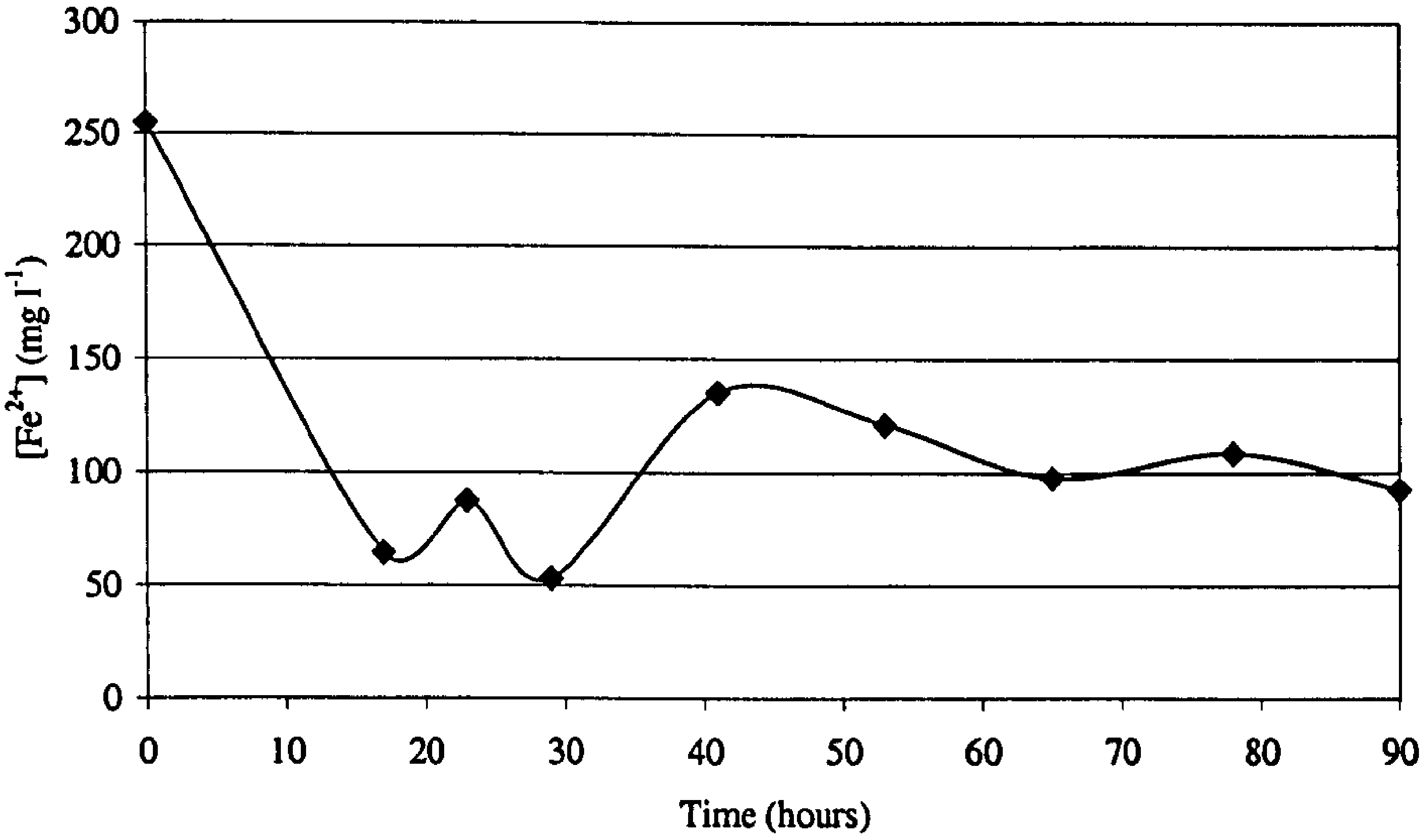


Figure 3.18: The biphasic iron oxidation effect exhibited by Ynysarwed mine drainage waters. Mean±s.e., n=3.

3.4.3 Discussion

The results from the experiment outlined in this section suggest that a previously undescribed phenomena was occurring. The mechanism for iron oxidation in Ynysarwed waters, in particular following the addition of phosphate solution, appeared to be in two phases. An initial phase of rapid iron oxidation possibly due to the addition of inorganic nutrients enhancing the microbial community, which in turn increases the rate of iron oxidation. It is thought that the production of ferric iron provides additional substrate for iron reducing bacteria which after 30 hours, slightly increases the availability of ferrous iron. In the second phase, the iron oxidising bacteria may continue to proliferate, increasing the rate of iron oxidation above that of iron reduction and the second downward trend is observed between 40 and 90 hours.

3.5 Summary

Bench-scale investigations have shown that the rate of iron oxidation in two physiochemically different mine-waters, Ynysarwed and Wheal Jane, is limited by microbial numbers. The growth of these microorganisms in these metalliferous waters, one circum-neutral, the other acidic, has been shown to be limited by the availability of inorganic nutrients, in particular phosphate. Further research has shown that the critical phosphate concentration for increasing the rate in these mine waters was in the region of 0.004M in Ynysarwed water and 0.008M in Wheal Jane water although, more detailed investigations would be required to provide a more precise result.

Chapter 4

A Comparison of the Impact of Soil Type and the Presence or Absence of Plants on the Remediation of Ynysarwed and Mona Mine Drainage Waters

In the previous chapter, investigations were undertaken to ascertain the factors affecting the rate of iron oxidation, a process which produces the characteristic smothering red/brown sediment in mine drainage waters. These investigations only dealt with one part of the (acid) mine drainage problem which also includes acidity and high sulphate concentrations. In this and the subsequent chapters, these other constituents, along with that of high iron concentrations, will be considered, thus tackling the whole problem of (acid) mine drainage.

In recent years, wetland construction for the remediation of (acid) mine drainage waters has gained momentum/impetus. Not only do these aesthetically pleasing areas facilitate habitat restoration but, they assist metal precipitation and water neutralisation (Skousen *et al.*, 1994). This is of great importance especially when treating problematic waters such as (acid) mine drainage. The success of wetlands constructed for this purpose has been mixed. For example, researchers on pilot constructed wetlands systems in West Virginia, U.S.A. (Kolbash and Romanoski, 1989) and Minnesota, U.S.A. (Frostman, 1993) have reported successful metal removal throughout test periods of several years, whilst wetlands such as those treating waters from Wheal Jane (U.K.) have been more problematic.

Microcosms were used in a laboratory-based investigation to ascertain the impact of planting and soil type on the remediation of Ynysarwed and Mona waters. Studies such as that of Eger and Wagner (1995) and Rabenhorst *et al.* (1992) have undertaken substrate investigations using horse manure, straw manure and yard manure amongst others, although, there appears to be less published research on varying soil types for the treatment of waters affected by mine drainage in the British Isles.

One mechanism through which the soil affects the remediation strategy, is through the importance of sulphate reducing bacteria (SRB's) such as *Desulfovibrio* spp., as a cost-effective method for increasing alkalinity in (acid) mine drainage waters (Clarke, 1995). It has been shown that under anaerobic conditions SRB's use sulphate as a terminal acceptor resulting in hydrogen sulphide production (Wendt-Potthoff and Neu, 1998). This alkalinity generation facilitates the precipitation of metal ions in solution. However, for this process to occur, a suitable organic substrate for the sulphate reducing bacteria must be provided. Previous research (e.g. Dvorak *et al.* (1992)) has shown that mushroom compost (Machemer and Wildeman, 1992) and wood-chippings to be suitable soils and are therefore used in this research, substituting wood-chippings for bark-chippings due to cost effectiveness and availability. For the purpose of this research, the other two soils used in the microcosms were peat and gravel. Peat, acts as a non-labile organic substrate/soil and gravel, as a control.

The composition material of mushroom compost varies depending upon the 'school' of farming, but normally, horse manure (straw based) or synthetic manure (made from hay and crushed corn cobs) is used, with gypsum (calcium sulphate) and nitrogen additions. The compost obtained for the purpose of these experiments was made using traditional Lancashire methods, from horse manure, straw, gypsum and nitrogen additions.

4.1 Materials and Methods

Microcosms were set up in quadruplicate as detailed in chapter 2 (section 2.2.2) using the following soils (approximate values in brackets indicate mass of soil required to give 110mm soil depth in planted and unplanted microcosms): mushroom compost (approx. 800g) obtained from the Curshaw family farm, Southport, Merseyside; 'Westland' Irish Moss Peat (approx. 680g); bark-chippings (approx. 640g) and gravel approximately 6mm diameter (approx. 2880g). Indigenous plant samples (*Eriophorum augustifolium*) were collected from a natural wetland, which forms part of the Mynydd Parys complex (Anglesey, North Wales - see section 2.1.1). These plant samples (figure 4.1) were bagged in equal quantities (approximately 20 plants), in the field, for ease of manipulation into the microcosms once back in the laboratory.

Water samples were taken from the microcosms on a regular basis, filtered and stored at 4°C, until analysis for anions, cations and dissolved iron could be undertaken (metal analysis was undertaken immediately).

ORIGINAL IN COLOUR

Figure 4.1: Preparation of plant material in the field to aid manipulation into microcosms back in the laboratory.

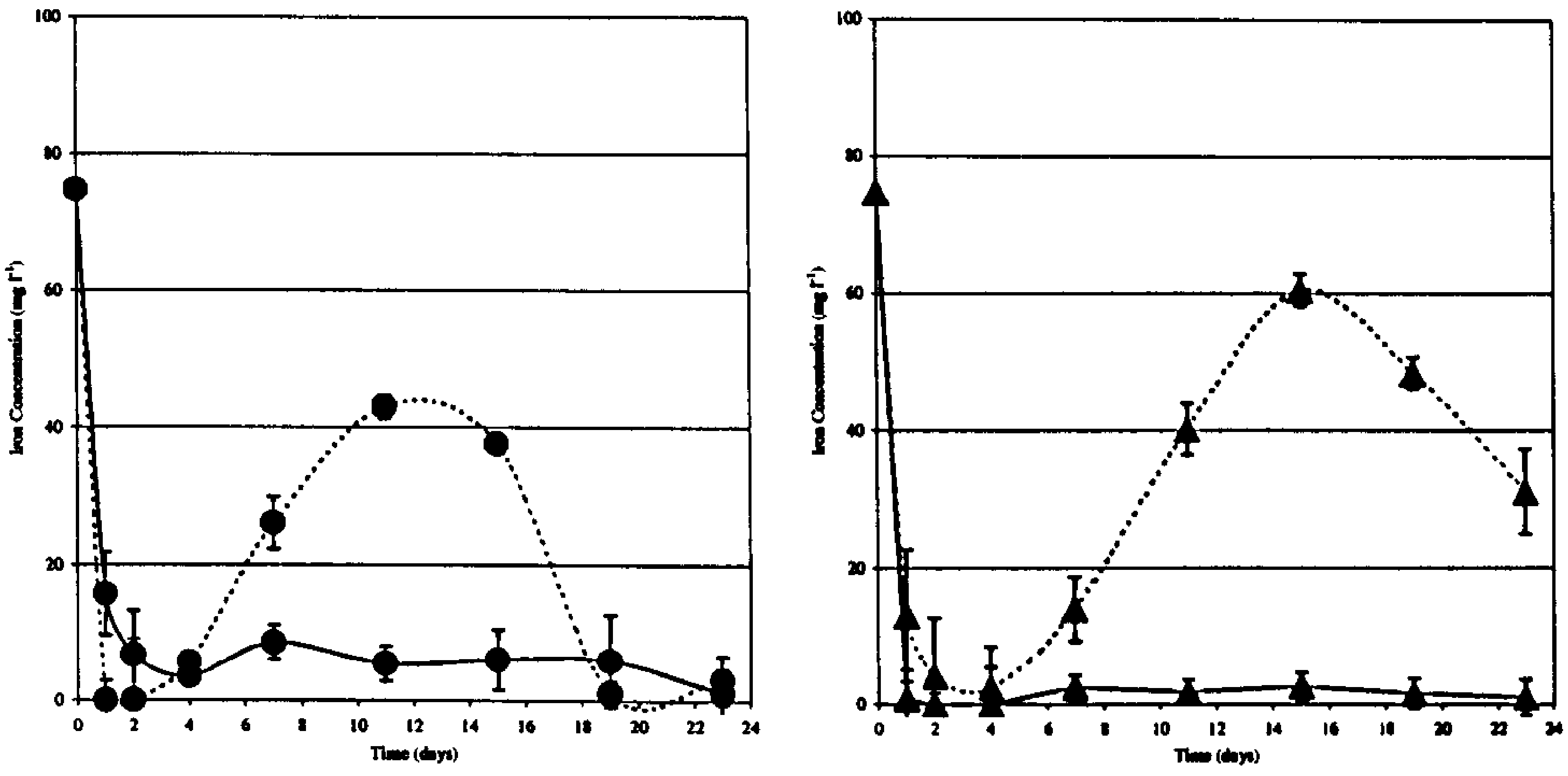
	Ynysarwed	Mona
pH	6.3	2.9
Temp. (°C)	14.1	10.4
$[Fe^{2+}]$ (mg l^{-1})	300	280
$[SO_4^{2-}]$ (mg l^{-1})	2000	2250
$[Al^{3+}]$ (mg l^{-1})	23.00	11.39
$[Mn^{2+}]$ (mg l^{-1})	8.00	6.93

Table 4.1: Summary of major physico-chemical variables obtained from a typical analysis of Ynysarwed and Mona mine drainage water.

4.2 Results

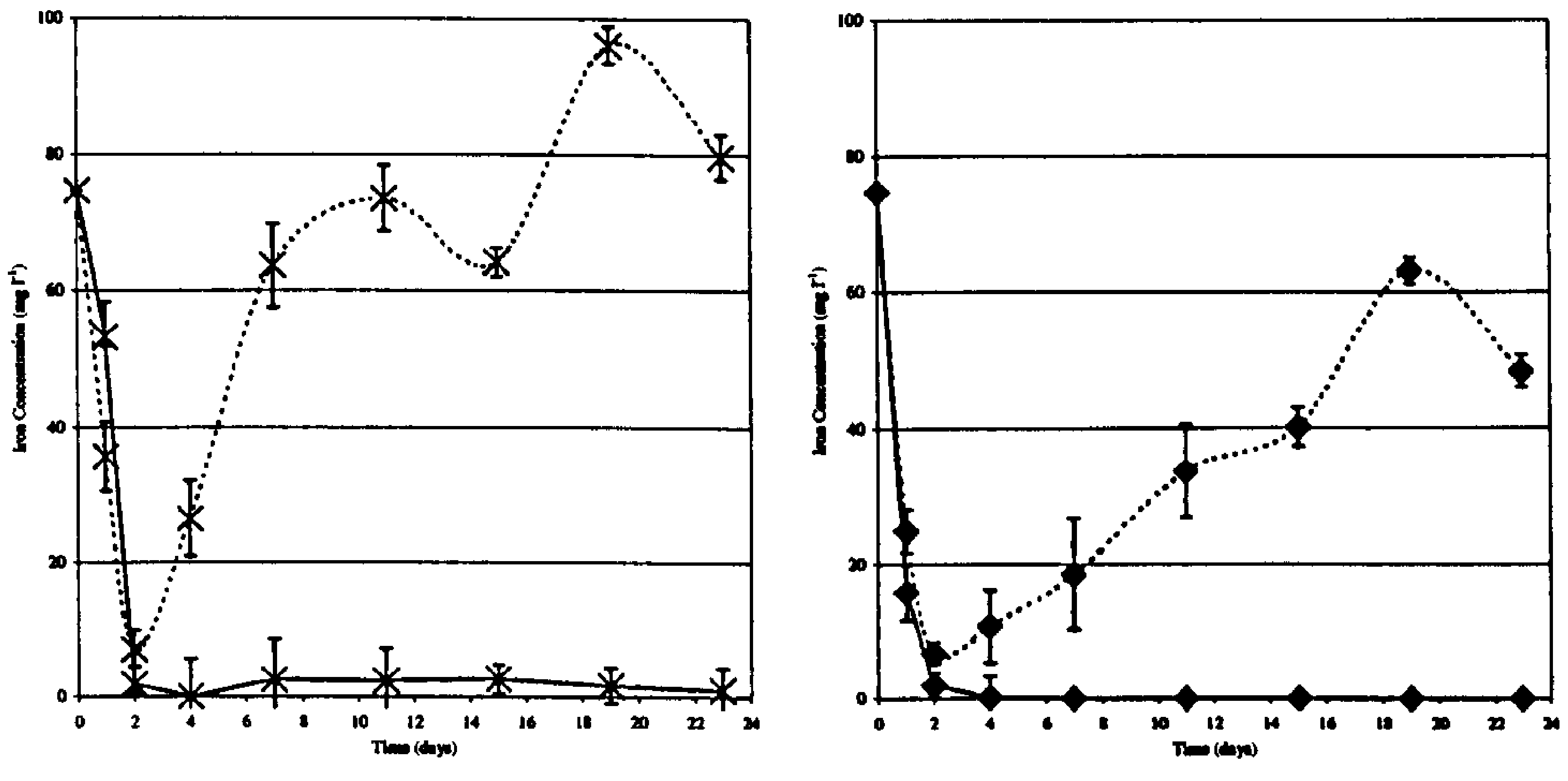
Table 4.1 compares the major physico-chemical characteristics of Ynysarwed and Mona waters. From this table it can be seen that the waters discharging at Mona adit and Ynysarwed are extremely similar in terms of their chemical characteristic and yet, the discharging waters from Mona adit are considerably more acidic (pH 2.9) than that of Ynysarwed (pH 6.3).

The effect of soil type and planting on interstitial water dissolved iron content in microcosms, following the addition of the aforementioned waters, can be observed in figures 4.2 and 4.3. The one-way analysis of variance of these data showed that there is a significant difference between the iron concentration observed in the different experiments treating Ynysarwed and Mona mine water ($F_{7,280}$=16.136, P<0.001; $F_{7,280}$=14.765, P<0.001, respectively). *Post hoc* (LSD) on those experiments treating Ynysarwed mine water showed that two distinct groups were evident. Those that contained unplanted soils had a significantly (P=0.05) lower



(a) Peat

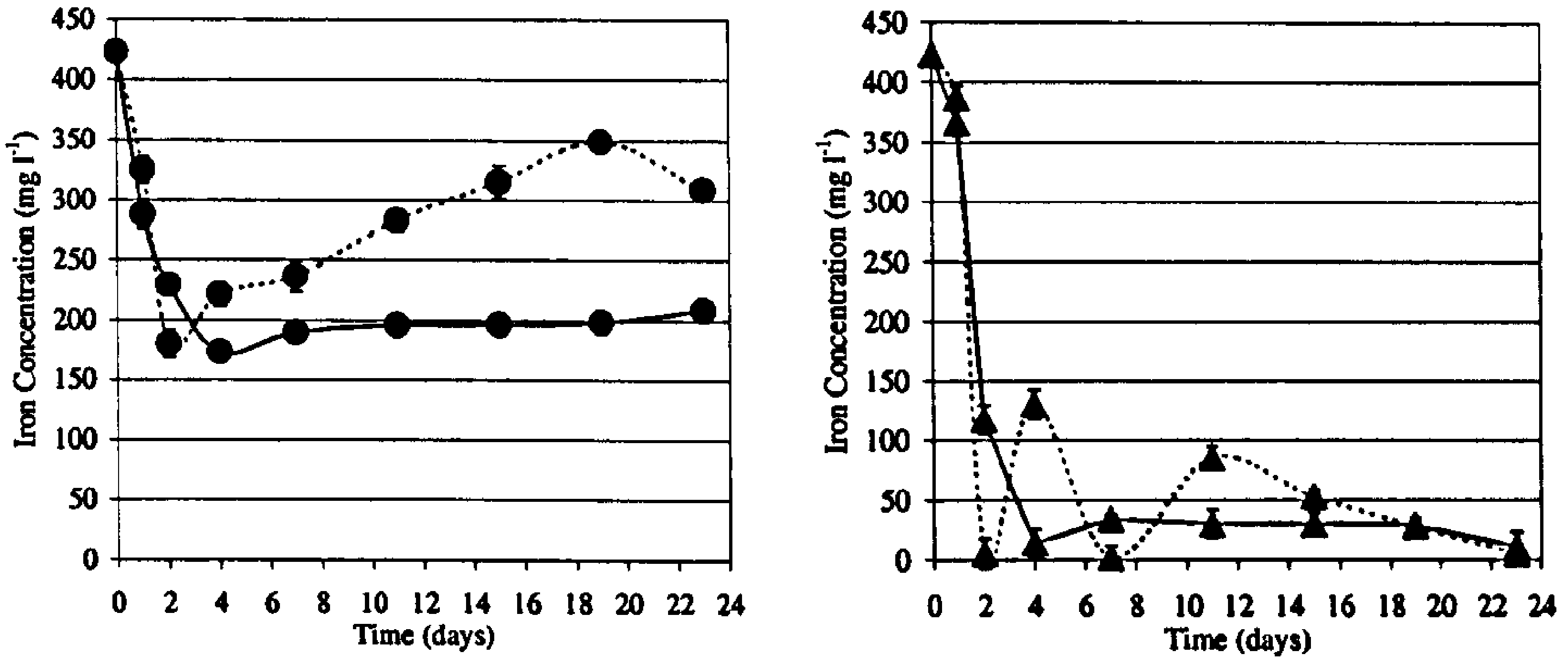
(b) Gravel



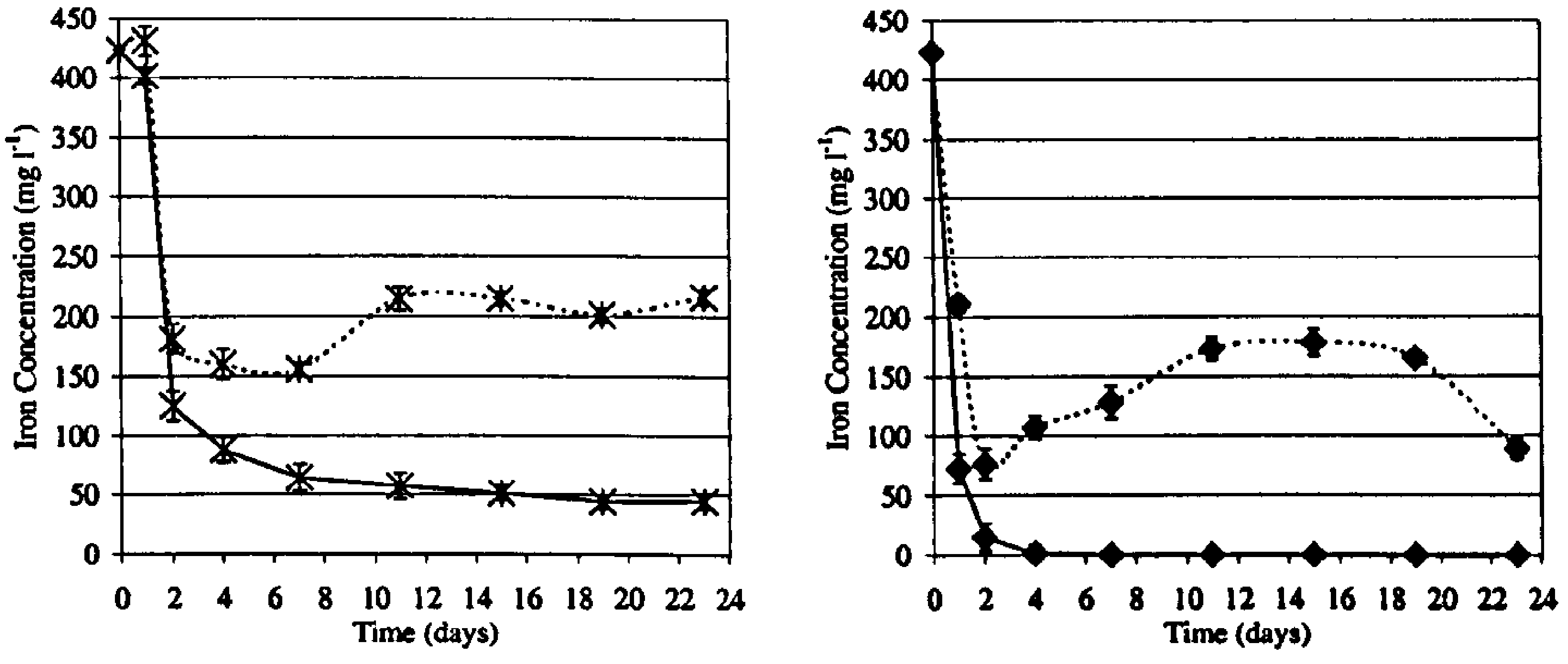
(c) Bark-chippings

(d) Mushroom Compost

Figure 4.2: The effect of soil type and planting on interstitial water dissolved iron concentrations in microcosms treating Ynysarwed mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat (b) Gravel



(c) Bark-chippings (d) Mushroom Compost

Figure 4.3: The effect of soil type and planting on interstitial water dissolved iron concentrations in microcosms treating waters obtained from Mona adit (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.

mean than those with planted soils. For those experiments treating Mona mine drainage water, four groups were evident, which are summarised with ascending mean dissolved iron concentration as follows:

$$\mathrm{MO}\quad \underline{\mathrm{GO}\quad \mathrm{PG}\quad \mathrm{BO}\quad \mathrm{PM}}\quad \underline{\mathrm{PO}\quad \mathrm{PB}}\quad \mathrm{PP}$$

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

In all of the microcosms there is a rapid decrease in dissolved iron concentration within the first twenty-four hours, though this is followed by a re-release of up to one hundred percent of the dissolved iron by the planted microcosms. This phenomenon was most marked in the microcosms treating Ynysarwed discharge waters and was akin to that found earlier in the research outlined in chapter 3. After the fluctuation, the unplanted substrates were seen to maintain fairly consistent low dissolved iron concentrations whereas those that were planted maintained higher dissolved iron concentrations to the end of the experimental period.

In terms of iron removal, mushroom compost is clearly seen (figures 4.2(d) and 4.3(d)) to be the most beneficial of the unplanted soils tested, whereas peat was the least effective (figures 4.2(a) and 4.3(a)). This was confirmed using *post hoc*

(LSD) analysis following two-way analysis of variance. The results are as follows:

Ynysarwed:

$$\underline{\text{MO}\quad\text{GO}}\quad\text{BO}\quad\text{PO}\quad\text{PP}\quad\text{PG}\quad\text{PM}\quad\text{PB}$$

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

For experiments treating Mona mine water, the order is identical to that presented for the one-way ANOVA presented earlier. However, all experiments were found to be significantly different ($P<0.05$).

More importantly, mushroom compost was the only soil, of those tested, to completely remove the iron from solution over the test period, taking four days. Plants were shown to have a negative effect compared to their unplanted counterparts, when used to remediate aqueous iron from these microcosms. After an initial decrease in iron concentration in these planted microcosms, re-mobilisation occurs. From these figures (figures 4.2 and 4.3), it can also be seen that when the circa-neutral mine drainage water is added to planted and unplanted gravel based microcosms (figure 4.2(b)), a significant difference between the dissolved iron concentrations in the microcosms is observed over the experimental period. In contrast, when acidic mine water is added (figure 4.3(b)) no significant difference is observed between the mean dissolved iron concentrations in the planted and unplanted microcosms, over the same period.

Figure 4.4 and 4.5 shows the effect of Ynysarwed and Mona mine drainage addition to the aforementioned substrates on the interstitial water sulphate concentration.

The one-way analysis of variance on the interstitial sulphate concentrations in microcosms treating Ynysarwed and Mona mine water showed that there is a significant difference between the soil types ($F_{7,280}=47.967$, $P<0.001$; $F_{7,280}=111.705$, $P<0.001$, respectively) over the experimental period. Least Significant difference analysis showed the following results (at $P=0.05$):

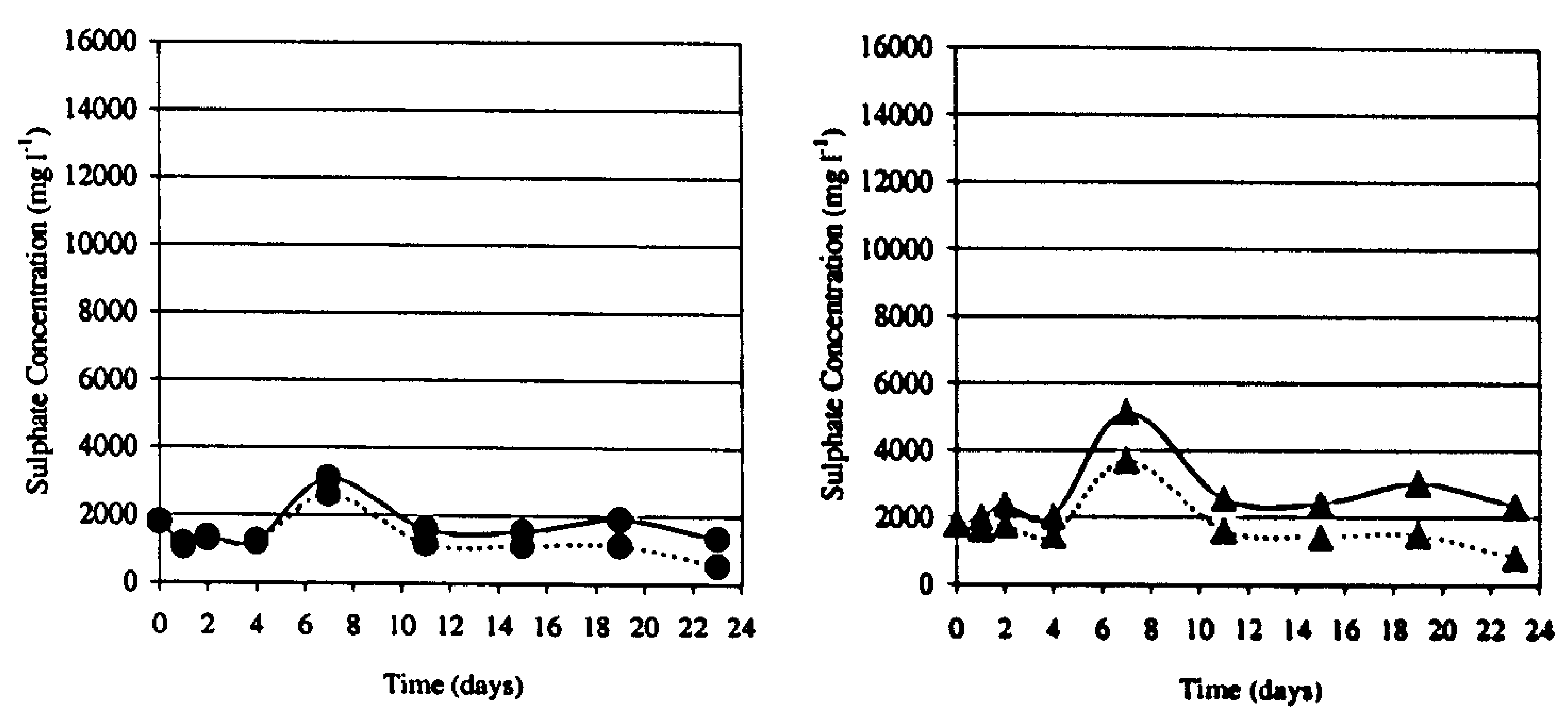
Ynysarwed:

$\text{PP} \quad \underline{\text{PB} \quad \text{PO} \quad \text{PG}} \quad \text{BO} \quad \text{GO} \quad \text{PM} \quad \text{MO}$

Mona:

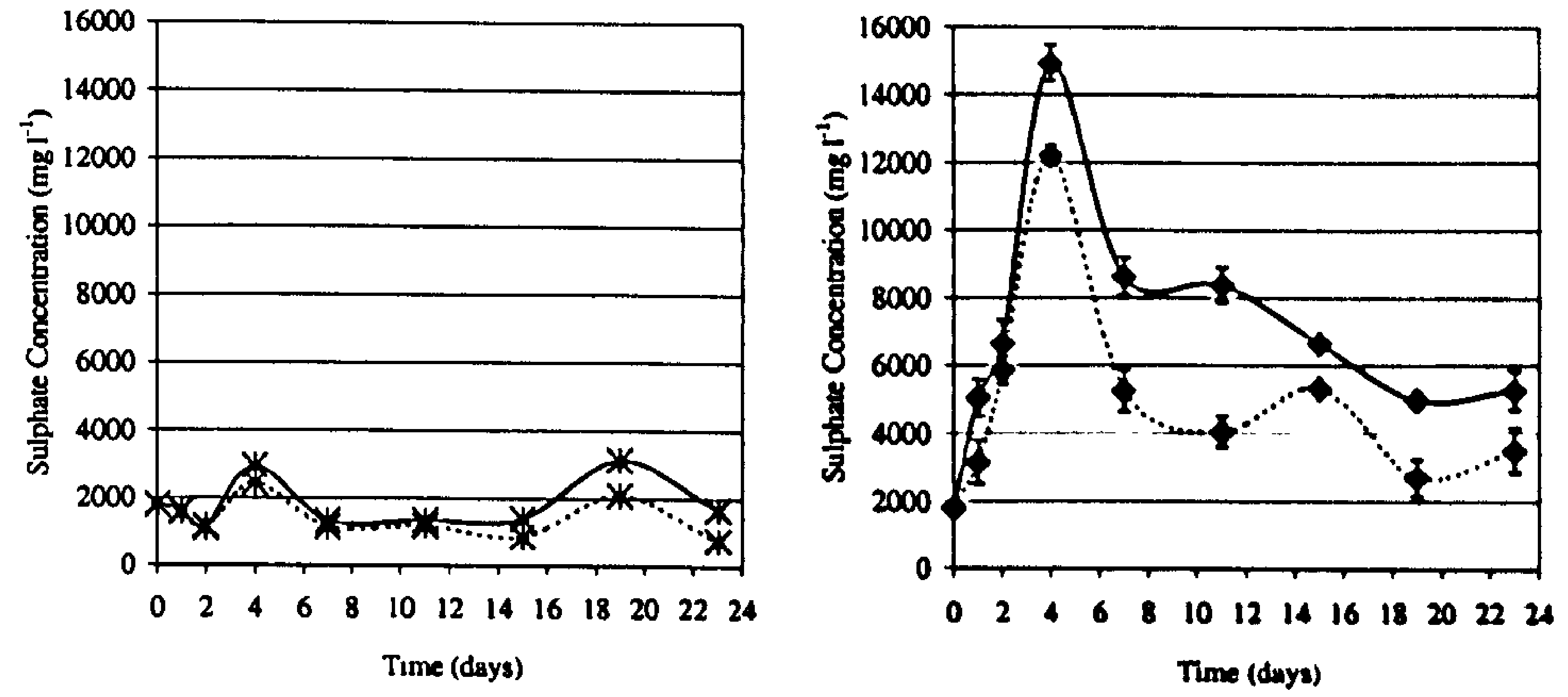
$\underline{\text{PP} \quad \text{PG}} \quad \underline{\text{PB} \quad \text{BO} \quad \text{PO} \quad \text{GO}} \quad \text{PM} \quad \text{MO}$

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel,



(a) Peat

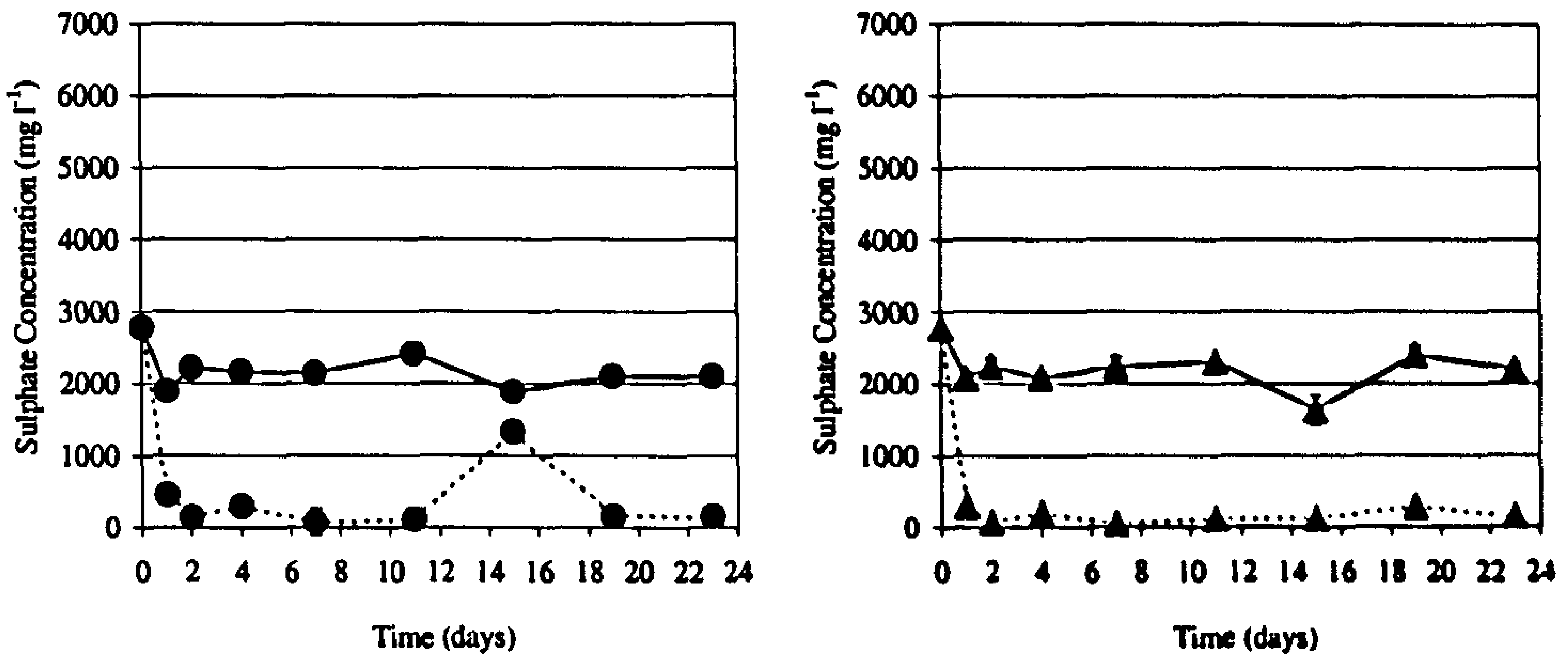
(b) Gravel



(c) Bark-chippings

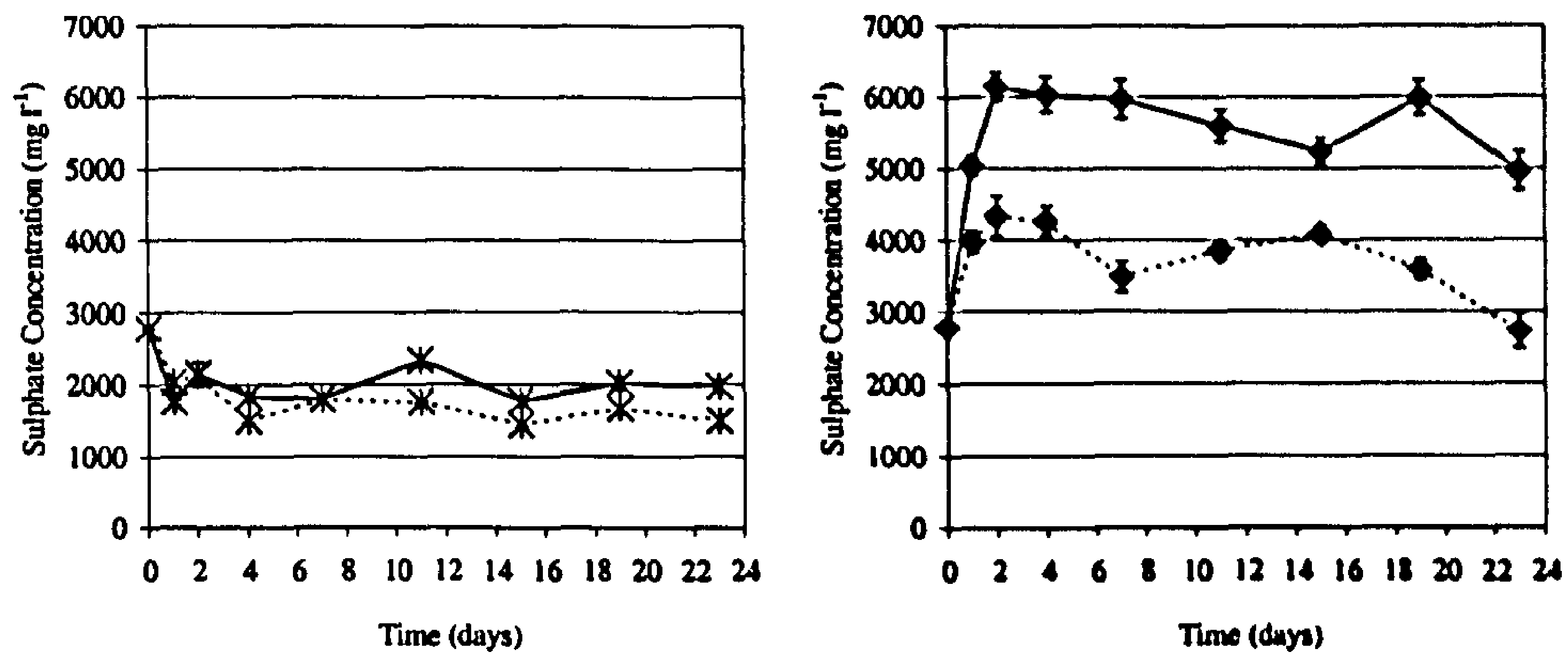
(d) Mushroom Compost

Figure 4.4: The effect of soil type and planting on interstitial water sulphate concentration, in microcosms treating Ynysarwed mine waters. (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

(b) Gravel



(c) Bark-chippings

(d) Mushroom Compost

Figure 4.5: The effect of soil type and planting on interstitial water sulphate concentration, in microcosms treating mine waters obtained from Mona adit. (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.

BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

The concentration of interstitial water sulphate observed on 'day 0' is effectively that concentration which is entering the microcosm. It can be seen from these figures that after 24 hours there is a decrease (7–47%, $P<0.05$ (Ynysarwed) and 26–89%, $P<0.001$ (Mona)) in interstitial water sulphate concentration in all of the microcosms except those containing mushroom compost in which an increase, by a magnitude of 2.81 (Ynysarwed) and 1.81 (Mona) times, is observed. In comparison, these mushroom compost based microcosms contained the highest concentrations of interstitial water sulphate, reaching their highest concentrations on day four (Ynysarwed) and day two (Mona). In the case of unplanted mushroom compost microcosms treating water obtained from Mona (figure 4.5), this sulphate concentration does not increase further before day eight and then decreases until day fifteen. This water is seen to have an increase in concentration prior to the end of the experiment, but the general trend is that of a plateau effect (i.e. neither an increase or decrease in concentration) post 48 hours, at a concentration of approximately 5900 mg l^{-1}. This effect, although less prominent, is similar to that found in the planted mushroom compost treating Mona mine waters. In mushroom compost based microcosms treating Ynysarwed water (figure 4.4) the trend that occurs in the unplanted soil almost mirrors that of the planted substrate. Here it can be clearly seen that the effect of planting on interstitial water sulphate concentrations is to yield a lower sulphate concentration than its planted counterpart (34%, P=0.0061). This phenomenon follows for all of the soils tested (25–93%; P<0.0001) with greater percentage differences in sulphate being observed in microcosms containing peat, whilst the lower percentages were found in microcosms containing mushroom compost.

Figures 4.4 and 4.5 also show that when the interstitial water sulphate concentrations are examined from those microcosms containing peat, they are consistently lower (Ynysarwed P<0.01, 6df; Mona P<0.0001, 6df) than those found in the other microcosms treating the same water, whenever the peat is planted and would therefore, be the preferred microcosm in terms of sulphate removal. This effect is more pronounced in microcosms treating Mona water, although no significant difference was found between microcosms containing planted peat and planted gravel following two-way analysis of variance and post hoc (LSD):

Ynysarwed:

$$\text{PP} \quad \underline{\text{PB} \quad \text{PO} \quad \text{PG}} \quad \text{BO} \quad \text{GO} \quad \text{PM} \quad \text{MO}$$

Mona:

PP PG PB BO PO GO PM MO

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

The effect of soil type on the interstitial water pH within the different microcosms, with respect to time, when mine drainage obtained from Ynysarwed and Mona respectively, is added, is presented in figures 4.6 and 4.7. Both figures show a similar trend in terms of alkalinity generation. For this purpose, the most advantageous soil has been shown to be that of planted mushroom compost when used to treat both waters, though the magnitude of alkalinity generation is greater in those microcosms treating Mona AMD. Figure 4.7 also shows that all of the planted and unplanted soils had an ameliorating effect on the Mona acid mine drainage water but when these soils treat the circa-neutral Ynysarwed water (figure 4.6), the unplanted soils yield a more acidic output for the first 11 days. This level of acidity tends to remain fairly stable over this period, following the initial pH decrease in the first 24 hours.

Figures 4.8 and 4.9 shows the extracellular enzyme activity of the enzymes β-glucosidase and sulphatase on soils treating water obtained from Ynysarwed and Mona respectively. It should be noted that the assay was undertaken on the last

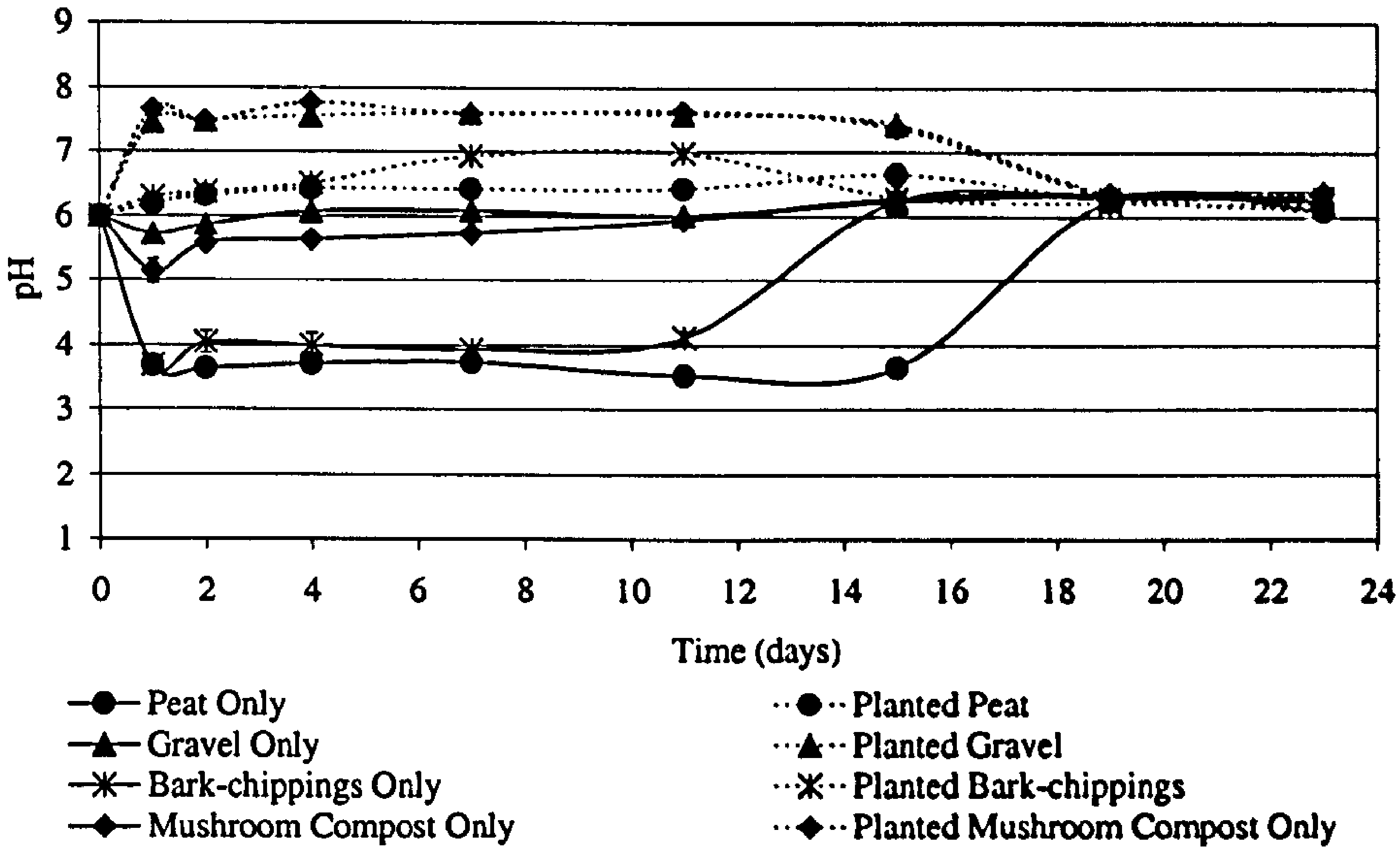


Figure 4.6: The effect of soil type and planting on interstitial water pH, in microcosms treating Ynysarwed mine waters. (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.

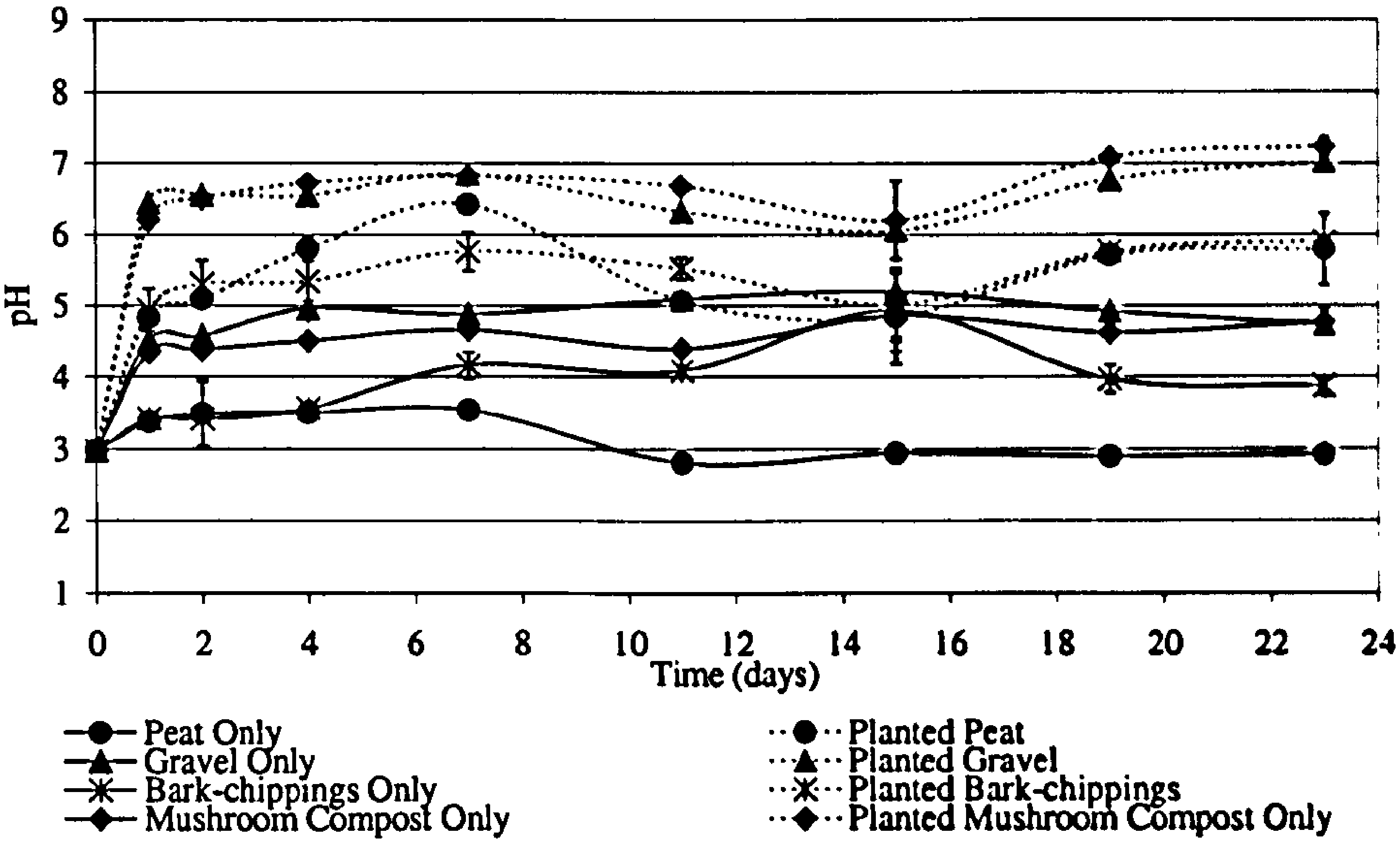


Figure 4.7: The effect of soil type and planting on interstitial water pH, in microcosms treating mine waters obtained from Mona adit. (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.

day of sampling using the fluorogenic substrates methylumbelliferyl (MUF) as outlined in chapter 2, section 2.5. From these figures, it can be seen that in mushroom compost, there is a higher amount of sulphatase activity compared to the other soils tested. These results coincide with the high levels of aqueous sulphate found in the microcosms containing mushroom compost.

One-way analysis of variance showed a significant difference in sulphatase activity in microcosms treating Ynysarwed water ($F_{7,24}=102.631$, $P<0.001$). Least significant difference analysis showed the following relationships.

$$\underline{\text{GO}\quad\text{PG}\quad\text{BO}\quad\text{PB}}\quad\underline{\text{PO}\quad\text{PP}}\quad\text{PM}\quad\text{MO}$$

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

In microcosms treating Mona mine water, a significant difference was also found ($F_{7,24}=2436.465$, $P<0.001$) and least significant analysis showed mean activities were associated in the following way:

$$\underline{\text{PG}\quad\text{GO}\quad\text{BO}\quad\text{PB}\quad\text{PO}}\quad\text{PP}\quad\text{MO}\quad\text{PM}$$

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

Analysis of β-D-glucosidase activity in microcosms treating Mona mine water, shows that the highest levels were in those microcosms containing bark-chippings, followed by peat microcosms. This is not surprising as the quantity of accessible cellulose is probably greater in bark-chippings than in peat and β-D-glucosidase is involved in the break down of cellulose to glucose units. It is also possible that the β-D-glucosidase activity is being inhibited in those microcosms containing mushroom compost due to their high concentrations of sulphate, as described by Dinesh *et al.* (1999). In microcosms treating Ynysarwed mine water this effect is reversed with the higher β-D-glucosidase activity in peat microcosms rather than microcosms containing bark-chippings. Figure 4.8 also shows that there is increased enzyme activity in planted soils compared to their unplanted counterparts. One-way analysis of variance showed a significant difference when comparing the β-D-glucosidase activity in soils treating Ynysarwed mine water ($F_{7,24}$=492.194, P<0.001). Least significant difference analysis showed all experiments to be significantly different apart from planted and unplanted gravel, and those containing planted and unplanted bark-chippings.

GO PG MO PM BO PB PO PP

Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

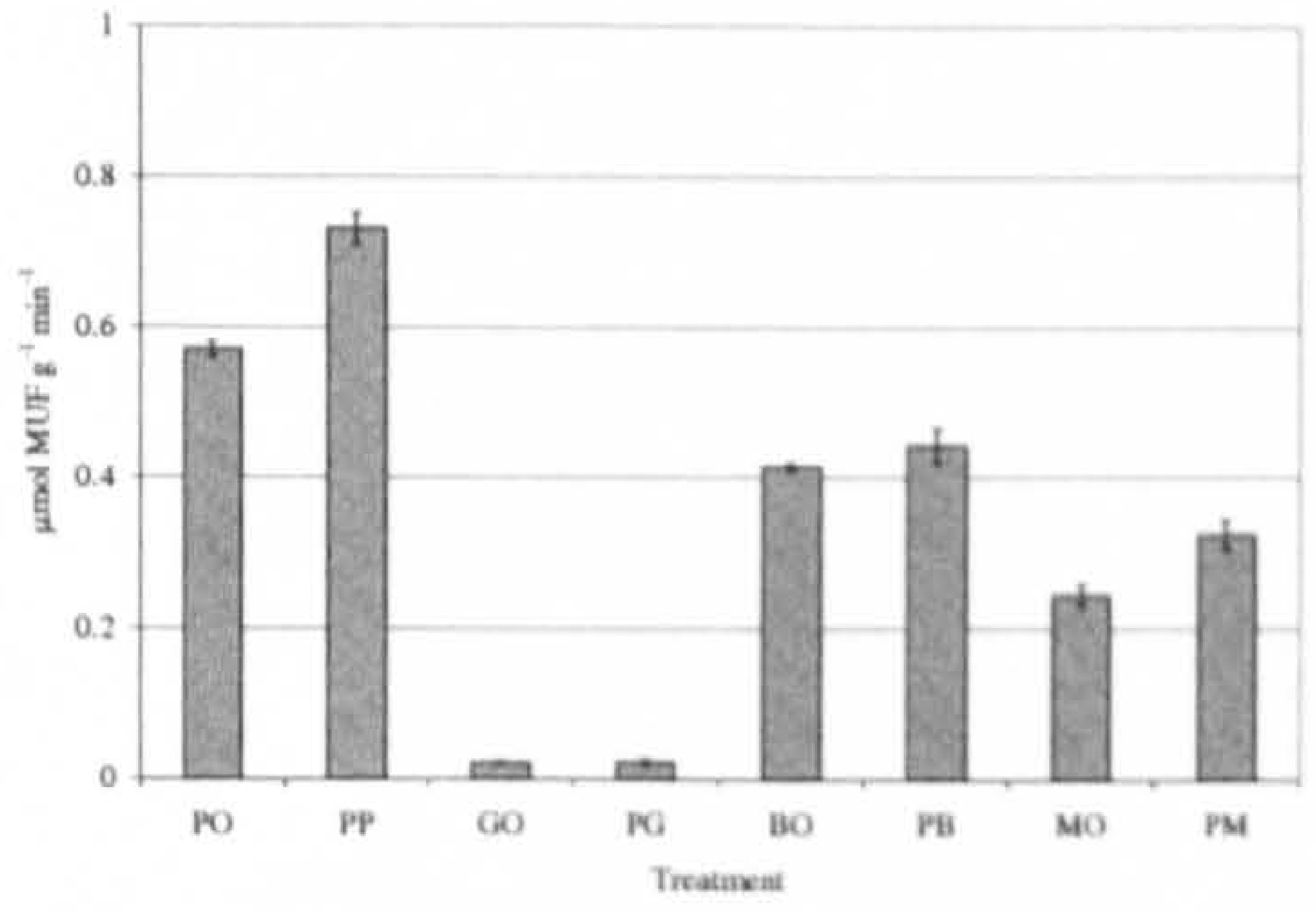
In microcosms treating Mona mine water, one-way analysis of variance showed there to be a significant difference in β-D-glucosidase activity ($F_{7,24}$=2261.173, P<0.001). Least significant difference analysis showed all experiments to be significantly different apart from planted and unplanted gravel.

$\underline{\text{GO} \quad \text{PG}}$ MO PM PO PP BO PB

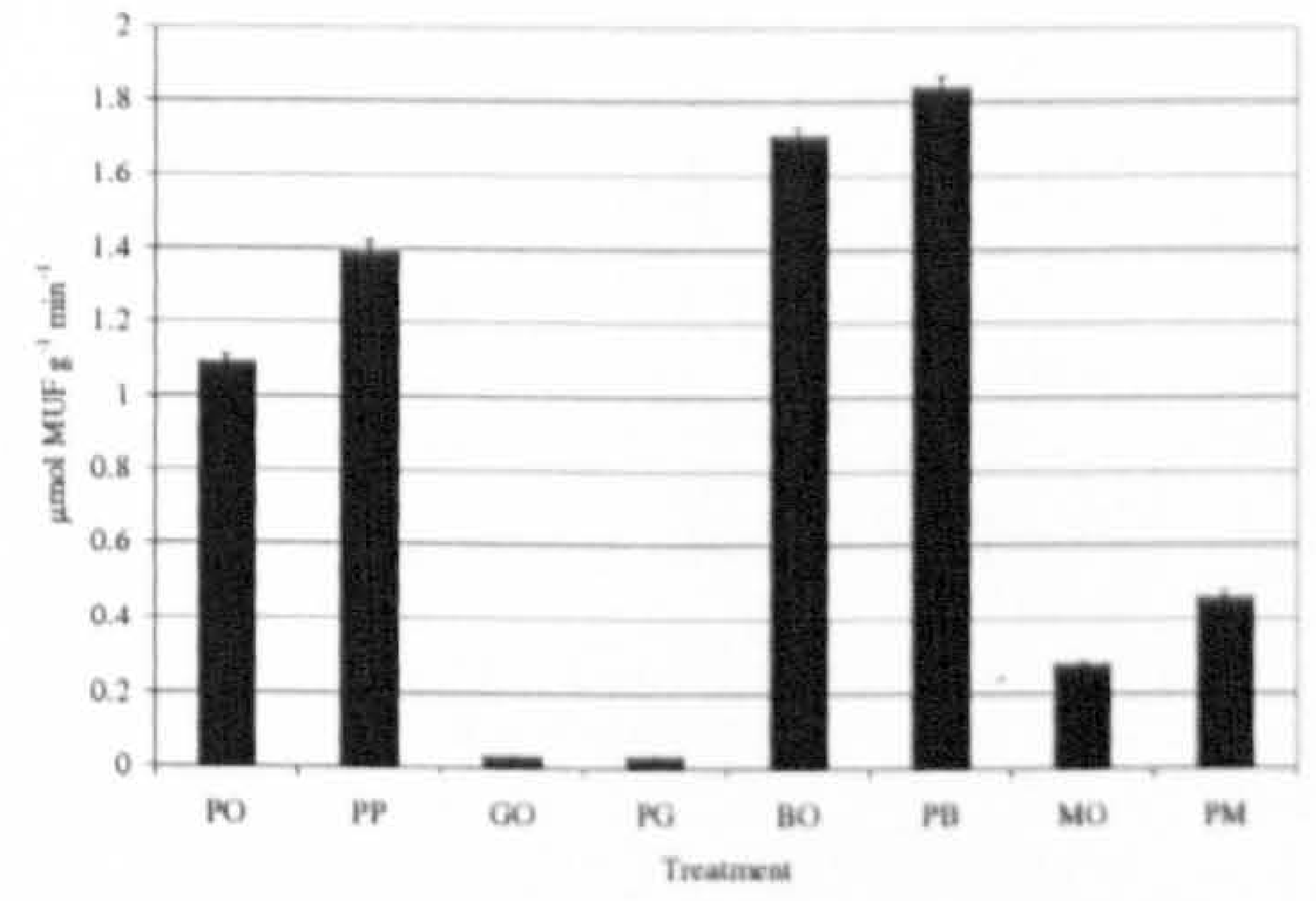
Where, PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost.

4.3 Discussion

The use of microcosms in the study of mine drainage remediation are useful in that they enable the interactions between planting and soil type to be investigated simultaneously, providing a large amount of site specific data. These systems also allow flow rate and other environmental conditions such as rainfall and temper-

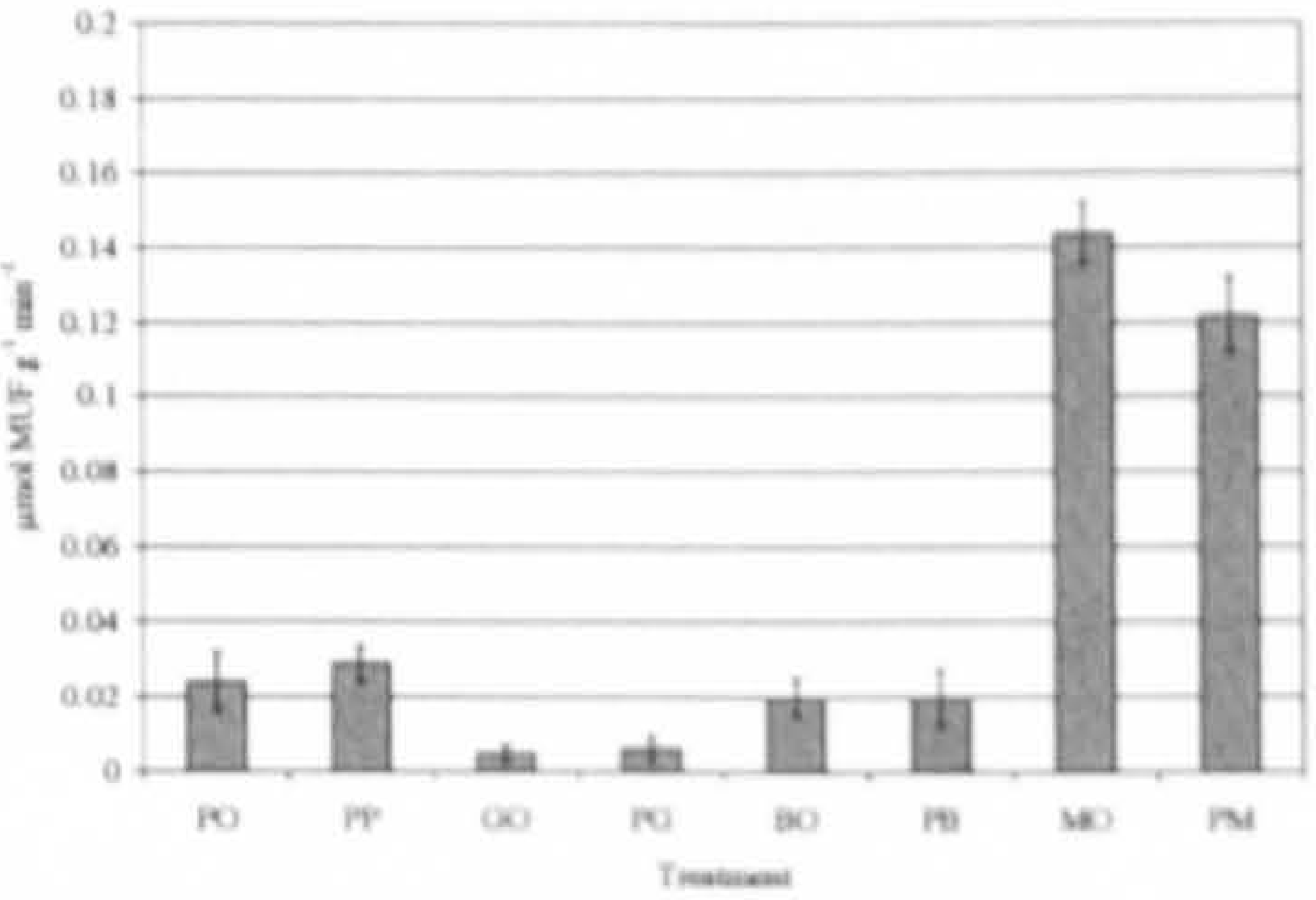


(a) Ynysarwed

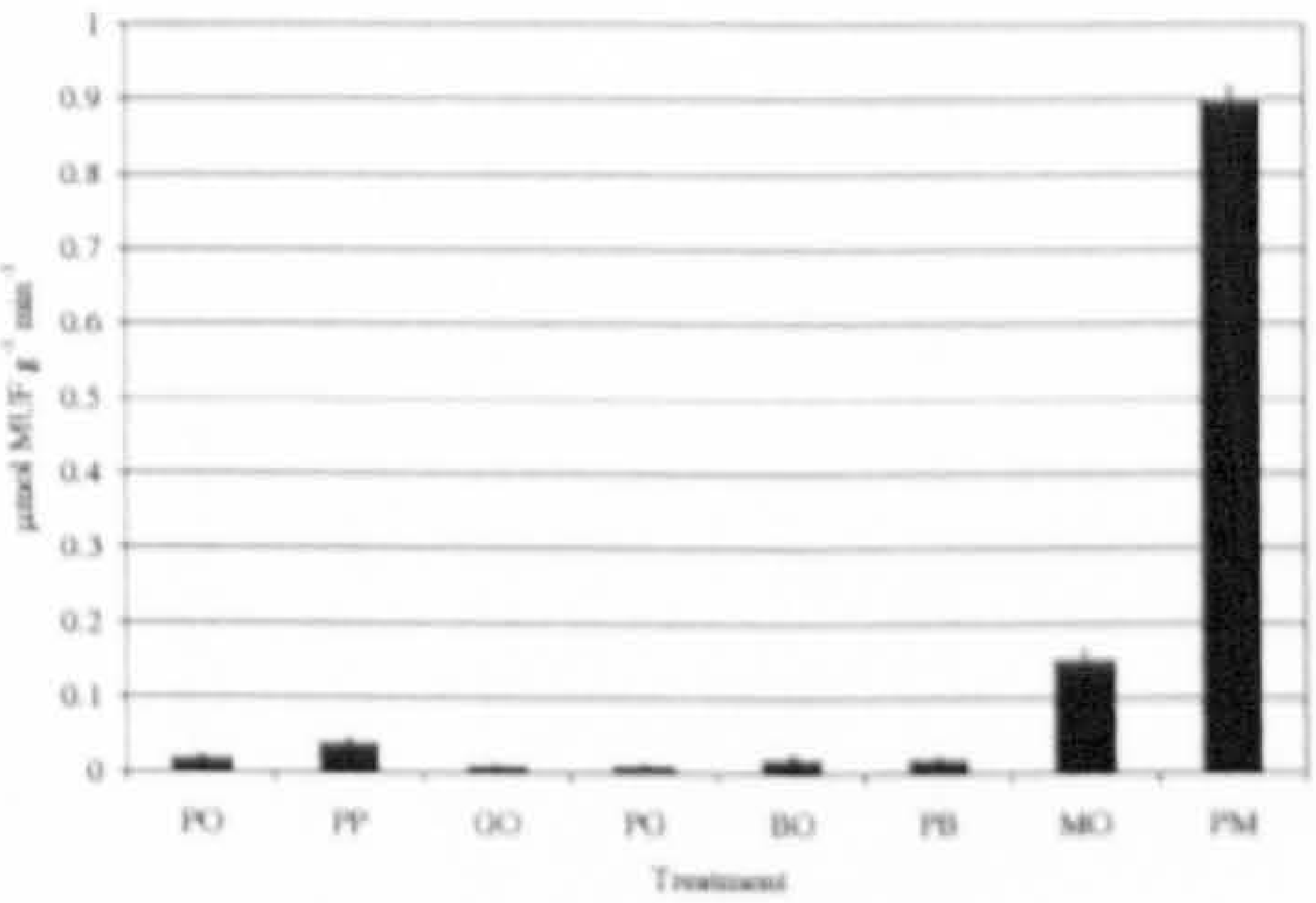


(b) Mona

Figure 4.8: The effect of soil type and planting on β-D glucosidase activity in microcosms treating mine drainage waters. Key: PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost (Mean±s.e., n=4).



(a) Ynysarwed



(b) Mona

Figure 4.9: The effect of soil type and planting on sulphatase activity in microcosms treating mine drainage waters. Key: PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost (Mean±s.e., n=4).

ature to be controlled. But they also have their drawbacks in that they are not subjected to the natural environmental affects of, for example, mixing by wind and dilution from the over-land and through flow of water. The benefits out-weigh the drawbacks and microcosms are consequentially a valuable tool in the development of cost-effective remediation strategies. Therefore, bench-scale wetland microcosms were used in this research to investigate the affect of soil type and planting on the remediation of Ynysarwed and Mona mine drainage waters.

The waters obtained from Mona are more acidic in nature than those found at Ynysarwed (pH 2.9 compared to pH 6.3 at Mona) and yet their other chemical characteristics are very similar (table 4.1). Addition of these waters to planted and unplanted microcosms containing mushroom compost, bark-chippings, peat or gravel, showed that mushroom compost was the best soil for the remediation of these waters in terms of alkalinity generation and metal removal. These findings, on the ability to remediate iron, could potentially exceed those found by Kolbash and Romanoski (1989), where only fifty percent (compared to one hundred percent over the twenty three day study period found in this research) of iron was removed in the first growing season in a sterile mushroom compost *Typha* spp. wetland which had an underlying layer of limestone. From the results outlined above (figures 4.2 to 4.7), it is apparent that for the treatment of Ynysarwed and Mona waters, a residence time of four days within the mushroom compost wetland/treatment system is required to ameliorate the problems posed by acidity and high iron and sulphate concentrations.

The effect of planting has been shown, through investigations detailed in this chapter, to have an affect on aqueous metal concentrations albeit a negative one. Re-mobilisation of iron was observed in all planted microcosms (figures 4.2 and 4.3). This phenomena was most marked in those planted microcosms treating Ynysarwed water and was not dissimilar to that detailed in chapter 3 (section 3.4, figure 3.18). Re-mobilisation of metals, in particular iron, has also been observed by Tarutis Jr. and Unz (1995). Wieder (1994) noted whilst investigating diel changes in the ratio of ferrous to ferric iron in wetlands, that iron was being re-mobilised and suggested that either sulphides, especially FeS_2, were being oxidised in the substrates or that anaerobic bacterial iron reduction was occurring. Lovley and Phillips (1986) suggest that the bacterial reduction of iron may form an integral part in the decomposition of organic matter and iron geochemistry in waterlogged soils, although, the microorganisms may only be capable of reducing a small proportion of the total iron available. Indeed, Vile and Wieder (1993) suggest that iron and sulphate reduction may initially be important in the treatment of mine drainage using wetland systems. However, as the ferric compounds increase with time within the wetland, a potential net release of iron from the wetland, may be observed (Vile and Wieder, 1993). Lindsay (1991) suggests that the production of siderophores by microorganisms and the release of acids and reducing agents in the rhizosphere, may lead to the re-solubilisation of iron. This reason may indeed explain why the re-release phenomena was more pronounced in the planted-test microcosms in this research (see figures 4.2 and 4.3). The re-mobilisation of metals, in particular cobalt, copper and lead, was also observed by Bloomfield *et al.* (1976).

Planting and soil type was also shown to have an effect on the extracellular enzyme activity of the enzymes sulphatase and β-D glucosidase in microcosms treating Ynysarwed and Mona mine drainage waters (figures 4.8 and 4.9). Soil enzymes play a crucial role in the degradation of carbohydrates found in the soil by enhancing their availability for other organisms (Eivazi and Tabatabai, 1988). For example, β-D-glucosidase breaks down cellulose into glucose units and sulphatase mobilises organically bound sulphate for the chemotrophic organisms, namely sulphate reducing bacteria. Indeed, the highest levels of sulphatase activity was observed in microcosms containing mushroom compost (figure 4.9) which also exhibited the highest concentrations of sulphate (figures 4.4(d) and 4.5(d)). Planting also had an beneficial effect of enzyme activity. Higher levels of β-D glucosidase activity was observed in the planted microcosms compared to their unplanted counterparts. β-D glucosidase will release labile carbon, by the aforementioned process, for use by, for example, sulphate reducing bacteria (SRB).

If β-D glucosidase activity is compared alongside that of sulphate concentration, it can be clearly seen that high levels of β-D glucosidase (figure 4.8) occur in planted peat soils which in turn provide the lowest concentrations of sulphate (figures 4.4(a) and 4.5(a)). Therefore, the enzymes sulphatase and β-D glucosidase play an important role in the remediation of the problems posed by mine drainage, especially in terms of iron and sulphate.

The role of plants in this system was found to be a surprising one. Research has shown that experimental wetland systems have successfully removed metals such as iron for several years (Frostman, 1993; Hedin, 1997), although unsuccessful treatment wetlands do occur such as Wheal Jane (Pers comm. 1998 Wheal Jane Site Manager and 2002 H. Prior Reading University). However, this research has shown that plants have a negative effect in terms of metal removal. This clearly raises a question about whether the systems studied by Frostman (1993) and Kolbash and Romanoski (1989) (removal of 50% of iron entering the wetland in less than a growing season) could have been more effective had the plants been removed. In contrast, in terms of sulphate removal, plants have a substantial beneficial effect. They also dramatically improve the pH within the microcosms. This was shown to be of greatest evidence in planted peat soils. To explain why these planted microcosms provide the best treatment for high sulphate and pH maintenance, two possible theories can be proposed. The plants may be providing labile dissolved organic carbon (DOC) which the sulphate reducing bacteria can utilise or the plants may be taking up the sulphate as a nutrient thus producing the same affect i.e. the observed decrease in sulphate concentration in the planted microcosms.

In these experiments indigenous microflora from a natural wetland receiving acid mine drainage from Dyffryn Adda (part of the Mynydd Parys complex) was used. Prior to the experiments outlined above, plants of the same species as those used in this research were obtained from areas further away from the adit. Experiments with these plants were unsuccessful due to their death. Reasons for this were originally thought to be a result of the trauma associated with re-location and transplantation, but careful repetition, using a control in local water, would suggest that the high concentrations found in acid mine drainage (metals and sulphates) may have caused their death. Tolerant ecotypes have been recorded by researchers such as Dunbabin and Bowmer (1992) and Ye *et al.* (1997), and naturally some of these ecotypes are more tolerant than others (Dunbabin and Bowmer, 1992). Therefore, further research is needed to test if the plants used in this research have evolved as a tolerant ecotype.

4.4 Summary

Bench-scale wetland microcosm studies using mine waters obtained from Ynysarwed and Mona, have ascertained that of the soils tested (peat, gravel, bark-chippings and mushroom compost), mushroom compost is the most valuable soil type in terms of metal, in particular iron, removal and alkalinity generation. In terms of decreasing sulphate concentration, peat planted with indigenous flora (*Eriophorum augustifolium*) from a natural wetland Mynydd Parys, Anglesey, is the best soil type. Therefore, plants have been shown to be beneficial for the removal of sulphate from (acid) mine drainage waters and the improvement of pH. However, plants were found to be detrimental to the removal of metals, in particular iron, from these waters.

Chapter 5

Mechanisms Driving the Effects of Plants and Mushroom Compost on the Remediation of Mine Waters from Mona

In the previous chapter (4), unplanted mushroom compost was shown to be the most beneficial soil, of those studied, for the remediation of mine drainage waters in terms of metal removal, particularly iron, with the classically used planted treatment systems having a negative effect. Results also showed that this unplanted soil was highly effective at raising the pH and thereby remediating acid conditions. Investigations are therefore required to ascertain whether the remediative advantages possessed by mushroom compost (high sulphatase activity and high calcium sulphate concentrations) are the reasons why mushroom compost removes iron from mine drainage water more effectively than peat soils.

Research also showed, in the same chapter, that plants had a negative effect on the removal of metals, in particular iron, from mine drainage waters. They did however, dramatically reduce the high sulphate concentrations found in these waters and maintain a circum-neutral pH, thus reducing the acidity. It was suggested that the observed effect in the planted microcosms was due to one of two affects, either the plants are producing labile dissolved organic carbon (DOC) as a substrate for sulphate reducing bacteria (SRB) or the plants themselves are utilising sulphate as a nutrient.

It has already been noted that the mushroom compost utilised in these experiments has been derived from a mixture of horse manure and straw and that it is common practice to add during its production gypsum (calcium sulphate). In an attempt to compare the relative merits of peat and mushroom compost, it was decided to add calcium sulphate to peat to confer upon it the same characteristics that are inherent in mushroom compost containing gypsum. In short, to to test whether the observed effect in microcosms containing mushroom compost is due to labile carbon in manure, calcium sulphate or sulphatase activity.

5.1 Remediation Mechanisms in Mushroom Compost

5.1.1 Materials and Methods

Unplanted peat based microcosms were set up to treat Mona acid mine drainage in-accordance with the protocol outlined in chapter 2 (section 2.2.2). Aqueous additions of sulphatase and calcium sulphate were added separately to each set (four) of test microcosms. Calcium sulphate was added at a concentration which reflected that found in mushroom compost. Sulphatase was added at a concentration of 500 units (note, one unit is defined as that which will "hydrolyse 1.0mmole of p-nitrocatechol sulphate per hour at (pH 5) at 37°C (30 min.) assay" (Sigma catalogue)). Unplanted microcosms containing mushroom compost were also set up for comparison.

Water samples were taken on a regular basis, handled as described in chapter 2 (section 2.2.2) and analysed for ferrous iron, anions, cations and pH within 24 hours.

5.1.2 Results

Dissolved interstitial water iron was monitored over an 11-day period in a variety of treatment combinations; the results of which are presented in figure 5.1. All soils were associated with a marked reduction in dissolved iron during the first 24 hours. After a period of fluctuation, all samples maintained reasonably consistent dissolved iron concentrations after 4 days. The following summarises soil combinations from the least effective (number 4) to the most effective (number 1) at decreasing the iron concentration:

Rank Order	Treatment
4	Peat
3	Peat + Sulphatase
2	Peat + $CaSO_4$
1	Mushroom Compost

Mushroom compost is clearly seen to be the most effective at decreasing the concentration of dissolved iron. Although, when $CaSO_4$ and sulphatase are added to microcosms containing peat, an increased amount of iron removal is observed than in microcosms containing peat alone. One-way analysis of variance on these data has shown there to be no significant difference between the experiments over

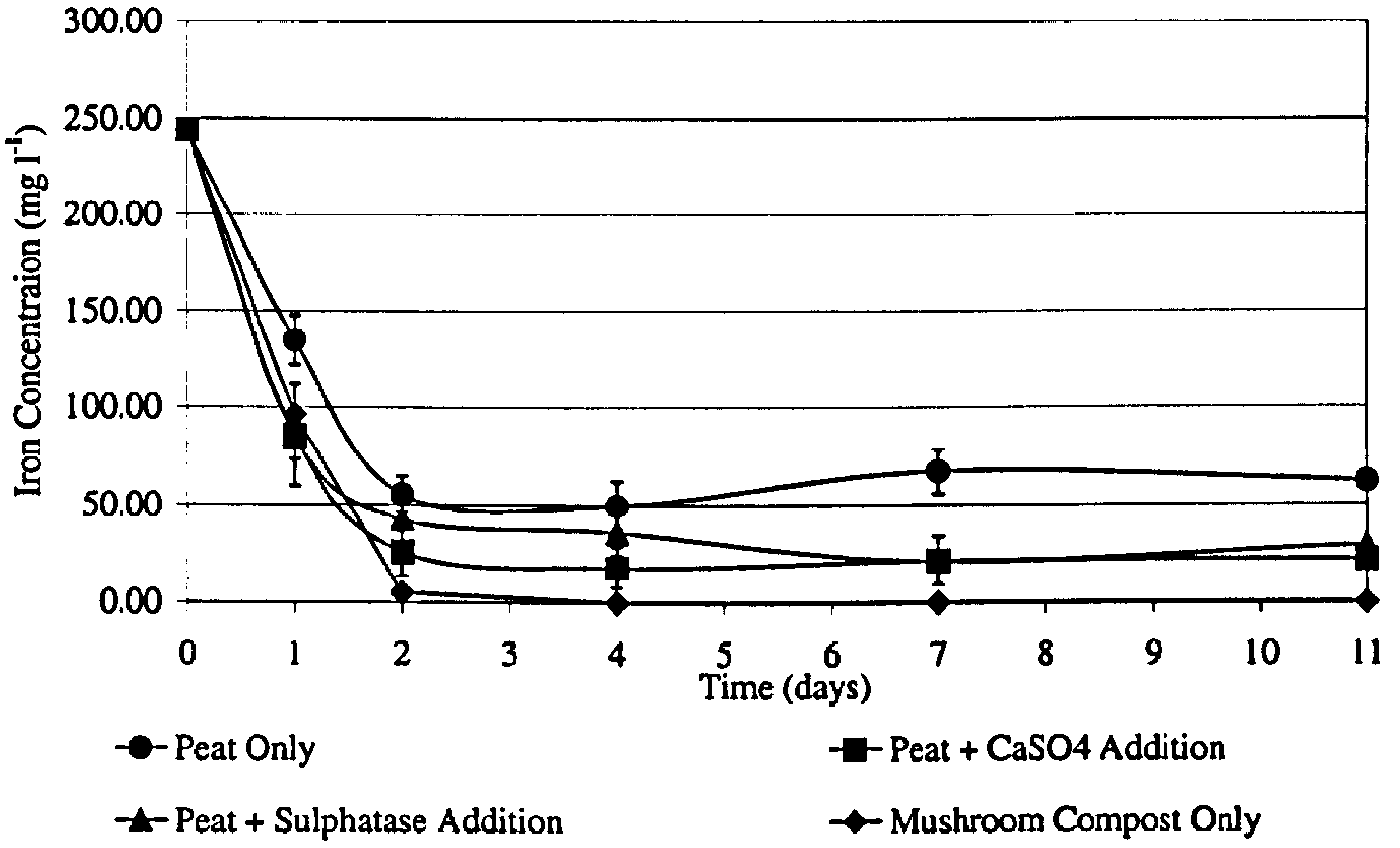


Figure 5.1: The effect on dissolved iron concentration, of sulphatase and $CaSO_4$ additions to unplanted peat microcosms, compared with unamended unplanted mushroom compost. Mean±s.e., n=4.

the experimental period. Although, two-way analysis of variance showed there to be a significant difference in the effect of experiments on dissolved iron concentration on each of the sampling days ($F_{3,92}$=29.393, P<0.001). Further analysis of these data using *post hoc* testing (LSD) at P=0.05, showed the following results:

Mushroom Compost   Peat + $CaSO_4$ Peat + Sulphatase   Peat Only

Statistically there was no significant difference between the dissolved iron concentration in the $CaSO_4$ and sulphatase peat microcosms, but these microcosms were extremely significantly different from peat microcosms without any additions.

Figure 5.2 shows how the interstitial water pH changes with respect to time when acid mine drainage is exposed to microcosms containing peat and mushroom compost. It also shows the extent by which pH changes in microcosms containing peat, modified by the addition of sulphatase and $CaSO_4$.

From this graph (figure 5.2) it can be seen that over the first 24 hours, there is an increase in pH in all the samples, indicating that the acid mine drainage water is being remediated with respect to its acidity. Mushroom compost is seen to be far more effective at raising the pH than peat and microcosms containing peat maintain the interstitial water pH at approximately 3. This has been shown to be consistent with previous trends (chapter 4). Analysis of variance (one-way) on

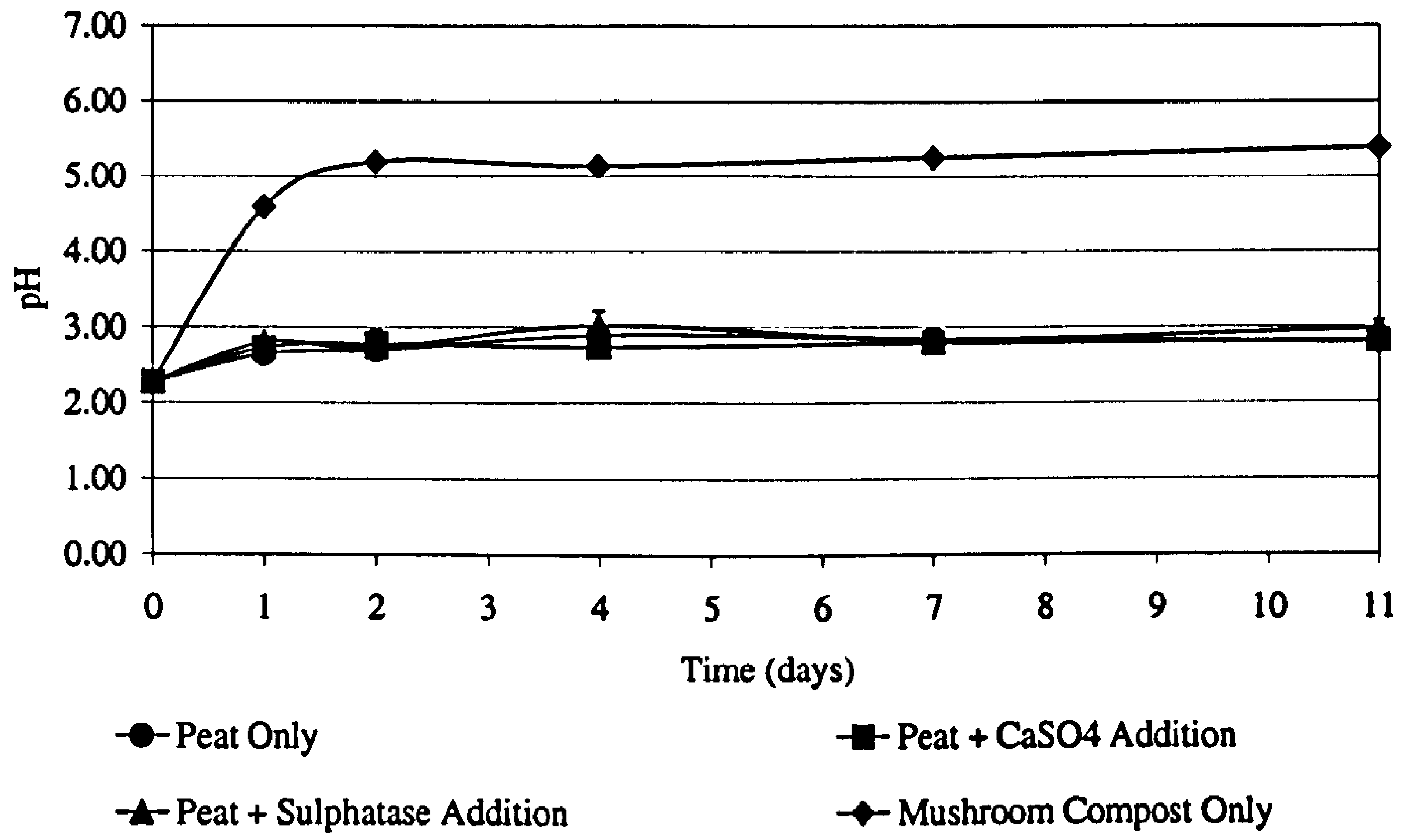


Figure 5.2: The effect of increasing the abundance of sulphatase and $CaSO_4$ in peat, on interstitial water pH. Mean±s.e., n=4.

these data showed there to be a significant difference between the pH observed in the different experiments ($F_{3,92=63.042}$, $P<0.001$) and post hoc analysis showed that there was no significant difference between the peat containing microcosms, plus/minus additions, over the experimental period:

Peat + $CaSO_4$ Peat Peat + Sulphatase Mushroom Compost

Following two-way analysis and subsequent post-hoc testing (LSD at $P=0.05$) it was found that only peat and peat with $CaSO_4$ addition were not significantly different on different sampling days:

Peat + $CaSO_4$ Peat Peat + Sulphatase Mushroom Compost

In figures 4.8 and 4.9 (chapter 4), the results showed the enzyme activity of mushroom compost was exceptionally high. It was therefore, important to determine and compare the sulphatase activity of unplanted mushroom compost and peat soils in the presence and absence of sulphatase. Figure 5.3, shows sulphatase activity in amended peat soils over time. Following the addition of acid mine drainage to mushroom compost, a marked increase in the enzyme activity was observed between day 0 and 11 of a magnitude of 3.2 times ($P<0.0001$). In comparison peat demonstrates a very low level of sulphatase activity which gradually declines over time ($P<0.05$ between day 0 and 11) and when the sulphatase concentration in the peat is artificially raised, this is initially reflected in the data. However, the activity of the additional sulphatase also declines with time (over 11 day period, $P<0.0001$). Statistical analysis using one-way analysis of variance showed there to be a significant difference between the sulphatase activity in these microcosms ($F_{2,45=30.864}$, $P<0.001$). Further testing using post hoc analysis (LSD at $P=0.05$) showed there to be no significant difference between the activity found in peat microcosms only and those with sulphatase addition:

$$\underline{\text{Peat} \qquad \text{Peat + Sulphatase}} \qquad \text{Mushroom Compost}$$

Two-way analysis of variance and subsequent post hoc testing (LSD at $P=0.05$) separated the experiments further and proved there to be a significant difference between all the experiments on each of the sampling days.

The effect of acid mine drainage addition, to peat and mushroom compost, on the interstitial water sulphate concentration, is shown in figure 5.4. 'Day 0' is effectively an analysis of the AMD that is entering the microcosms. After 24 hours, it can be seen that the sulphate concentration increased in those microcosms containing mushroom compost and peat with sulphatase or with calcium sulphate amended mine drainage water, the most significant increases being observed in the microcosms containing mushroom compost and peat with sulphatase addi-

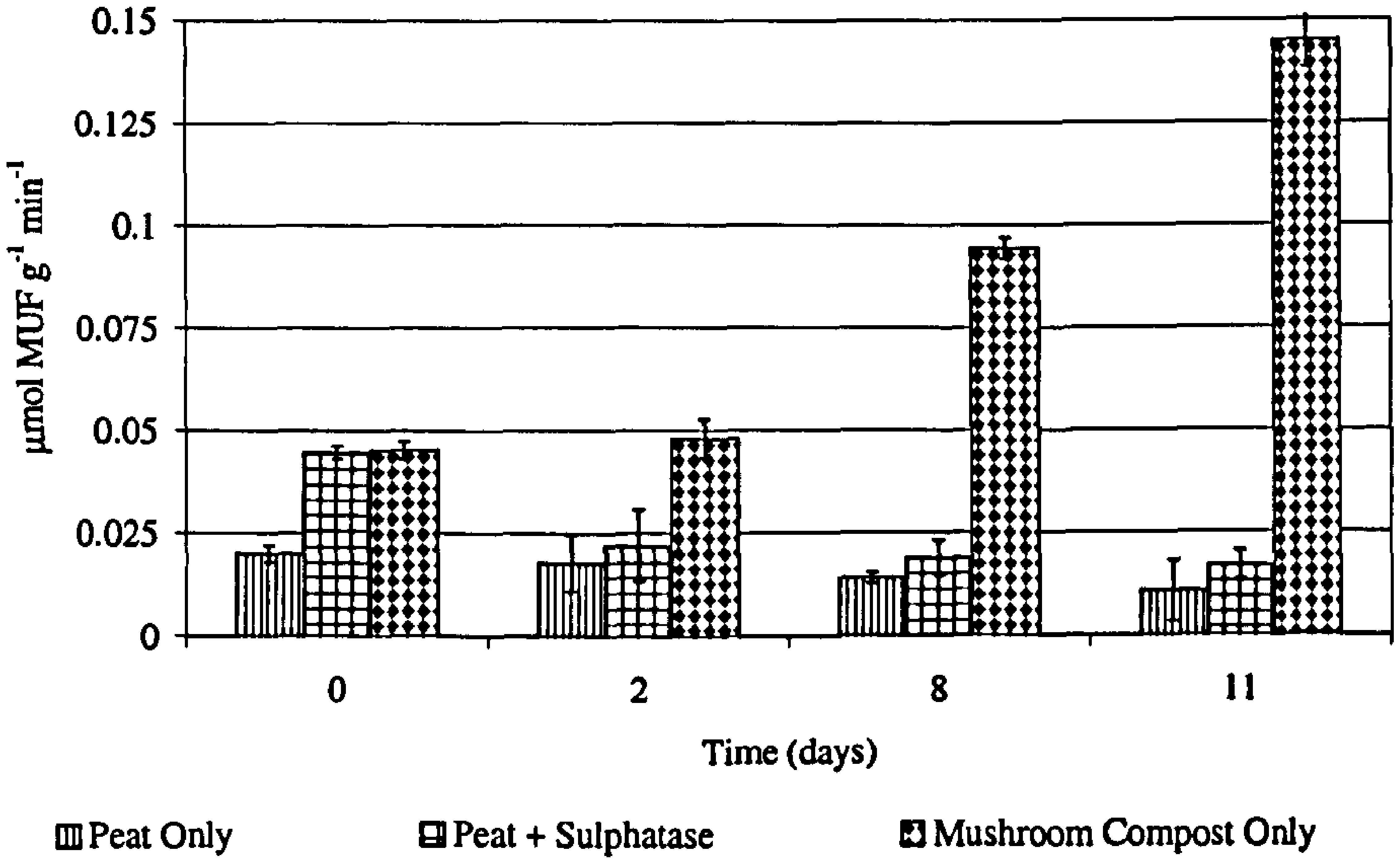


Figure 5.3: The effect, on sulphatase activity, of amending the peat microcosms with sulphatase compared with the activity in unamended peat and mushroom compost microcosms. Mean±s.e., n=4.

tions ($P<0.01$). Microcosms containing unamended peat, displayed little change in sulphate concentration over the test period and those with the addition of $CaSO_4$ maintained a similar concentration after day 4. Statistical analysis using one-way and two-way analysis of variance showed there to be a significant difference between the interstitial sulphate concentrations found in the different experiments ($F_{3,68}=6.175$, $P<0.001$; $F_{3,48=122.298}$, $P<0.001$, respectively). Least significance analysis (at $P=0.05$) showed the interstitial sulphate concentration to be significantly different only in those microcosms containing mushroom compost, compared to the other experiments:

$\underline{\text{Peat + CaSO4} \qquad \text{Peat + Sulphatase} \qquad \text{Peat}}$ $\qquad$ Mushroom Compost

It can be seen from figure 5.4 that the highest levels of interstitial water sulphate are found on day 7 in the mushroom compost microcosms. Similar results were obtained when investigating the effect of soil on water obtained from Mona Adit; results of which were discussed in chapter 4. As discussed in the aforementioned chapter, these results have shown that mushroom compost exhibited high levels of sulphatase activity.

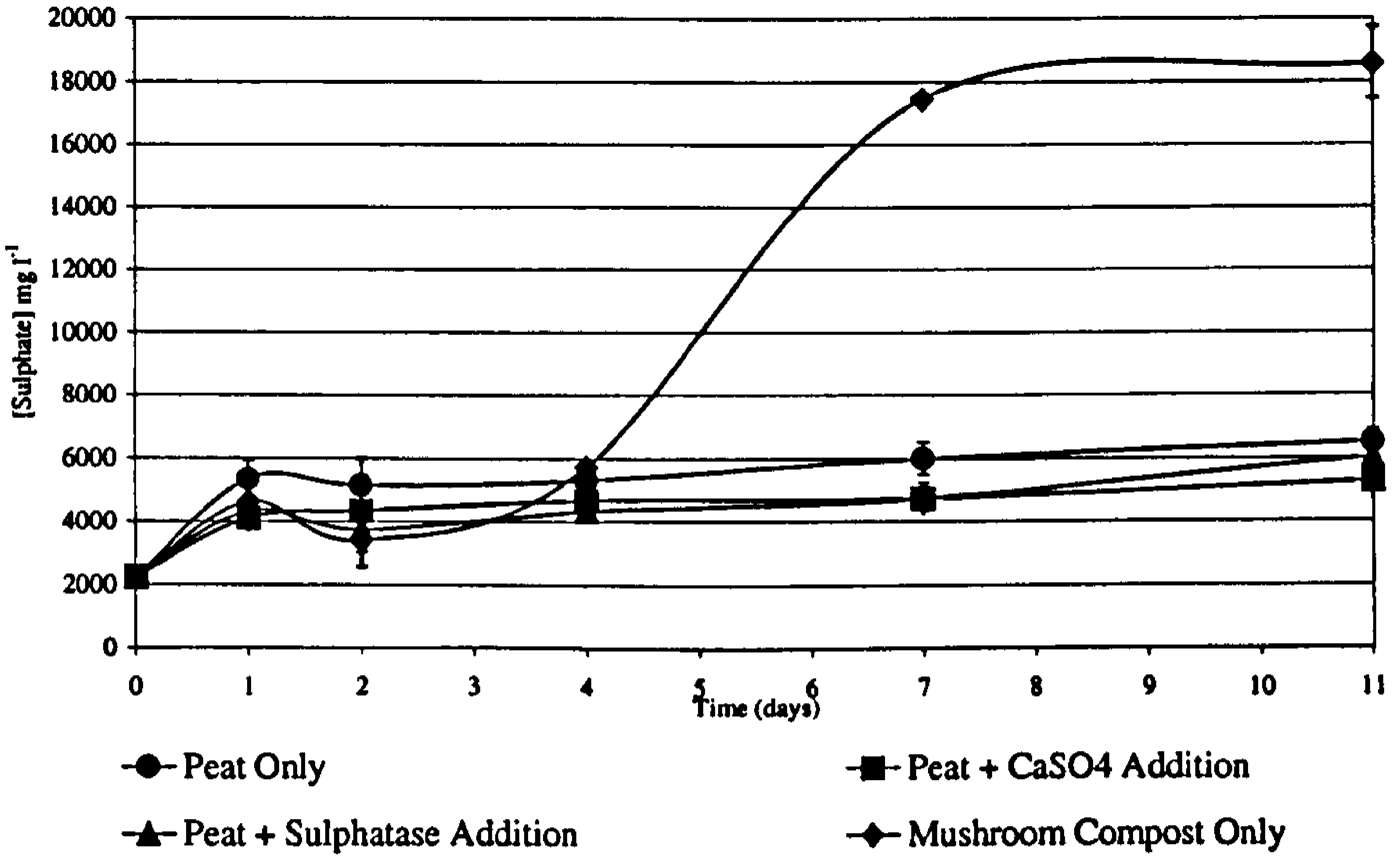


Figure 5.4: The effect on sulphate concentration of Mona acid mine drainage addition to unplanted peat and mushroom compost microcosms, compared with peat microcosms amended with $CaSO_4$ and sulphatase. Mean±s.e., n=4.

5.1.3 Discussion

In chapter 4, microcosm studies showed that mushroom compost was the most valuable soil type, of those tested, for the removal of metals from ferruginous mine drainage waters. By comparison, peat soils were not as effective at metal removal, in particular iron, but had value in terms of decreasing the concentration of sulphate found in these waters. It has been previously stated that, in the manufacture of mushroom compost, calcium sulphate is added in the form of gypsum and that microcosms containing mushroom compost also have higher levels of sulphatase activity (figure 4.9). Therefore, microcosm studies were undertaken to ascertain whether the increased levels of $CaSO_4$ and sulphatase were providing mushroom compost with the remediative advantage, in terms of iron removal, over peat.

Aqueous additions were made to unplanted microcosms containing peat and mushroom compost treating Mona mine drainage water, in the form of sulphatase and $CaSO_4$, to levels found in mushroom compost. The results of these experiments have shown that by increasing the sulphatase and calcium sulphate concentrations in peat microcosms, peat is capable of remediating the problems posed by the high iron concentrations found in Mona mine drainage water (figure 5.1), to a significantly better degree than peat alone. Although mushroom compost was still shown to be the most valuable soil in terms of metal removal, the performance of peat can be improved with sulphatase and $CaSO_4$ additions. It is possible that the labile dissolved organic carbon (DOC) present in the mushroom compost is the cause of the greater efficiency displayed by those microcosms. This will be investigated later in this thesis (chapter 7).

The results from the enzymatic study show that a marked increase in enzyme activity was observed following the addition of acid mine drainage to mushroom compost. This may be attributed to an expanding population of microflora within the microcosms. In comparison, microcosms containing peat demonstrate a very low level of enzyme activity. When the concentrations are artificially enhanced, this is initially observed in the data (figure 5.3). However, the results also show that when additional sulphatase is added, activity also declines with time, returning back to the original low levels observed in peat microcoms. In environments such as the mushroom compost the microorganisms release sulphatase which cleaves sulphate from organic matter, this in turn may be utilised by the sulphate reducing bacteria (SRB). Hence, the enzymic activity was observed to be higher in the microcosms containing a higher organic content. Although, it should be noted that sulphate can also be derived from the calcium sulphate (gypsum) added to the mushroom compost.

5.2 The Role of Plants in Treating Mine Drainage Waters

5.2.1 Materials and Methods

Planted microcosms containing peat were set up in the pre-described manner (section 2.2.2) to treat mine waters obtained from Mona. Aqueous additions of calcium sulphate and sulphatase were added to a set of four replicate test microcosms as detailed above (section 7.1.1). Once again, water samples were taken on a regular basis as described in section 2.2.2 and analysed within 24 hours.

5.2.2 Results

Interstitial water iron concentration was monitored over the 11-day experimental period in planted peat microcosms, with or without sulphatase and $CaSO_4$ additions. The results of the findings are shown in figure 5.5. Over the first forty-eight hours of this experiment, a marked decrease in iron concentration was observed in all of the experimental microcosms, followed by an increase (up-to 7 times, $P<0.05$). A decrease in iron concentration is then observed in all treatment microcosms for the remainder of the experimental period. The following summarises the most effective (1) and least effective (4) soil combination for the remediation of ferruginous mine waters:

Rank Order	Treatment
4	Peat + Plants
3	Peat + Plants + Sulphatase
2	Peat + Plants + $CaSO_4$
1	Mushroom Compost + Plants

From figure 5.5 and the above table, it can be clearly seen that mushroom compost is the most effective soil type at decreasing the high iron concentrations found in Mona mine drainage water. The addition of sulphatase and $CaSO_4$ to planted peat does enhance the remediative value in terms of the amelioration of iron. Statistical analysis using one-way analysis of variance has show there not to be a significant difference in the dissolved iron concentration between experiments. However, when taking the variation from day to day into account, further analysis using two-way analysis showed there was a significant difference ($F_{3,72}=23.045$, $P<0.001$) between all of the experiments over the experimental period and the results of LSD analysis (at $P=0.05$) are as follows:

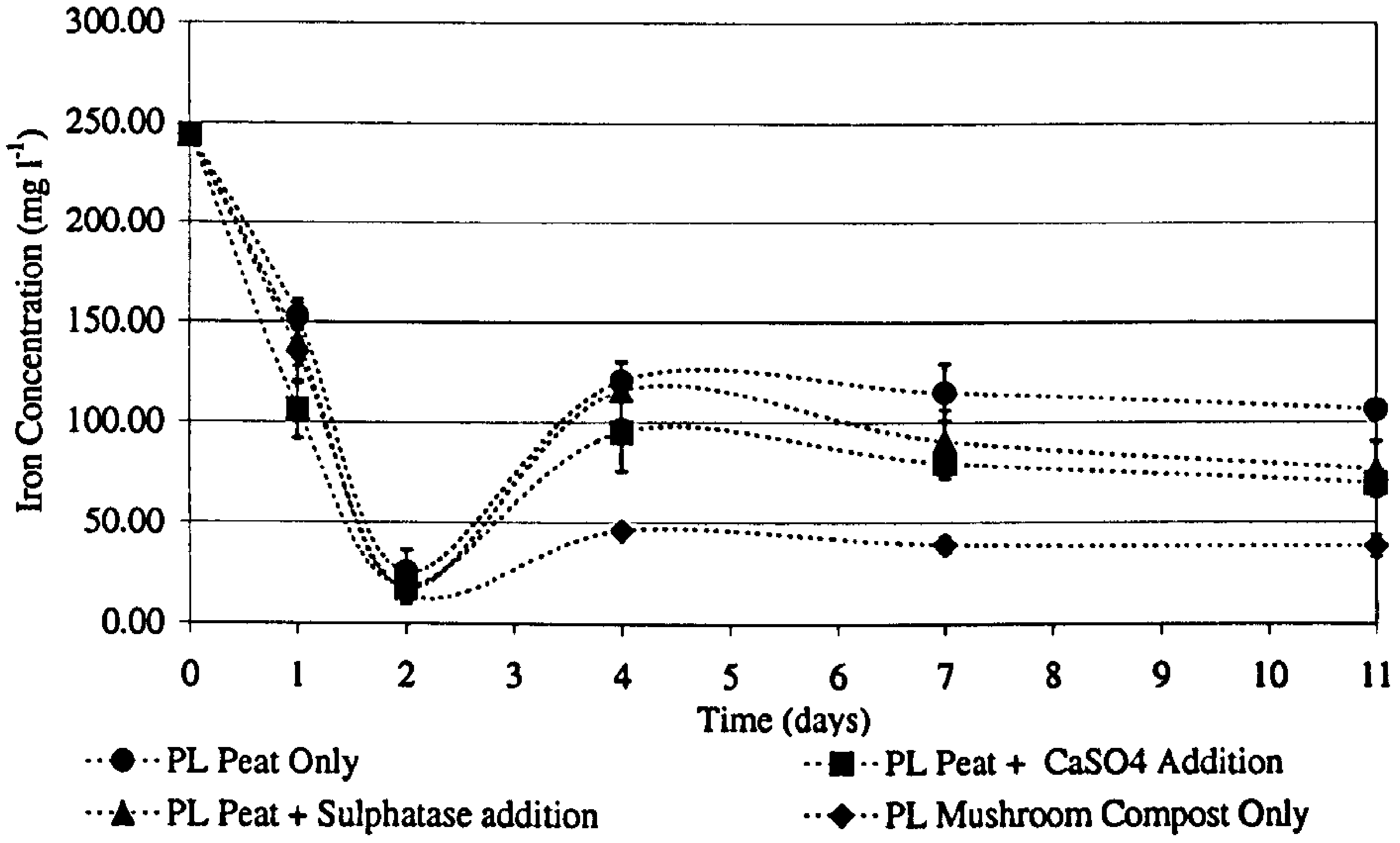


Figure 5.5: The effect on iron concentration in interstitial water, of sulphatase and $CaSO_4$ additions to planted peat microcosms, compared with unamended planted mushroom compost microcosms. Mean±s.e., n=4. Note: 'PL' denotes planting.

PL Mushroom Compost   PL Peat + $CaSO_4$ PL Peat + Sulphatase   PL Peat

Where PL denotes planting.

The effect on interstitial water pH over the experimental period of exposing planted peat and mushroom compost microcosms to Mona mine drainage water, can be seen in figure 5.6. One-way analysis of variance and subsequent post hoc testing (LSD at P=0.05) has shown there not to be a significant difference between the interstitial pH observed in the different experiments over the experimental period. Further analysis using two-way analysis of variance and post hoc testing (LSD at P=0.05) showed that there was indeed a significant difference between the experiments over the experimental period ($F_{3,72}$=190.237, P<0.001).

PL Peat   PL Peat + $CaSO_4$ PL Peat + Sulphatase   PL Mushroom Compost

Where PL denotes planting.

From figure 5.6 it can be seen that an increase in pH is observed in all soil types over the first twenty four hours, followed pH stabilisation to the end of the experimental period. This graph also shows the effect of sulphatase and $CaSO_4$

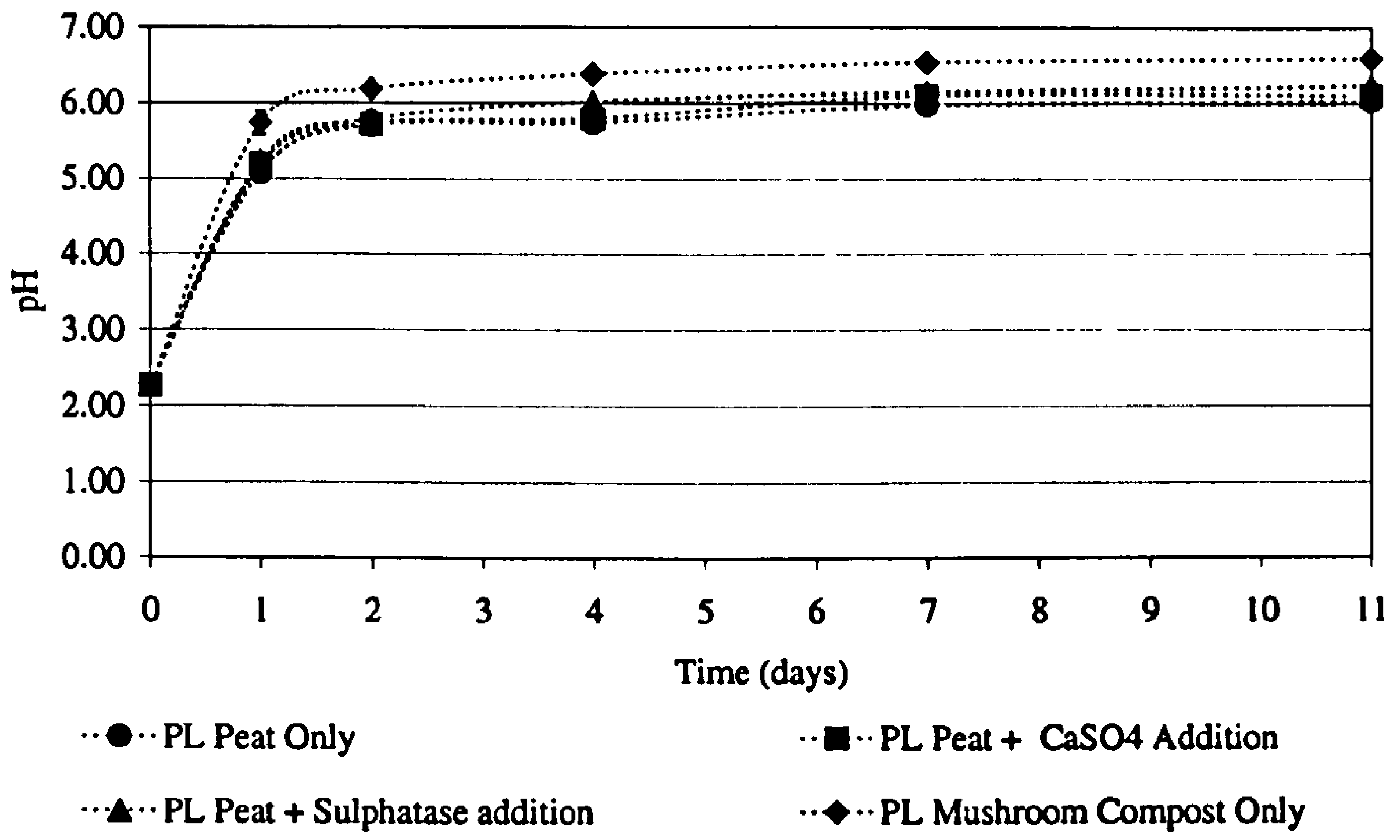


Figure 5.6: The effect of planting and increasing the abundance of sulphatase and $CaSO_4$ in peat, on interstitial water pH. Where 'PL' denotes planted soils. Mean±s.e., n=4.

additions to planted peat. It can be seen that the addition of sulphatase or $CaSO_4$ confers a slight remedative advantage over unamended planted peat soils. Once again, mushroom compost can be seen to be more effectively raising the interstitial water pH to a more neutral value (pH 6.6). If this figure is compared with that of 5.2, it can be see that once again planting confers an advantage with respect to pH remediation.

Figure 5.7, shows sulphatase activity in planted peat soils over time. It can be seen that when sulphatase is added to planted peat soils to a level equivalent to those

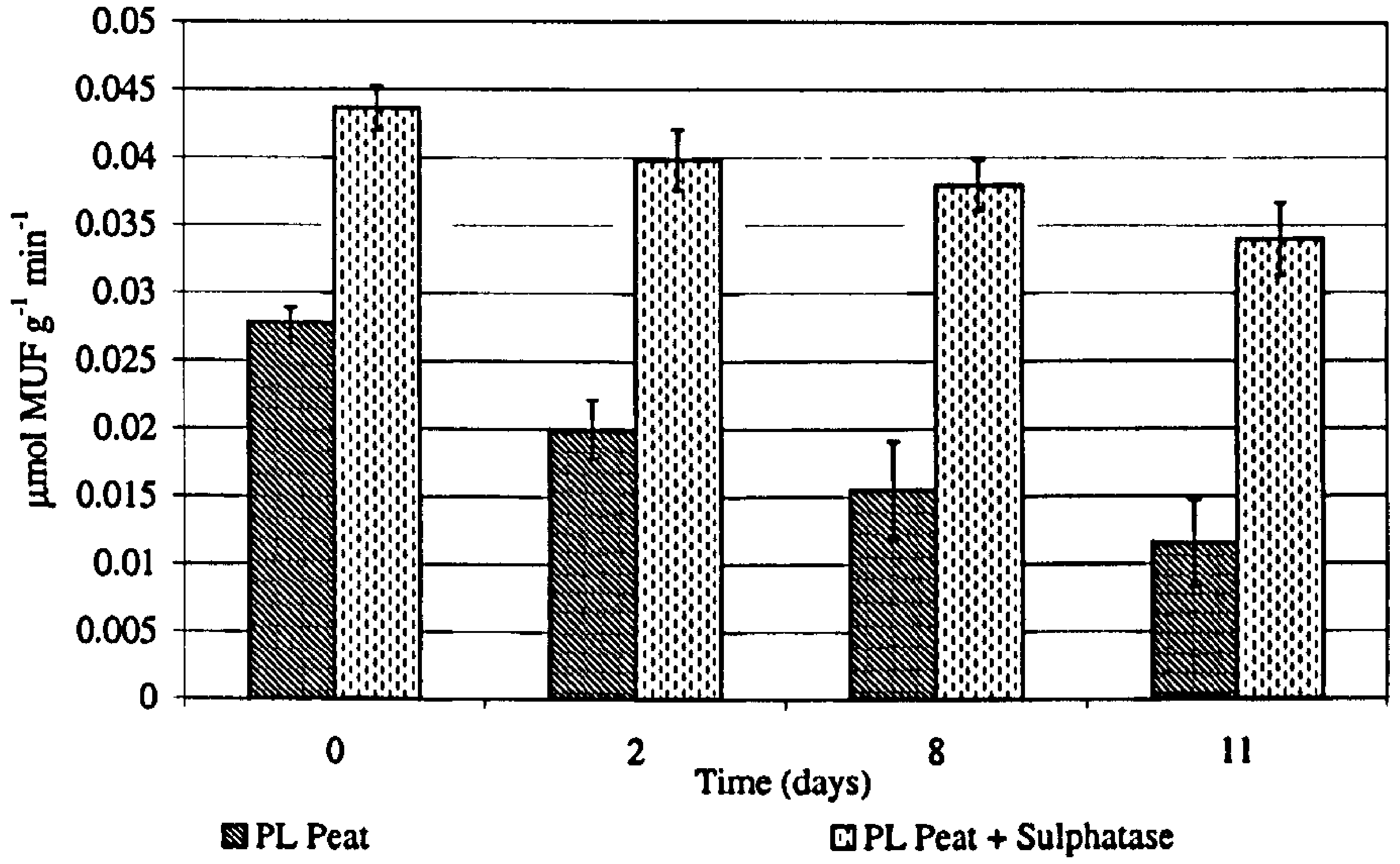


Figure 5.7: The effect, on sulphatase activity, of amending the peat microcosms with sulphatase. Where 'PL' denotes planted soils. Mean±s.e., n=4.

found in unplanted mushroom compost, activity slowly decreases with time. This decrease is also observed in the unamended planted soil. If this figure is analysed alongside that of figure 5.5, it can be seen that after forty-eight hours, little change in iron concentration is observed in the planted peat microcosm. Likewise, there is no significant difference in enzyme activity between the start and end (day 11) of the experimental period. In planted peat soils amended with additional sulphatase, there is a significant difference, using a t-test, between the enzyme activity at the start of the experiment (day 0) and at the end (day 11) (t=3.068, 6df, $P<0.05$).

previously seen (figure 4.5(a))

The sulphate concentrations for planted peat and mushroom compost soils over the eleven day experimental period, can be seen in figure 5.8. This figure also shows the effect of sulphatase and $CaSO_4$ additions to these microcosms. It can be seen that the highest levels of interstitial water sulphate can be seen in microcosms containing mushroom compost. Following the addition of Mona mine drainage water to planted peat microcosms an initial increase in sulphate is only seen in those microcosms to which additions of sulphatase and $CaSO_4$ were made, reflecting these additions. Microcosms containing planted peat did not show this trend. Planted peat showed a reduction in sulphate concentration over the first 24 hours which continued over the experimental period. Indeed, planted peat only successfully remediated the high sulphate concentration (as previously seen (figure 4.5(a))) observed in these samples and displayed the lowest sulphate concentrations overall. After sulphatase is added to planted peat, an increase was observed in sulphate concentration on day 2, followed by a slow decrease over the remainder of the experimental period. This can be related to the enzyme activity as shown in figure 5.7 where no significant difference in activity was observed. Following the addition of $CaSO_4$ to planted peat microcosms, an initial increase in sulphate concentration was observed in the first twenty four hours, then a decrease was observed to a level not significantly different from planted peat only for the remainder of the experimental period (day 4 to 11). This was also shown statistically using one-way and two-way analysis of variance on these data. The

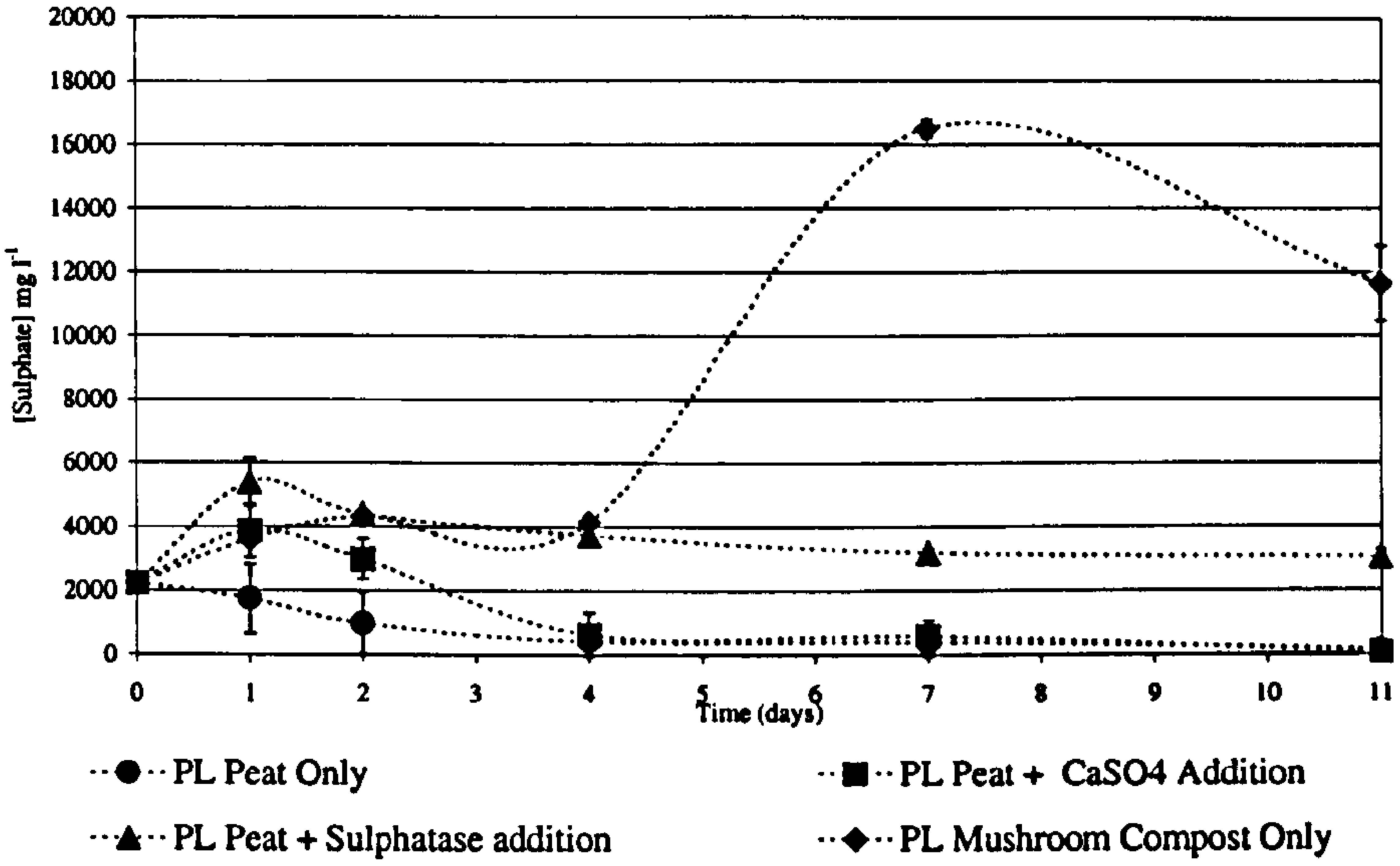


Figure 5.8: The effect of planting and increasing the abundance of sulphatase and $CaSO_4$ in peat, on interstitial water sulphate concentration, compared with planted unamended peat and mushroom compost. Mean±s.e., n=4.

analysis showed there to be a significant difference between the interstitial sulphate concentrations found in the different experiments over the experimental period ($F_{3,68}$=14.214, $P<0.001$; $F_{3,48}$=224.899, $P<0.001$, respectively). Post hoc analysis in both instances (LSD at P=0.05) showed the following results:

PL Peat	PL Peat + $CaSO_4$	PL Peat + Sulphatase	PL Mushroom Compost

Where PL denotes planting.

5.2.3 The Relative Contribution Of The Plant Material And Associated Microflora

Materials and Methods

Four planted microcosms containing mushroom compost treating mine water mine drainage water, were set up as previously described (section 2.2.2) and the aerial vegetation removed just below soil level, leaving the rhizomal ball. A further four microcosms treating Mona mine drainage water were constructed with no mushroom compost, just containing the rhizomal ball placed into the microcosm. Water samples were removed from the microcosms at regular intervals for dissolved iron and sulphate analysis.

Results

Figure 5.9 shows that when the rhizomal ball (no aerial vegetation) from the indigenous flora is added to mushroom compost based microcosms treating Mona acid mine drainage water, a more rapid rate of metal removal is observed, although sulphate concentration is increased by 2.5 fold (figure 5.10). In fact, complete remediation of iron occurred within two days. If the rhizomal ball (and its associated microflora) alone was placed in a microcosm, no change was observed in iron con-

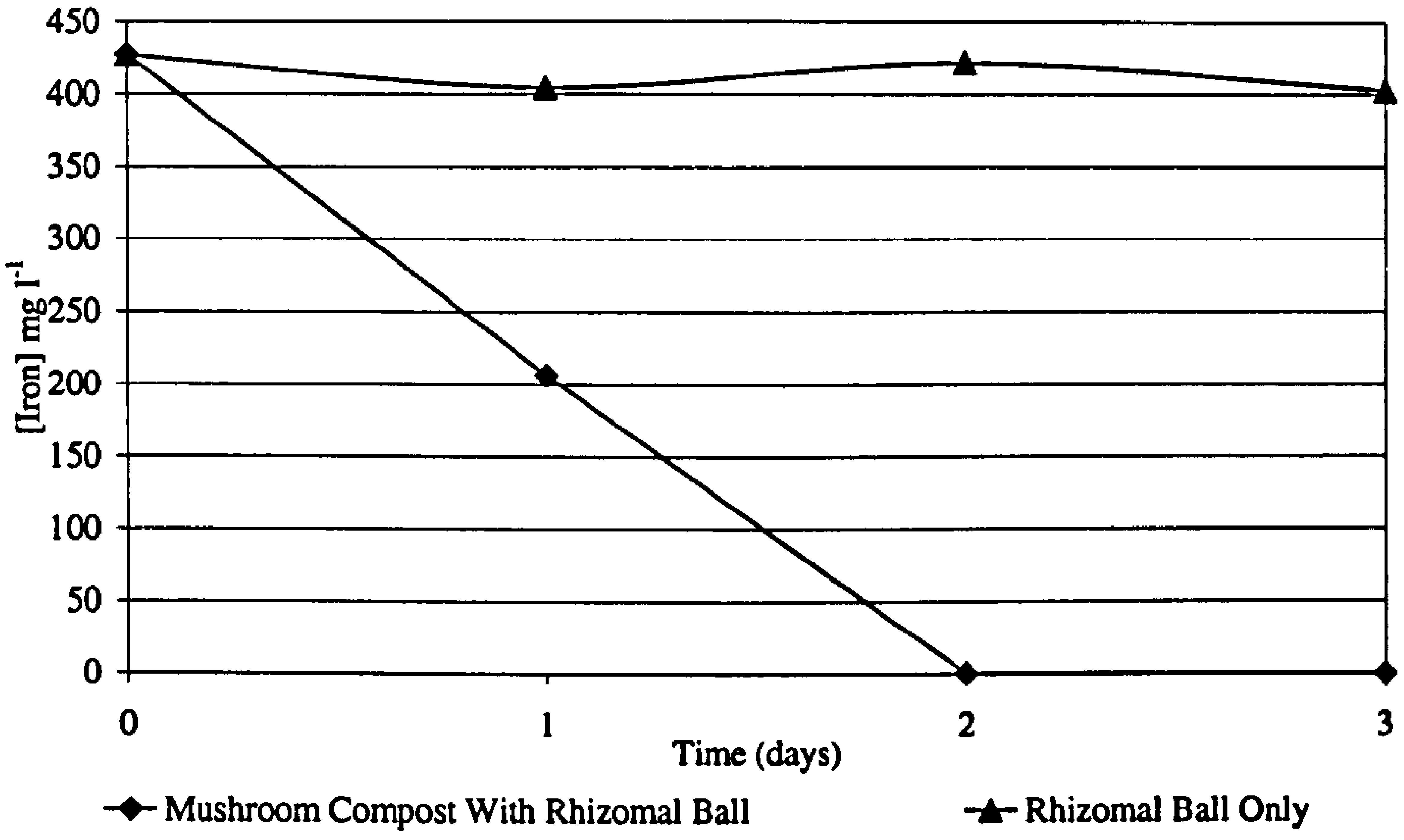


Figure 5.9: The role of the rhizomal ball and its associated microflora on the removal of dissolved iron from microcosms treating Mona mine drainage water. Mean±s.e., n=4.

centration, although, sulphate concentration was seen to reduce by 1339.15mg l^{-1} (figures 5.9 and 5.10).

5.2.4 Discussion

In chapter 4, it was shown that plants had a negative impact on metal removal but did remove sulphate and maintained a circum-neutral pH. Two possible theories for this were proposed. The plants may be utilising sulphate as a nutrient or they

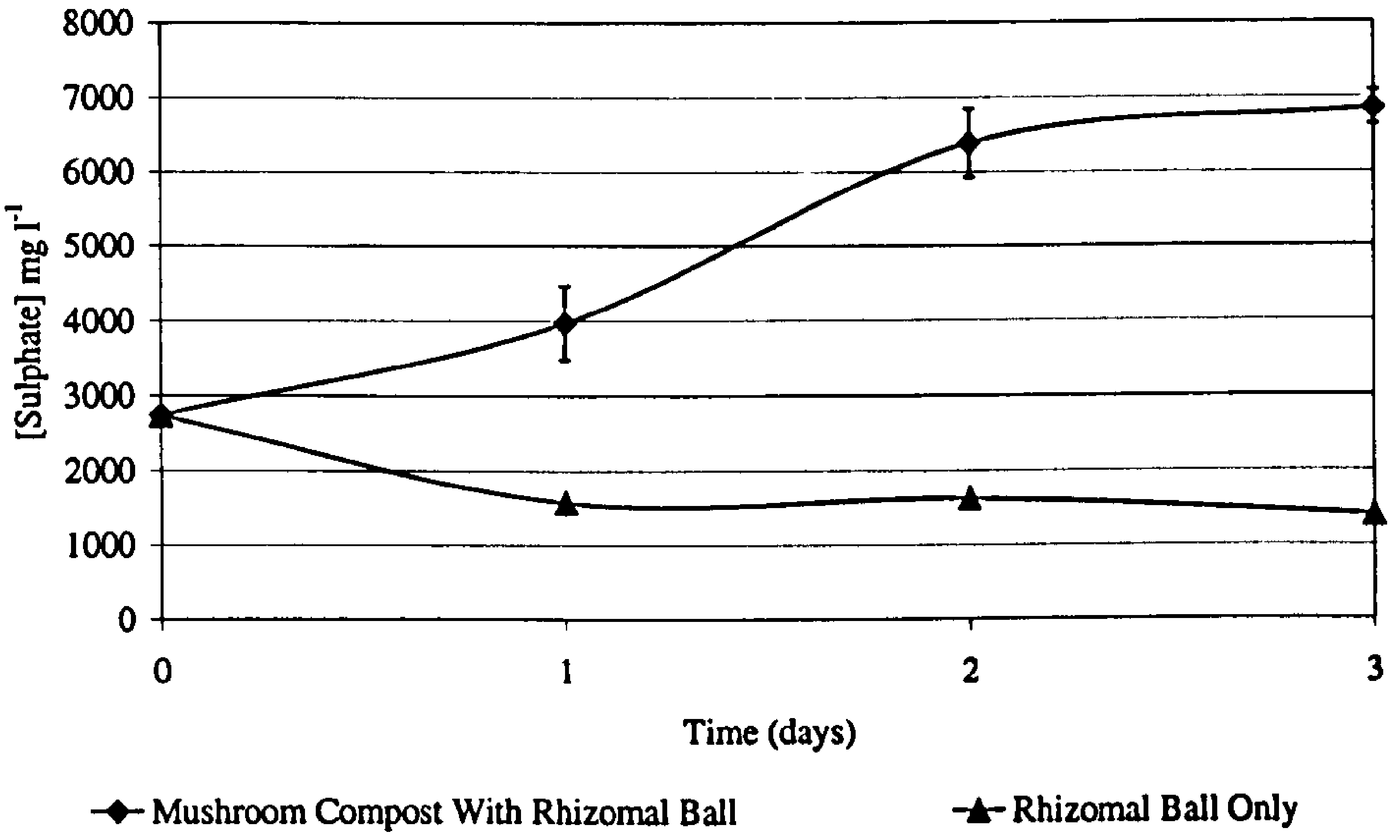


Figure 5.10: The effect of the rhizomal ball and its associated microflora on sulphate concentrations in microcosms treating Mona mine drainage water. Mean±s.e., n=4.

maybe producing labile dissolved organic carbon (DOC) which can be utilised by sulphate reducing bacteria (SRB's). Both these mechanism would display a decrease in interstitial water sulphate concentration and improve pH as observed previously (chapter 4) in the planted microcosms.

Therefore, in this section (5.2), the effect of adding additional $CaSO_4$ and sulphatase (sulphatase mobilises organically bound sulphate for SRB's) to biologically cancel out the effect of the indigenous plants, by preventing their removal of sulphate for nutritional purposes or by preventing the precipitation of metal sulphides by SRB's, was presented. The results have shown that in terms of iron removal (figure 5.5), the addition of $CaSO_4$ and sulphatase will cancel out some of the the effects of planting but, in terms of sulphatase addition, at the cost of the planted soils sulphate ameliorating capacities (figure 5.8). However, if additional $CaSO_4$ is added to these planted peat soils, these microcosms are still capable of effectively reducing the sulphate concentration to a concentration that is not significantly different to those observed in unamended planted peat soils, after day 4.

It has been found in this and previous experiments, that when interstitial water sulphate concentrations in peat microcosms are examined the sulphate concentrations are consistently lower whenever the peat is planted. The most statistically different planted treatment from its unplanted counterpart (figure 5.4) over the test period were unamended peat microcosms (t=3.349, P=0.0074, 10df). Microcosms containing mushroom compost and peat amended with sulphatase or calcium sulphate were not found to have a significantly different iron concentration between the planted and unplanted treatments.

In microcosms containing planted peat, a level of sulphatase activity, intermediate to that of peat and peat plus sulphatase, was observed. It may be that the indigenous plants used in these experiments have a high level of sulphatase producing activity. Further studies into these organisms are required and their ability to proliferate at low pH. Studies have shown (Dinesh *et al.*, 1999) that high sulphate concentrations, like those found in mushroom compost, can affect the growth of some microorganisms and as a consequence enzyme synthesis. There is little evidence to support this as the microbial population associated with the mine drainage and the indigenous plants, are indigenous to the extreme environments posed by acid mine drainage.

When the rhizomal ball (no aerial vegetation) from the indigenous flora is added to microcosms containing mushroom compost treating Mona mine drainage water, a rapid rate of iron removal is observed not too dissimilar to that found in unplanted mushroom compost microcosms (for example, figures 4.3(d) and 5.1). The removal of the aerial vegetation also enhanced the sulphate removal capabilities of mushroom compost (figure 5.10). Therefore, the negative effect of planting on metal removal may be due to competition for nutrients between plant. In this experiment, the plants' ability to photosynthesise and respire etc., is removed along with its aerial vegetation. This hypothesis will be investigated further in another chapter (chapter 7).

5.3 Summary

Microcosm studies showed that when the natural concentrations of sulphatase and $CaSO_4$ are enhanced in unplanted peat microcosms to levels similar to those found in mushroom compost, it was possible to improve the iron remediation ability (by $\sim$60%) of peat soils treating Mona mine drainage water. Although, mushroom compost was still the most effective soil type for iron removal. Experiments also showed that if these additions were made to planted peat soils, the detrimental effects of planting i.e. negative impact on metal removal, could be reduced by $\sim$30%. $CaSO_4$ was shown to be the most effective addition in that sulphate removal was not compromised.

Chapter 6

The Potential Wider Application of Mushroom Compost, Peat and Gravel in the Remediation of Contrasting Mine Waters

From the research detailed thus far in this thesis, mushroom compost has been shown to be the most valuable soil, of those tested, in terms of iron removal and alkalinity generation. Planted peat on the other hand, was required to reduce sulphate concentration and maintain a circum-neutral pH. It was considered essential to test the hypothesis that the same pattern would be observed in mushroom compost and peat soils treating mine-waters with different physico-chemical characteristics i.e. the universality of the proposed soils, thus enabling a successful remediation strategy for the treatment of (acid) mine drainage to be proposed (chapter 8). Waters for this study were obtained from several mining regions around the British Isles. Testing of waters from more sites was intended but this was not fulfilled.

6.1 The Universality of the Proposed Soils

6.1.1 Materials and Methods

Water samples were collected from the mine drainage sites outlined in the next section. These water samples were then added to 7 cm (diameter) × 4.8 cm (height) microcosms, set up in quadruplicate, containing peat, mushroom compost or gravel (control) within 24 hours of collection. These unplanted microcosms were much smaller than those detailed shown in figure 2.7 (chapter 2, section 2.2.2) but set up in the same way. Small aliquots (3ml) of interstitial water were then removed on a daily basis for metal analysis (dissolved iron), sulphate analysis and pH determination. Previous results have shown that planting enhances the

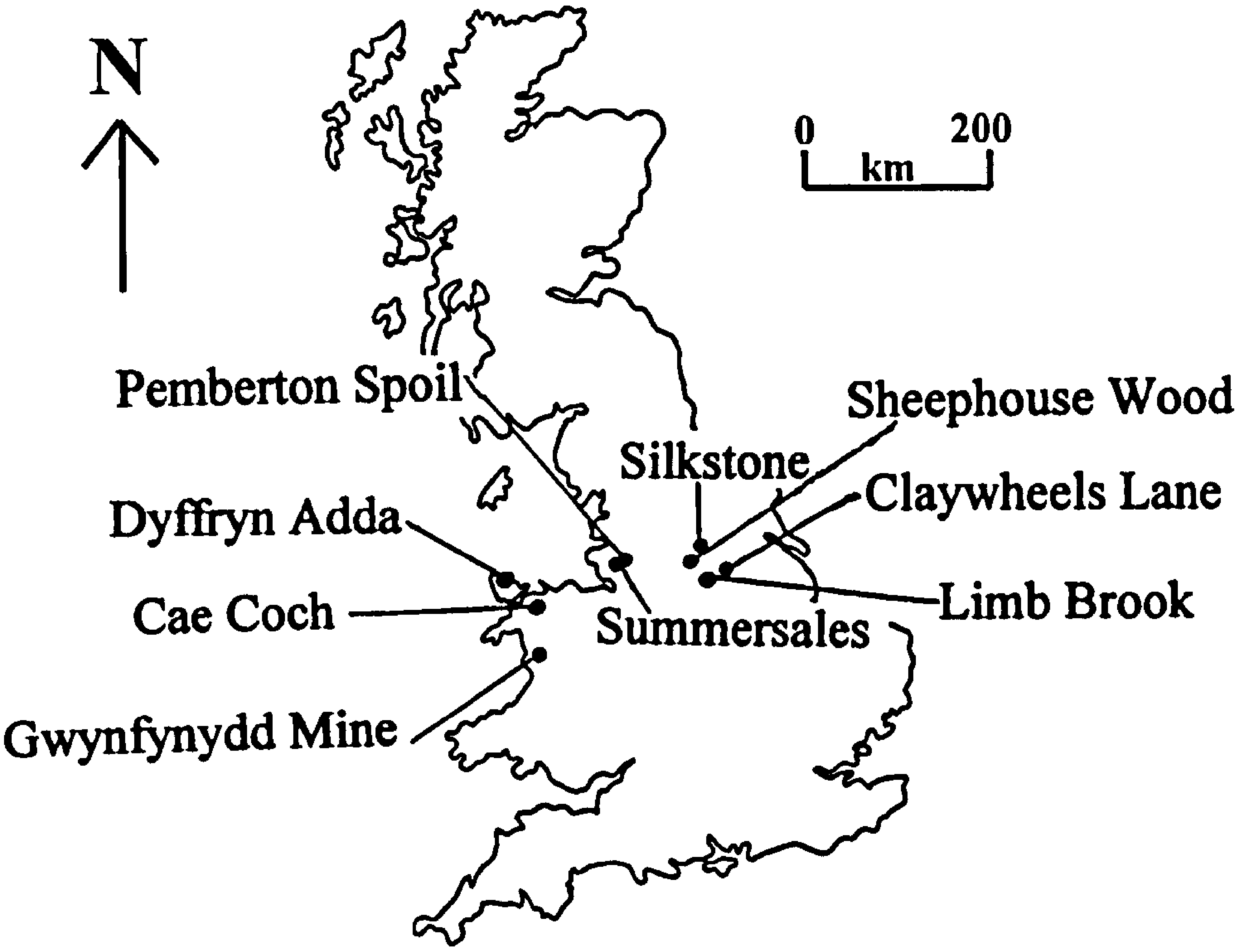


Figure 6.1: Map of Great Britain, showing the location of the mine drainage sampling sites.

effect in peat microcosms. As we were only interested in soil mediated properties in this part of the study, the experimental microcosms for this study were left unplanted.

The mine water sample sites are detailed below and their locations are shown in figure 6.1. Site selection was made after consultation with the local authorities and Environment Agency as many sites have become dangerous or hard to access.

Gwynfynydd Mine and Gwynfynydd Mine - Chidlaw Link Zone

Gwynfynydd Mine is located north of Dolgellau, North Wales, on the River Mawddach at grid ref. SH 737 281, with the latter site much deeper underground than the first. This site was used for gold mining until its abandonment in 1998. These waters were found to be of pH 5.3 and 7.28 respectively and contained 156mg l^{-1} and 0.53mg l^{-1} respectively of ferrous iron. Sulphate concentrations were found to be 89.97mg l^{-1} in Gwynfynydd Mine and 1364.07mg l^{-1} deep inside the mine at the Chidlaw Link Zone.

Cae Coch

This mine was first visited earlier in this research but for the purpose of this investigation, the water sample was taken from the adit rather than from within the mine itself (as in chapter 2). The mine (SH 775 653) is located near to the small village of Trefriw, which can be found on the B5106 in North Wales. This mine discharge eventually discharges into the River Conwy. The ferruginous waters discharging from the adit are of pH 2.8 and contain ferrous iron concentrations in the region of 3.77mg l^{-1}, although the total iron content was found to be much higher (approx. 550 mg l^{-1}). In the region close to the adit, the vegetation has decayed where the water has passed over leaving a small ‘scar’ on the landscape.

Dyffryn Adda

Dyffryn Adda mine water discharge can be found at SH 433 905 and forms part of the Mynydd Parys complex (NE Anglesey, North Wales SH 444 905). These discharging waters provide the source of mine drainage for the plants used in other parts of this research, outlined in previous and subsequent chapters. Here, the discharging waters that eventually discharge into the Afon Goch (north) river, are typically pH 3.4 and have an iron and sulphate content of 233.56 mg l^{-1} and 524.31 mg l^{-1} respectively.

Claywheels Lane

The minewater at this site, discharges from a pipe embedded in the bank of the River Don which runs adjacent Claywheels Lane, in the Hillsborough area of Sheffield (SK 319 919). The discharging waters are of coal origin of pH 5.79 and have an aqueous iron content of 47.33mg l^{-1}.

Summer Sales sites1-4

Water samples were collected from the Summer Sales discharge at four sites located at SD 578 041, SD 550 036, SD 550 035 and SD 551 034. These sites discharge into Smithy Brook. The Smithy Brook discharges originate from two coal collieries located in the Wigan area of Lancashire, Summersales (formerly known as Sumners Hall Drift) and Winstanley (formerly known as Billange and Winstanley Colliery). The pH of these sites ranges from 6.38–6.94, with iron and sulphate concentrations of 1.05–109.31mg l^{-1} and 113.63–856.35mg l^{-1} respectively.

Pemberton Spoil

'Pemberton Spoil' (SD 560 035) also discharges into Smithy Brook and is, once again, a consequence of the Summersales and Winstanley collieries. The discharge is strictly a culverted watercourse which has had a spoil heap placed on top resulting in the recognition as a mine drainage site. Its is otherwise known as Hawkley sough. The pH of the discharging water is 6.68, with 5.77mg l^{-1} of iron and 344.68mg l^{-1} of sulphate.

Limb Brook

Limb brook mine water discharge can be found in Ecclesall woods (SK 318 818), Sheffield, alongside the public footpath, arising from a small spring. It discharges into Limb brook via a small channel rich in ochreous deposits. Due to its location and popularity with walkers, the site is of high profile in the local area. The discharging water has a pH of 7.19 and an aqueous iron content of 4.97mg l^{-1}.

Silkstone

Silkstone minewater discharge is located in the village of Silkstone (SE 291 054), near Barnsley, alongside Silkstone Beck. Here, there is no pipe or conduit but a very definite point of emergence at the base of a wall. It can be found alongside the village allotments where locals speak of typically subsiding land. The Silkstone discharging minewater has a circum-neutral pH (6.8) and dissolved iron loadings of 2.14 mg l^{-1}.

Sheephouse Wood

The Sheephouse Wood minewater discharge is located in the heavily industrialised valley of Stocksbridge, north west Sheffield, at the base of which runs the River Don. The discharging water is from an adit (SK 253 992) which is channelled around the Underbank reservoir via a partially culverted discharge channel, where it eventually discharges into the Little Don. The minewater at this site was found to be pH 7.2 with total iron loadings of 1.53 mg l^{-1}.

Sample Site	pH	$[Fe^{2+}]$ (mg l^{-1})	$[SO_4^{2-}]$ (mg l^{-1})
Gwynfynydd Mine (G.M.)	5.3	156.00	89.97
G.M. - Deep inside mine	7.3	0.53	1364.07
Cae Coch	2.8	3.77	2893.58
Dyffryn Adda	3.4	233.56	524.31
Claywheels Lane	5.8	47.33	459.23
Summer Sales 1	6.7	109.31	856.35
Summer Sales 2	6.4	2.67	310.47
Summer Sales 3	6.8	1.05	753.78
Summer Sales 4	6.9	3.91	113.63
Pemberton Spoil	6.7	5.77	344.68
Limb Brook	7.2	4.97	44.52
Silkstone	6.9	2.14	ND
Sheephouse Wood	7.2	1.53	ND

Table 6.1: Pre-treatment iron and sulphate concentrations exhibited by the test waters, along with pH. It should also be noted that the results of Limb Brook water analysis also showed that this water body had high chloride levels (52.92mg l^{-1}). Note: ND = No Data.

6.1.2 Results

Table 6.1 compares the pH, iron and sulphate concentrations of the mine waters tested prior to their treatment in the microcosms.

From this table it can be clearly seen that the majority of water samples collected have a cirum-neutral pH with a vast range of dissolved iron and sulphate concentrations.

The remediation capabilities of mushroom compost, peat and gravel to ameliorate the test waters detailed above, are displayed in tables 6.2, 6.3 and 6.4. Results are shown as pH, iron and sulphate concentrations in interstitial water taken post

Sample Site	$[Fe^{2+}]$ (mg l^{-1}) at start	Treatment Soil Mushroom Compost	Peat	Gravel
Gwynfynydd Mine (G.M.)	156.00	UD	67.08	34.32
G.M. - Deep inside mine	0.53	UD	UD	UD
Cae Coch	3.77	UD	0.41	0.40
Dyffryn Adda	233.56	UD	137.80	84.08
Claywheels Lane	47.33	UD	12.31	5.21
Summer Sales 1	109.31	UD	53.56	22.96
Summer Sales 2	2.67	UD	UD	UD
Summer Sales 3	1.05	UD	UD	UD
Summer Sales 4	3.91	UD	0.51	0.23
Pemberton Spoil	5.77	UD	1.15	0.81
Limb Brook	4.97	UD	0.70	0.40
Silkstone	2.14	UD	UD	UD
Sheephouse Wood	1.53	UD	UD	UD

Table 6.2: Iron remediation in experimental microcosms containing mushroom compost, peat or gravel, treating minewater obtained from numerous British locations. Concentrations given for the interstitial water at start (day 0) and end of the experimental period (day 4). Where UD–Undetectable.

treatment, after the proposed residence time of four days.

Table 6.2 shows that once again, consistently complete iron removal, to undetectable levels, is exhibited by those microcosms containing mushroom compost within the forecasted retention time (four days). Microcosms containing peat are slightly less efficient than those containing gravel. These findings are consistent with those obtained earlier in this research (chapter 4). Although, soils treating mine drainage waters with a more alkaline initial pH, for example those obtained from deep inside Gwynfynydd Mine (G.M. – deep inside mine), showed high iron removal efficiencies irrespective of soil type. Statistical analysis shows that

Sample Site	$[SO_4^{2-}]$ (mg l^{-1}) at start	Treatment Soil Mushroom Compost	Peat	Gravel
Gwynfynydd Mine (G.M.)	89.97	1880.53	15.38	269.91
G.M. - Deep inside mine	1364.07	2578.56	390.78	1636.88
Cae Coch	2893.58	4129.61	207.66	3182.94
Dyffryn Adda	524.31	1950.43	93.81	681.60
Claywheels Lane	459.23	3161.65	314.32	780.69
Summer Sales 1	856.35	3641.99	602.54	1027.62
Summer Sales 2	310.47	2308.03	243.73	714.08
Summer Sales 3	753.78	3296.99	178.88	829.16
Summer Sales 4	113.63	2386.28	52.47	171.40
Pemberton Spoil	344.68	3745.11	316.78	413.62
Limb Brook	44.52	3146.85	17.66	84.59
Silkstone	ND	-	-	-
Sheephouse Wood	ND	-	-	-

Table 6.3: Sulphate remediation in experimental microcosms containing mushroom compost, peat or gravel, treating minewater obtained from numerous British locations. Concentrations given for the interstitial water at start (day 0) and end of the experimental period (day 4).

if the starting iron concentration (day 0) is compared with that at the end of the experimental period (day 4), then peat decreases the iron concentration by 57% ($P<0.0001$) compared to 78% ($P<0.0001$) by gravel in microcosms treating mine water from Gwynfynydd Mine (pH 5.3). By comparison, if these same soils are treating Dyffryn Adda mine water (pH 3.4), peat decreases the iron concentration by 41% ($P<0.05$) compared to 64% ($P<0.001$) in gravel.

In terms of sulphate concentration (table 6.3), microcosms containing peat consistently displayed effective remediation. Peat soils treating Cae Coch mine water (pH 2.8) ameliorated the most sulphate compared to the other test waters (93% $P<0.0001$). When treating neutral waters, for example inside Gwynfynydd Mine (pH 7.3), 71% ($P<0.001$) was removed. In contrast, those microcosms containing gravel and mushroom compost, added to the total sulphate interstitial water concentration, the latter soil yielding the highest values of up-to 71 times ($P<0.0001$) the original concentration, for example in microcosms treating mine waters from Limb Brook (pH 7.2). When mushroom compost is used to treat a more acidic water, for example Dyffryn Adda (pH 3.4) only 3.72 times ($P<0.0001$) the original sulphate concentration is added. Therefore, it would appear that more acidic waters yield a less dramatic increase in sulphate concentration (see also figures 4.4 and 4.5).

Table 6.4 shows the pH-changing capabilities of mushroom compost, peat and

Sample Site	pH at start	Treatment Soil		
		Mushroom Compost	Peat	Gravel
Gwynfynydd Mine (G.M.)	5.3	6.1	3.2	6.2
G.M. - Deep inside mine	7.3	7.1	4.0	6.8
Cae Coch	2.8	5.2	3.0	5.3
Dyffryn Adda	3.4	5.8	3.7	5.7
Claywheels Lane	5.8	6.3	3.3	6.0
Summer Sales 1	6.7	6.9	3.6	6.3
Summer Sales 2	6.4	6.6	3.4	6.4
Summer Sales 3	6.8	6.9	3.7	6.7
Summer Sales 4	6.9	7.0	3.7	6.7
Pemberton Spoil	6.7	6.7	3.5	6.5
Limb Brook	7.2	6.9	3.9	6.9
Silkstone	6.9	6.9	3.5	6.8
Sheephouse Wood	7.2	7.0	4.0	6.9

Table 6.4: pH change in experimental microcosms containing mushroom compost, peat or gravel, treating minewater obtained from numerous British locations. Values given for the interstitial water at start (day 0) and end of the experimental period (day 4).

gravel to ameliorate the test waters outlined above. Results are in terms of pH, taken post treatment, after the proposed residence time of four days.

From this table 6.4 it can be clearly seen that microcosms containing mushroom compost and gravel show greater potential in remediating the problems of acidity posed by acid mine drainage waters, than peat soils. Closer examination of the data shows that mushroom compost has generally a slightly higher increase in pH than gravel, proving once again the importance of mushroom compost as a remediating soil.

6.1.3 Discussion

Following the success of utilising mushroom compost and (planted) peat soils for the remediation of mine drainage waters, it was considered vital to investigate the universality of the proposed soils i.e. do the soils suggested (mushroom compost and peat) have that value in the remediation of any waters, irrespective of their origin? This is important so as to ensure that any recommendations that arise from this study can be applicable to a range of (acid) mine drainage waters. Although, many investigations have been undertaken on acid mine drainage worldwide, it would appear this investigation is the first of its kind, investigating the value of the proposed soils on remediation of mine waters from different origins.

It can be seen from tables 6.2, 6.3 and 6.4 that the soils tested were capable of remediating the waters outlined above to a satisfactory degree. Mushroom compost and peat consistently displayed the same ability to increase and decrease, respectively, the concentration of interstitial water sulphate, as previously found in this research when investigating Ynysarwed and Mona mine waters (chapter 4). Indeed, it would appear, in concordance with previous research in this thesis, that microcosms containing mushroom compost and gravel show a greater potential in remediating the problems of acidity posed by acid mine drainage waters, than peat soils (table 6.4).

On dismantling all microcosms containing mushroom compost, there was an extremely strong smell of hydrogen sulphide and very black zones within the soil, suggesting sulphate reduction was occurring. It can be suggested that this process is occurring due to the activity of sulphate reducing bacteria by mechanisms outlined in section 1.1 (equations 1.13 and 1.14), thus supporting the perceived role of SRBs (chapter 5) in the remediation of these waters.

According to the Environment Agency (pers. com.), the Environmental Quality Standards for iron is 1mg l^{-1} (dissolved). Post treatment of these waters using mushroom compost yields a discharging water firmly within that of the Environmental Quality Standard. In terms of sulphate, the E.C. Drinking water Directive stipulates a maximum limit of 250mg l^{-1}, although sulphate toxicity to organisms is modified by the cations with which it is associated (pers. com. E.A.). For example, some species of fish can withstand higher concentrations of Na_2SO_4 rather than $ZnSO_4$ (Dojlido and Best, 1993). In the majority of the mine drainage waters listed above treated in microcosms containing peat, sufficient reduction in sulphate concentrations was observed to meet with the E.C. Water Directive requirements. Of those that did not meet the requirements, it should be noted that the presence of plants in peat microcosms (chapter 4, figures 4.4(a) and 4.5(a)) decreased the interstitial water sulphate concentration to a greater degree than their unplanted counterparts. Therefore, planting those microcosms treating mine drainage waters such as those obtained from deep inside Gwynfynydd Mine, may provide a sulphate concentration which meets the aforementioned Directive requirements.

These data have also shown that microcosms containing mushroom compost and gravel are capable of increasing pH, with mushroom compost being the superior soil type. Once again, according to the Environment Agency (pers. com.), they use the guidelines set by the European Fisheries Advisory Committee to determine pH optima for our waterways. This pH (6–8) is considered of great importance especially on the migratory rivers of the salmonid stocks and could be the difference in their survival. The experimental microcosms detailed above (mushroom compost and gravel), met these requirements in respect of remediating these mine waters, with the exception of those microcosms treating mine waters obtained from Cae Coch and Dyffryn Adda. Microcosm treating these waters, yielded pH values of 5.2 and 5.8 respectively, when treated in microcosms containing mushroom compost, and 5.3 and 5.7 respectively, in those microcosm containing gravel.

Therefore, based on the diversity of starting conditions, mushroom compost will always be the best soil for metal removal, and pH improvement treating mine waters. Although, if the pH is neutral, any of the soils tested would be effective to a certain degree. In terms of sulphate removal, peat is consistently the most beneficial soil type.

6.2 The Impact of Soil Type and the Presence or Absence of Plants on the Remediation of Other Contaminating Metals

6.2.1 Materials and Methods

Planted and unplanted 130mm deep × 160mm diameter microcosms were set up in quadruplicate as detailed in chapter 2 (section 2.2.2) containing mushroom compost, gravel, bark-chippings or peat as detailed in chapter 4 (section 4.1). The plants used in the planted microcosms, were collected from a natural wetland (Mynydd Parys complex, Anglesey, North Wales - see section 2.1.1). Ynysarwed or Mona mine drainage water was added to the test microcosms.

Water samples were taken from the microcosms on a regular basis, filtered, acidified with 0.1M nitric acid and stored at 4°C until analysis could be undertaken. Metal (aluminium, cadmium, copper, zinc, cobalt and manganese) analysis was undertaken using Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES).

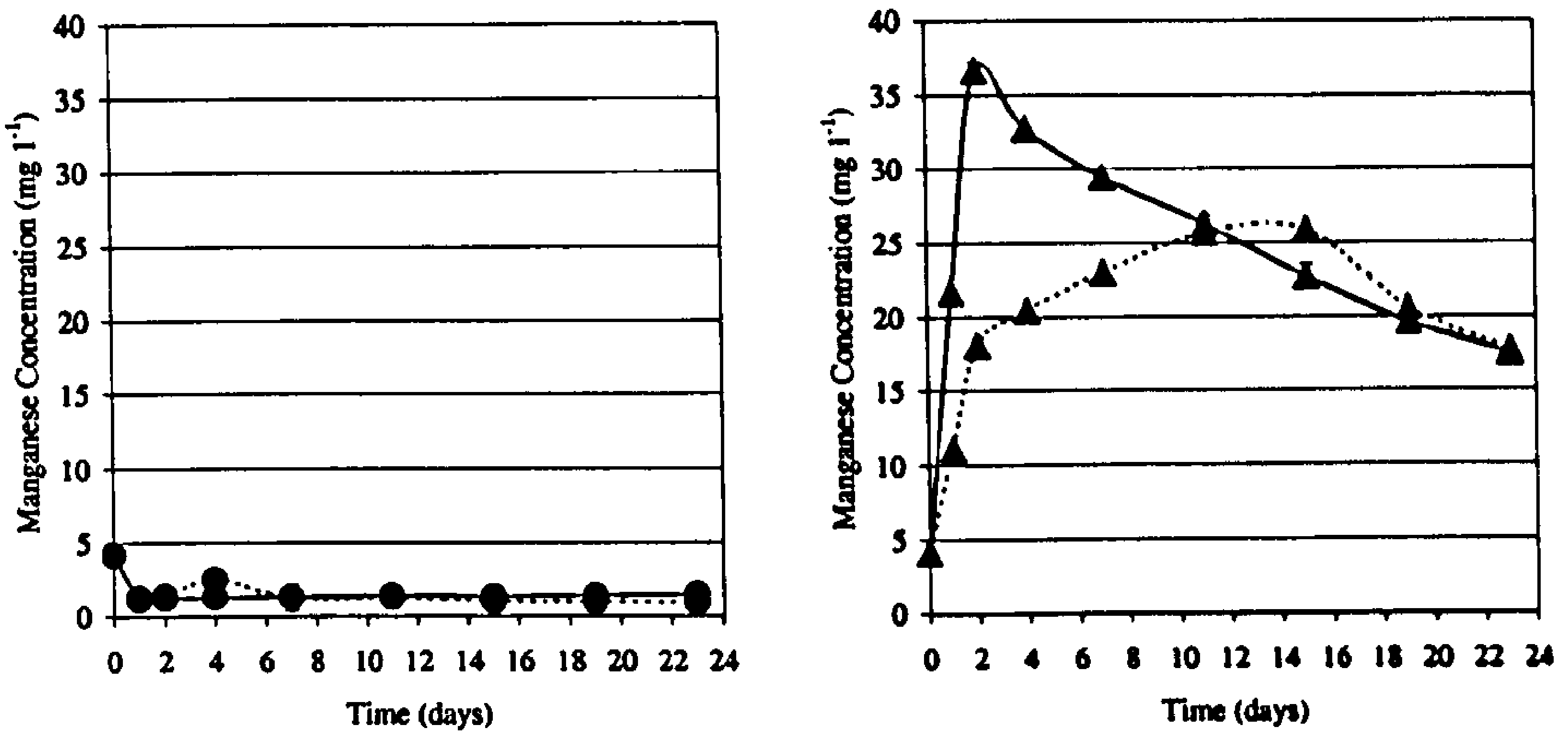
6.2.2 Results

The effect of soil type and planting on the interstitial water concentration on a variety of other polluting metals such as cadmium, aluminium, copper, zinc, cobalt and manganese are shown in figures 6.2, 6.3, 6.4, 6.5, 6.6, 6.7 and 6.8. The results of these investigations showed the ability of microcosms containing unplanted mushroom compost, to substantially reduce (by 99.8% $P<0.001$) the concentration of these metals found in the mine-waters within two to four days (see (d) figures 6.2 to 6.8). Each of the other soil types contributed beneficially to the remediation of other contaminant metals except for microcosms treating manganese found in Ynysarwed and Mona mine drainage water in planted and unplanted gravel and bark-chippings. From figures 6.2 and 6.7 ((b) and (c)) it can be seen that these soils exacerbate the problem, although the degree of increase is higher for the more neutral Ynysarwed waters than the acidic Mona mine waters, and gravel compared to bark-chippings. The magnitude by which this increase occurs in microcosms treating Ynysarwed and Mona waters is 8.8 times and 5.3 times respectively in gravel soils, and 6.5 times and 3.1 times in respectively bark-chippings (all values extremely significant at $P<0.0001$). In unplanted gravel soils (figures 6.2(b) and 6.7(b)) a initial increase within the first 2 to 4 days is followed by a period of decreasing concentration. This occurs when treating both water samples to the end of the experimental period.

Previous research detailed within this thesis (chapters 4 and 5) showed that planting had a detrimental affect on metal removal. These data (figures 6.2 to 6.8) show no evidence of this effect when treating other metals such as cadmium, copper, zinc and manganese, except during the treatment of cobalt. From figure 6.6 it can be seen that after an initial decrease in cobalt concentration, re-mobilisation occurs in all soils, but in planted gravel soils a rapid decrease in cobalt concentration is observed. Statistical analysis shows that the increase in cobalt concentration is extremely significant in all soil types, when a comparison is made between the lowest concentration and the maximum concentration using a t-test (peat $t=10.213$, 6df, $P<0.0001$; gravel $t=10.707$, 6df, $P<0.0001$; bark-chippings $t=8.762$, 6df, $P=0.0001$; mushroom compost $t=16.253$, 6df, $P<0.0001$).

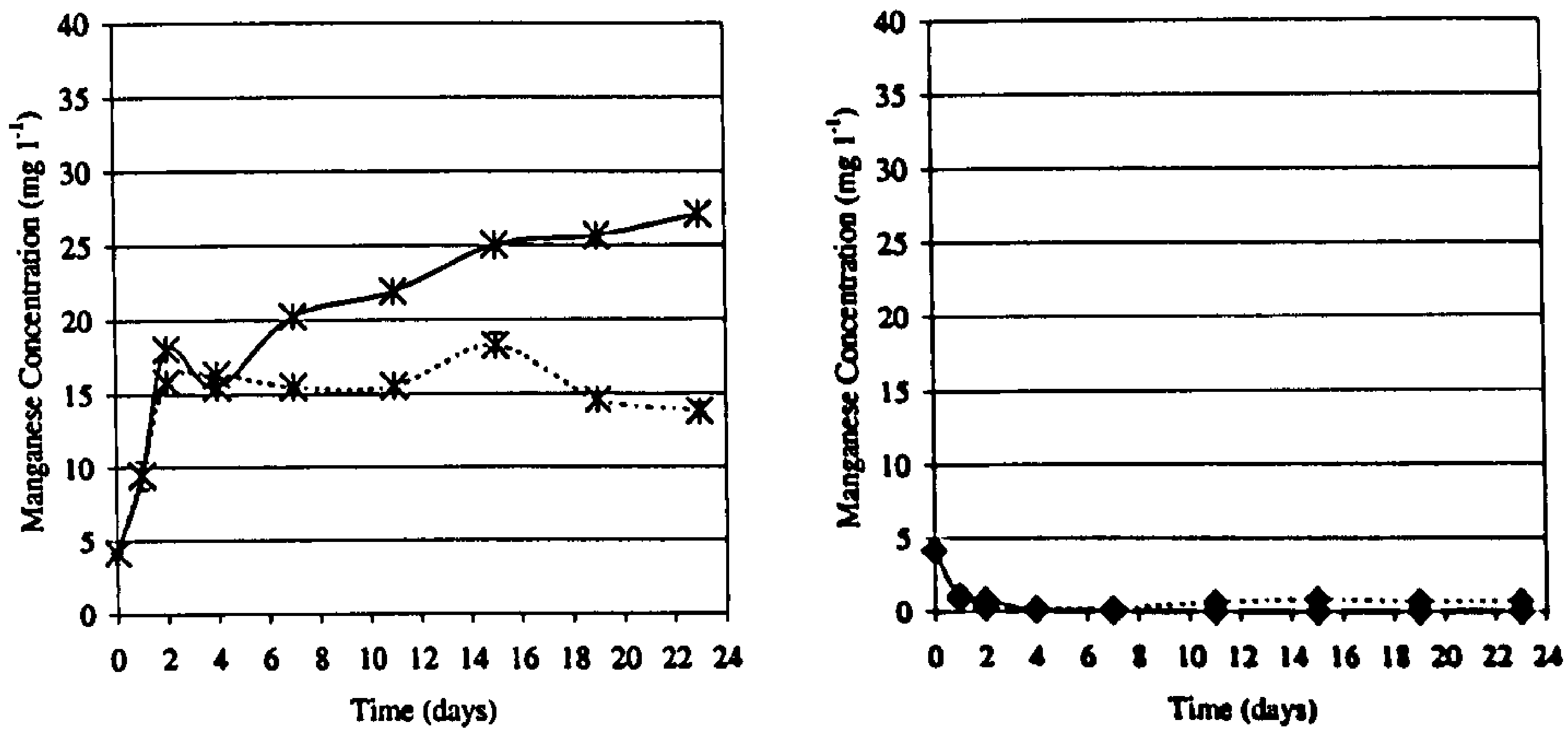
6.2.3 Discussion

The successful amelioration of iron by mushroom compost has been shown on a number of occasions throughout this thesis (for example 4.2(d), 4.3(d) and 5.1) but, even though the high concentrations of iron make this the most significant pollutant in these waters, mine drainage tends to possess high concentrations of other metals which in some cases are highly toxic and can have detrimental effects on the environment. These metals include aluminium, cadmium, copper,



(a) Peat

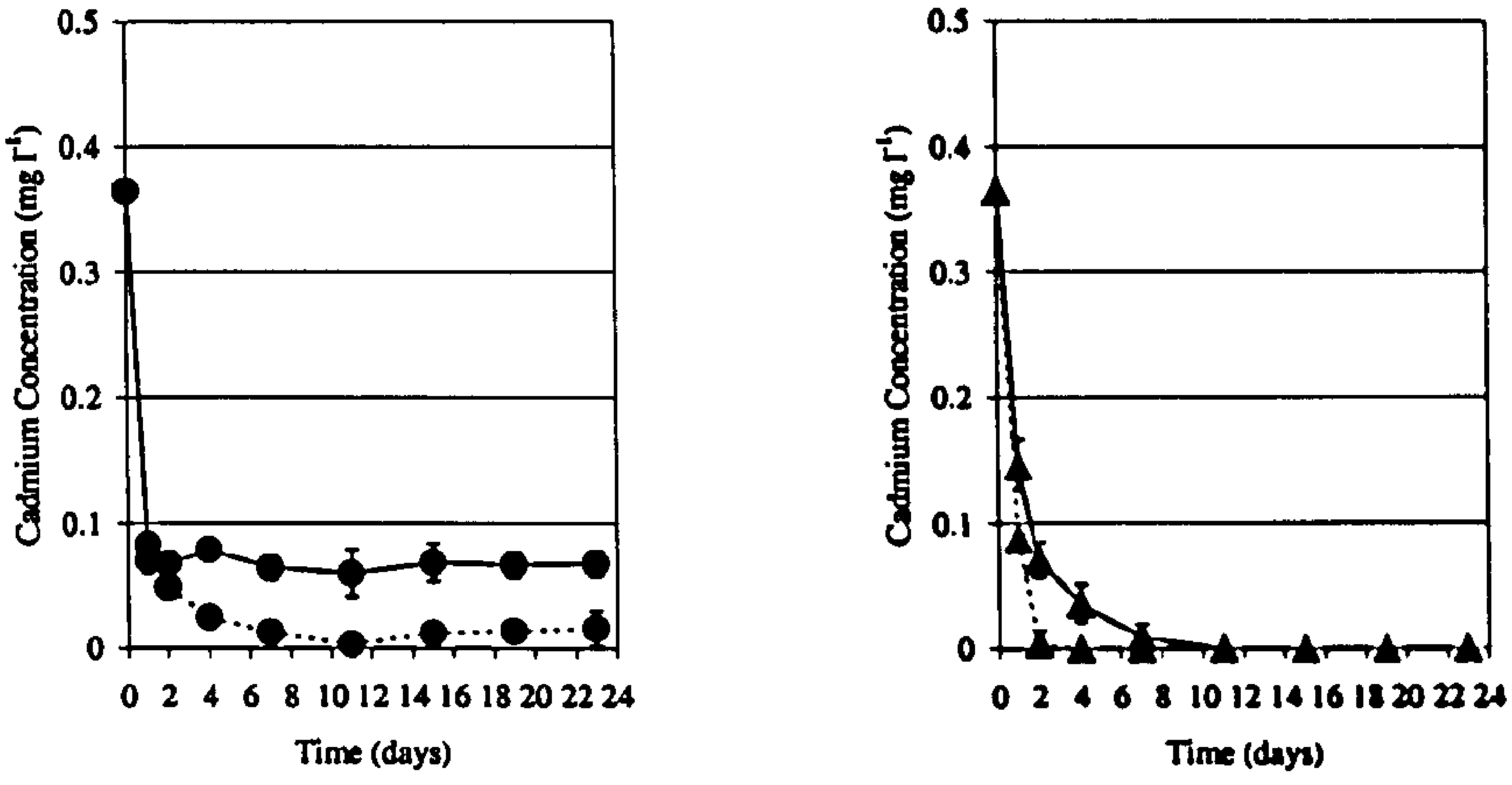
(b) Gravel



(c) Bark-chippings

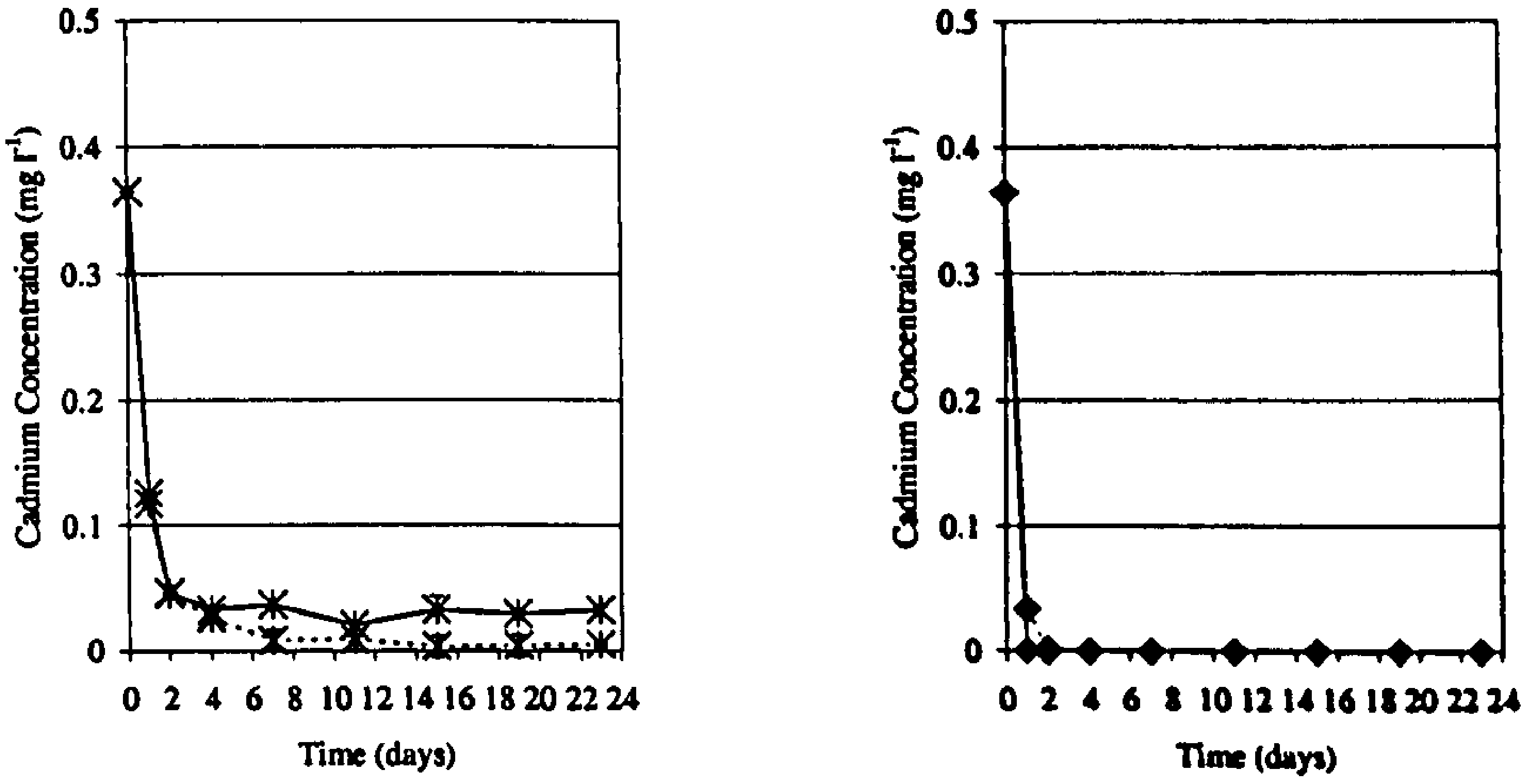
(d) Mushroom Compost

Figure 6.2: The effect of soil type and planting on interstitial water manganese concentrations in microcosms treating Ynysarwed mine waters. Mean±s.e., n=4. Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

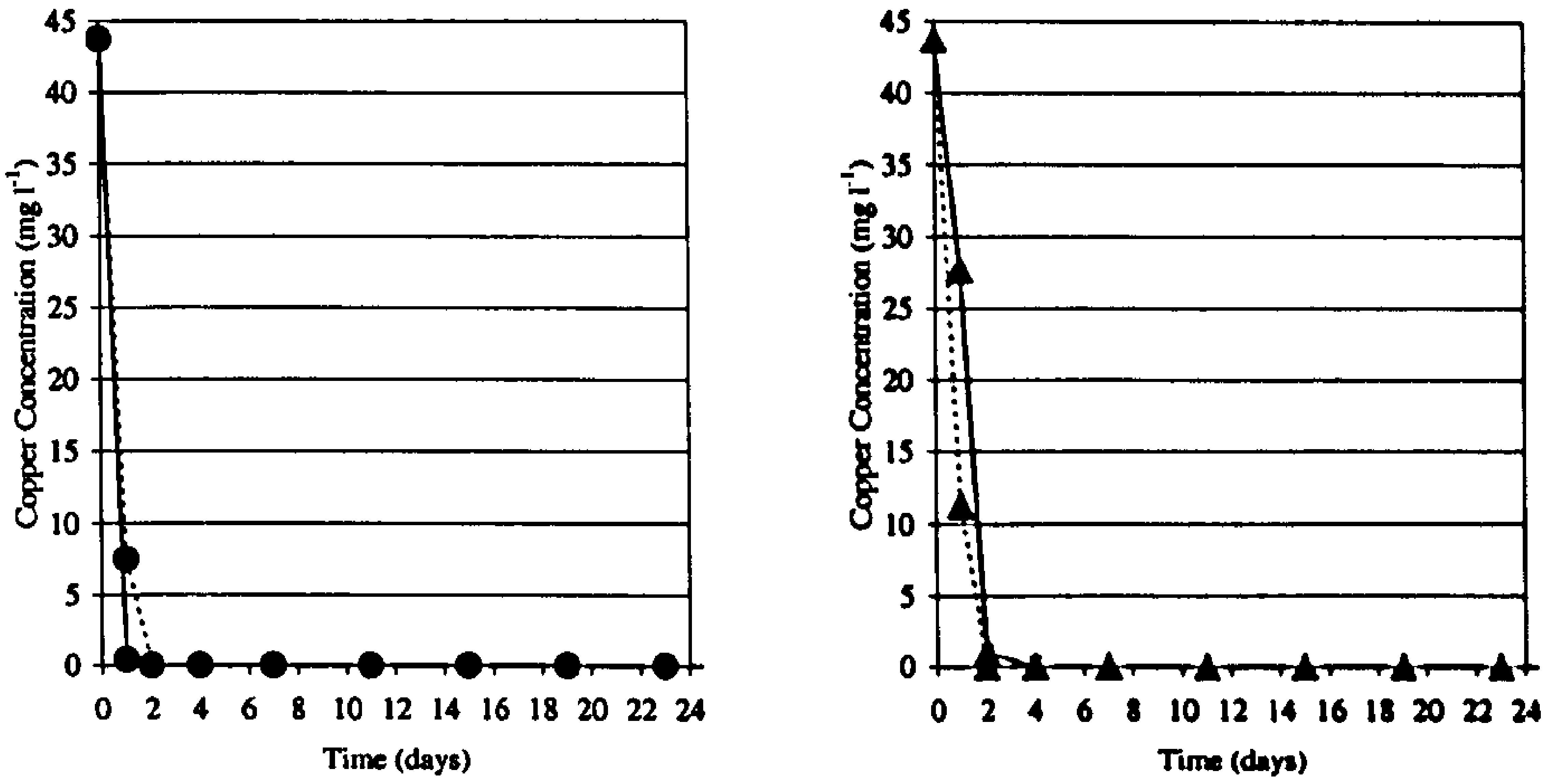
(b) Gravel



(c) Bark-chippings

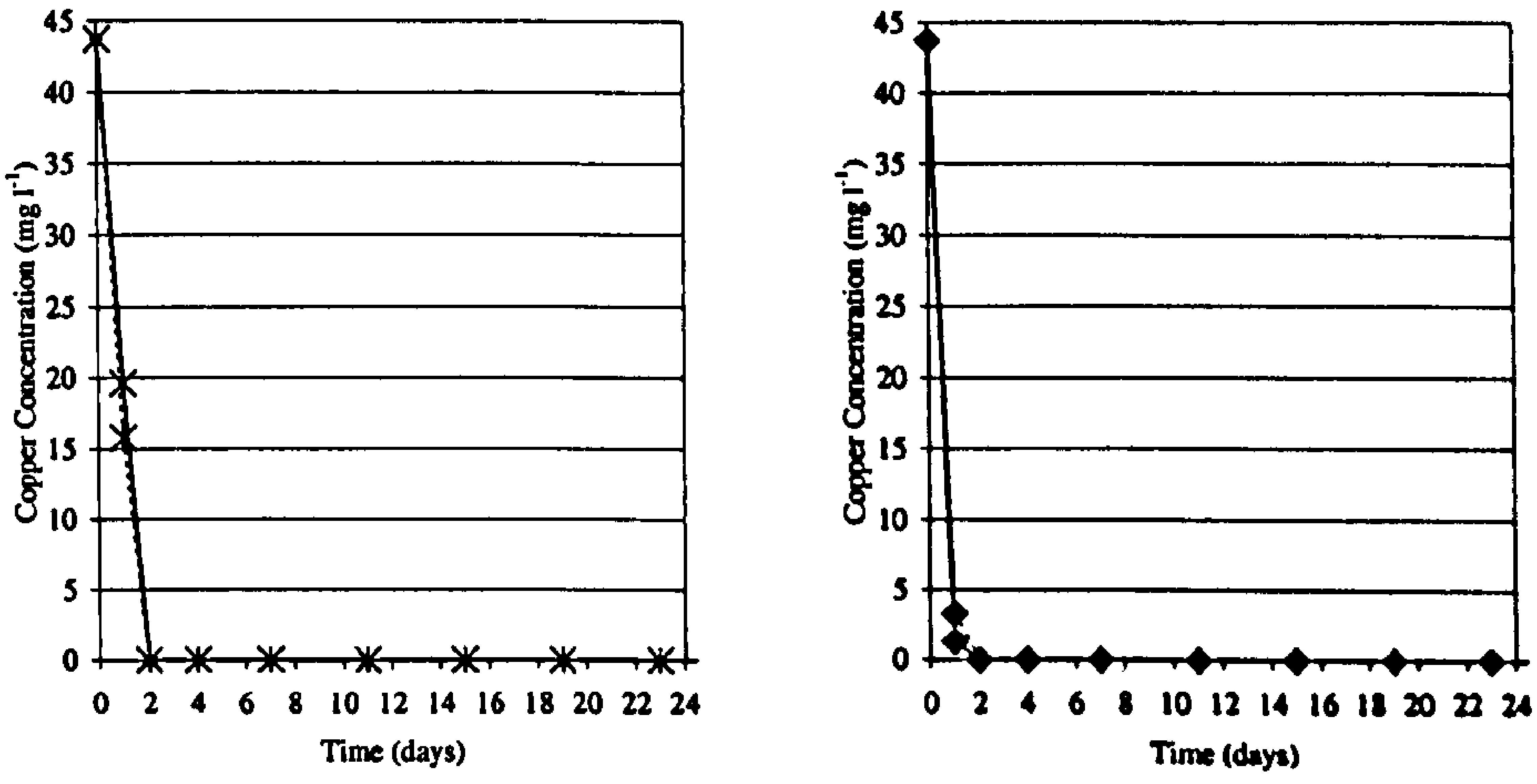
(d) Mushroom Compost

Figure 6.3: The effect of soil type and planting on interstitial water cadmium concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

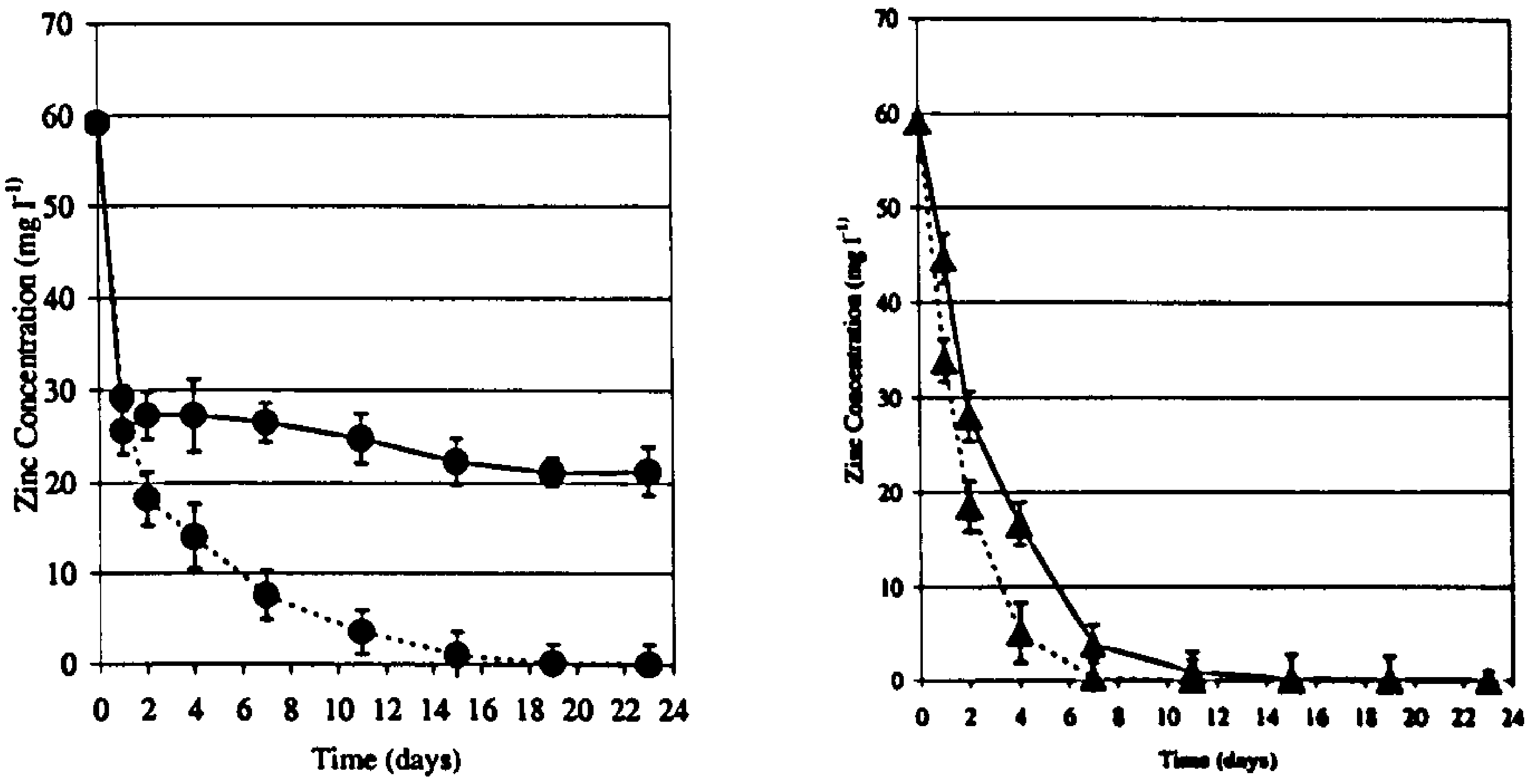
(b) Gravel



(c) Bark-chippings

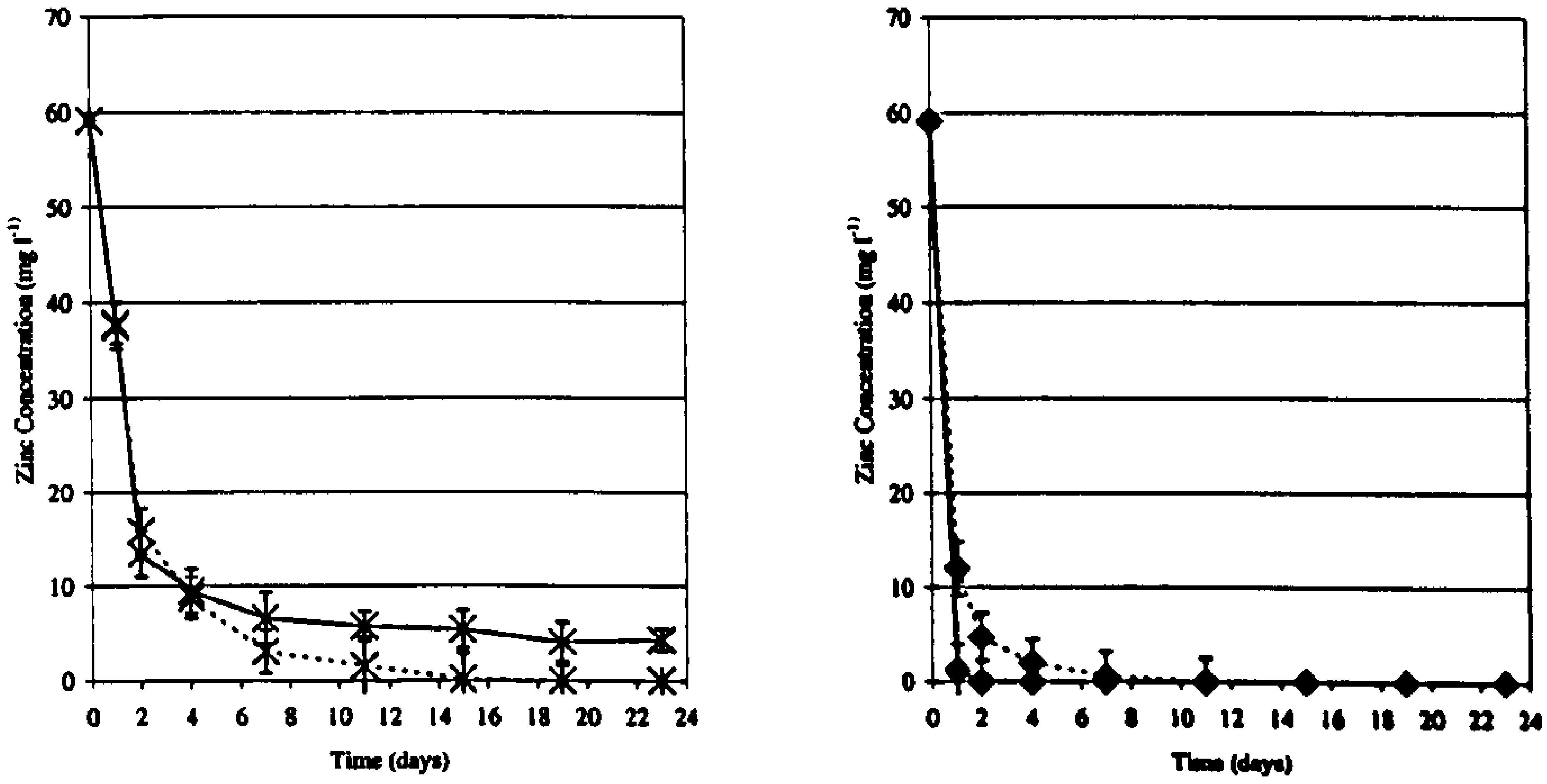
(d) Mushroom Compost

Figure 6.4: The effect of soil type and planting on interstitial water copper concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

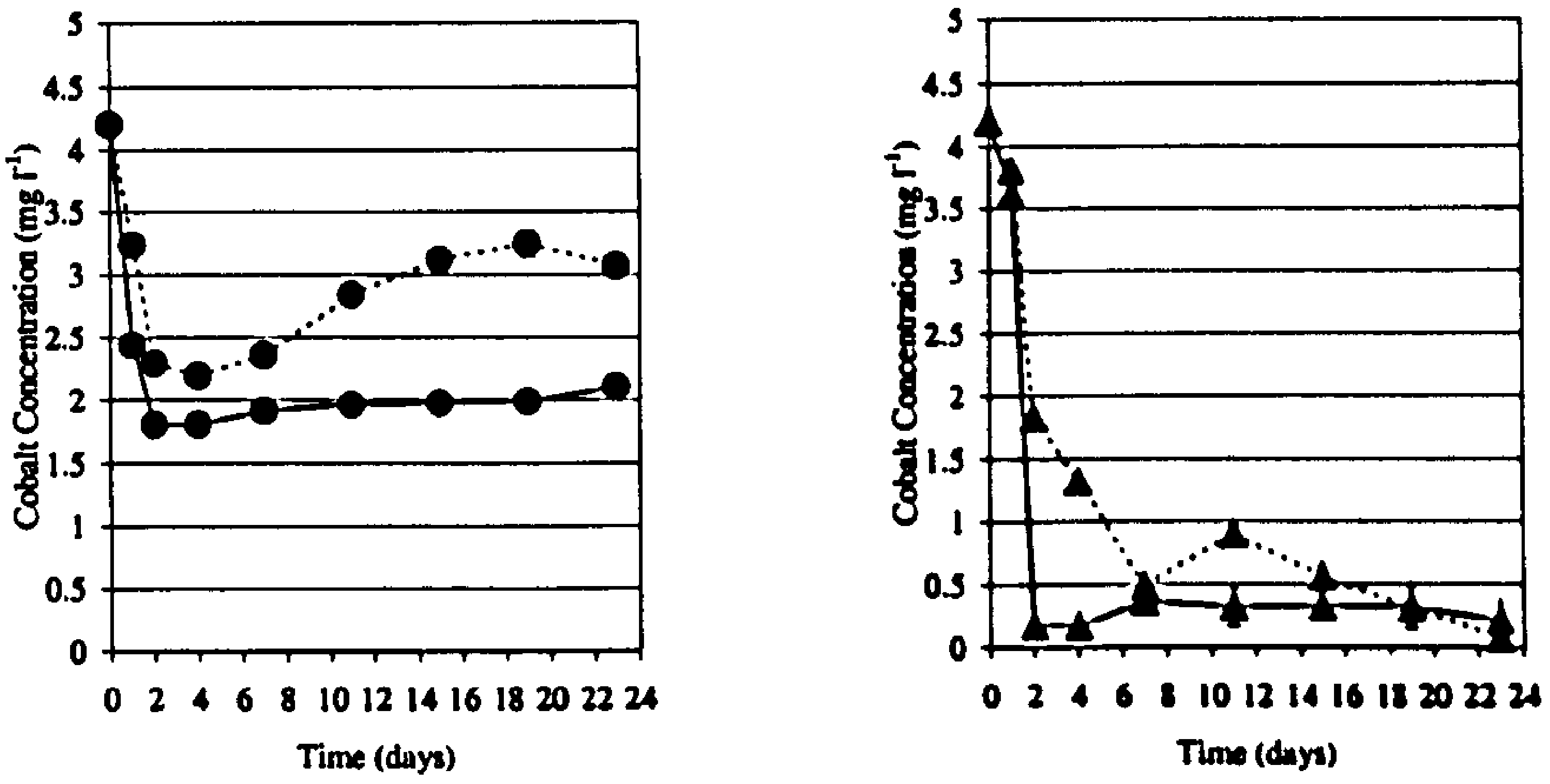
(b) Gravel



(c) Bark-chippings

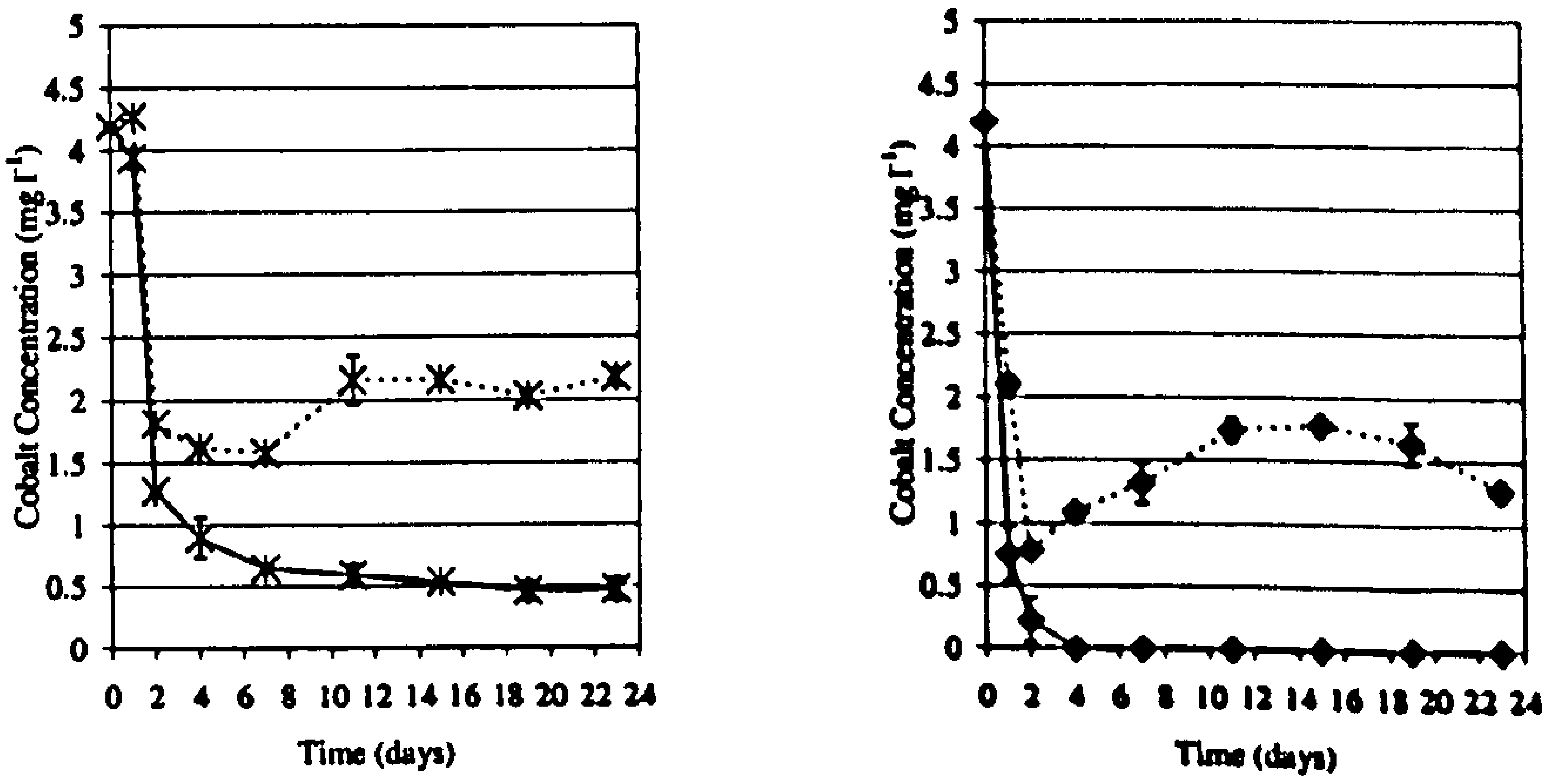
(d) Mushroom Compost

Figure 6.5: The effect of soil type and planting on interstitial water zinc concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

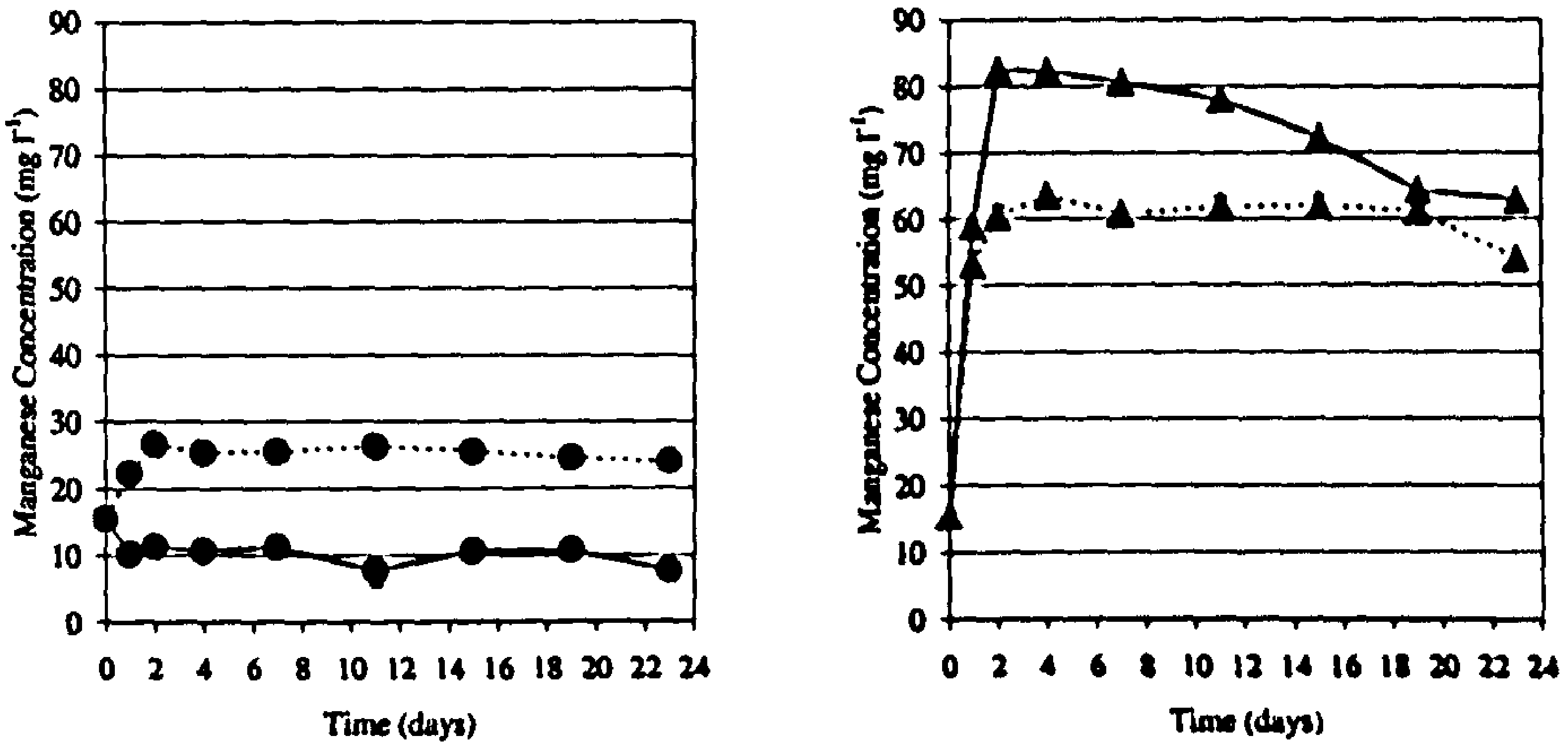
(b) Gravel



(c) Bark-chippings

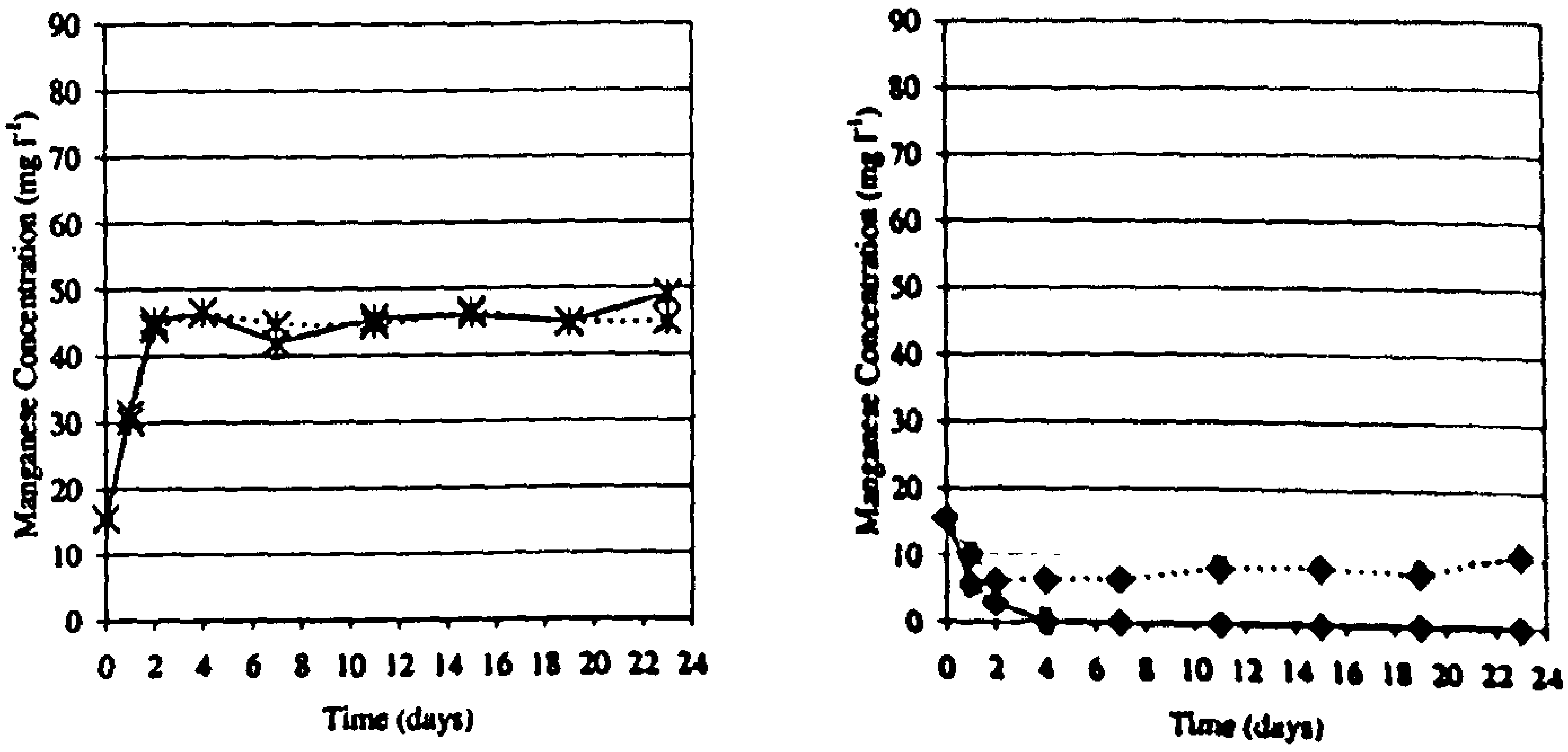
(d) Mushroom Compost

Figure 6.6: The effect of soil type and planting on interstitial water cobalt concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

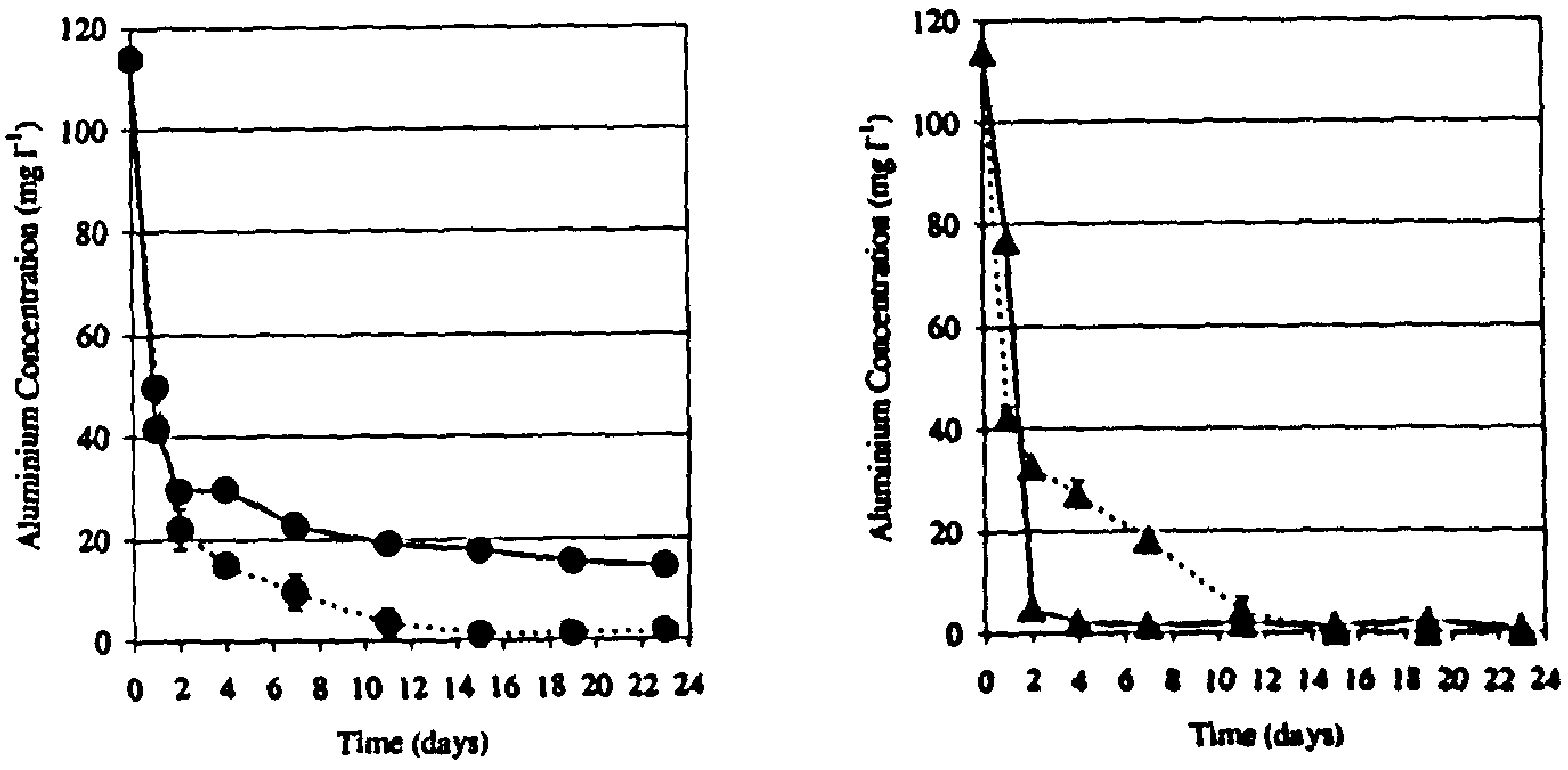
(b) Gravel



(c) Bark-chippings

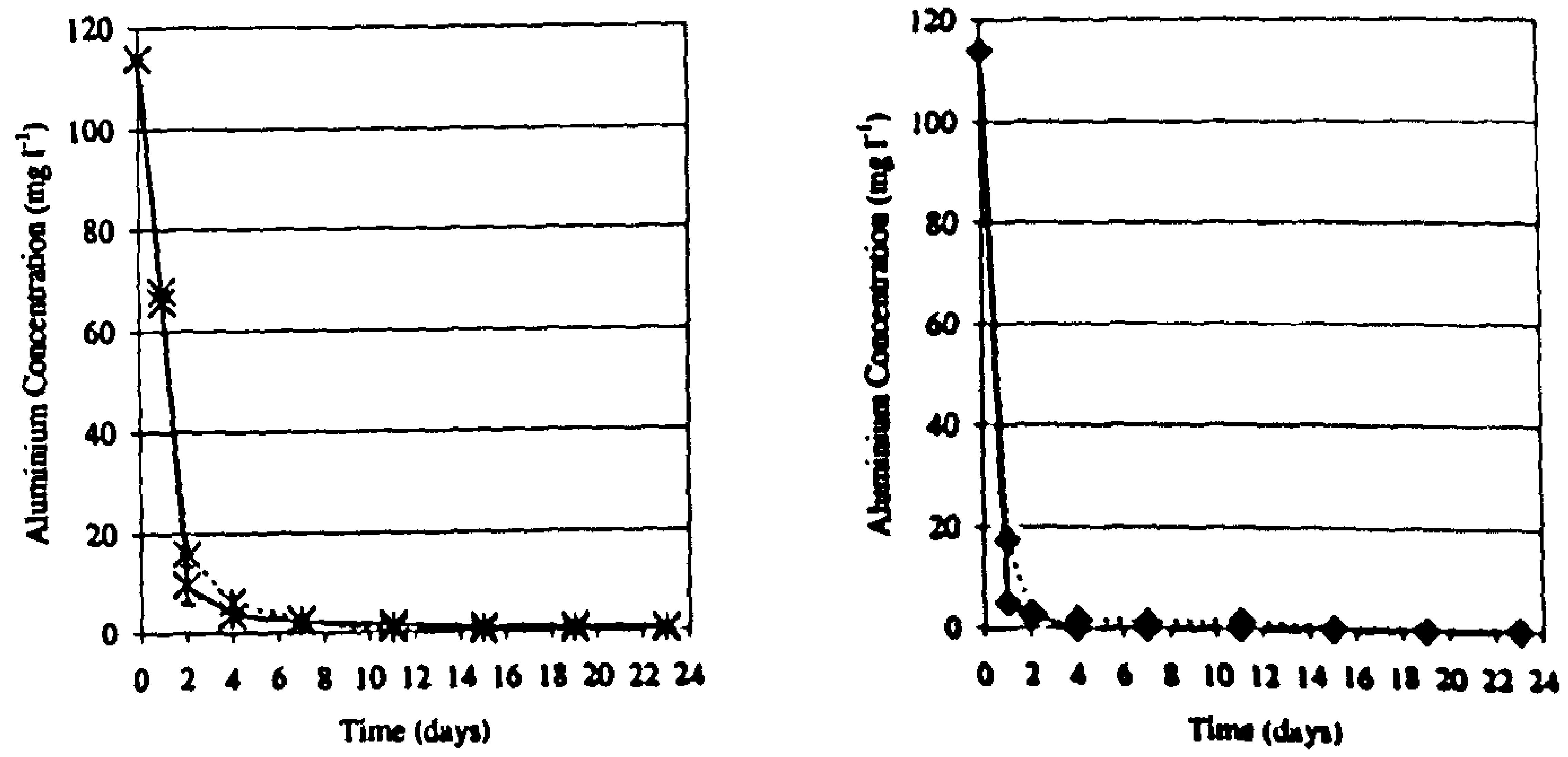
(d) Mushroom Compost

Figure 6.7: The effect of soil type and planting on interstitial water manganese concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.



(a) Peat

(b) Gravel



(c) Bark-chippings

(d) Mushroom Compost

Figure 6.8: The effect of soil type and planting on interstitial water aluminium concentrations in microcosms treating Mona mine waters (mean±s.e., n=4). Planted microcosms represented by dotted line, unplanted microcosms represented by solid line.

zinc, cobalt and manganese. Research has shown that mushroom compost can successfully decrease the concentrations of these pollutants to undetectable concentrations within the forecasted retention time of four days and was the most beneficial soil type for metal removal (figures 6.2 to 6.8). The effect of planting on iron concentrations has been shown previously to be detrimental, with re-mobilisation occurring. When investigating the effect of planting and soil type on the remediation of aluminium, zinc, cadmium and copper this phenomena did not occur, but was evident in planted microcosms treating cobalt (figure 6.6). Mobilisation of managanese was also observed when treated in planted and unplanted bark-chipping, gravel and planted peat (figures 6.2 and 6.7). This phenomena was also observed by Bloomfield *et al.* (1976), who observed the re-mobilisation of cobalt, copper and lead. No re-mobilisation of copper was observed in this study.

6.3 Mushroom Compost and Gravel as a Long-term Solution for Iron Removal From Mine Drainage Waters

6.3.1 Materials and Methods

Unplanted microcosms were set up using 100 ml separating funnels containing mushroom compost or gravel. Separating funnels were used to facilitate the draining of the microcosms, under gravity, after the proposed residence time of four days. For the purpose of this research synthetic mine drainage water adjusted to pHs similar to those found at Ynysarwed and Mona was used. These synthetic mine waters were made from MilliQ water with similar iron and sulphate loadings (using ferrous and ferric sulphate) to those found at the original sites (Fe^{2+}300mg l^{-1}, SO_4^{2-} 2000mg l^{-1}).

These microcosms were drained every four days and replenished with fresh synthetic mine drainage, simulating movement through the wetland. This study was undertaken for a period of 10 weeks.

6.3.2 Results

The results show that following the addition of synthetic mine drainage, the iron concentration increased immediately to 300mg l^{-1} as expected followed by a decline in concentration to approximately zero over the four day period. Complete metal removal continues to occur without a decrease in performance, over the experimental period (figure 6.9). From previous experiments outlined above, it was observed that the required residence time for the treatment of mine drainage by mushroom compost was four days (figure 4.3(d)). As a consequence, this dictated the sampling frequency of these test microcosms producing the result shown. In 'real time', i.e. if the sampling frequency was increased to match that of the other experiments, it is thought that the data would display similar profile over the four day period (see figure 4.3). Figure 6.10 shows the control data for this experiment. These microcosms were gravel based. As can be seen from this figure, full iron remediation occurs over a short period (32 days) but then a decrease in the remediation capacity of the gravel is observed.

6.3.3 Discussion

From the results outlined in this chapter and those found in chapter 4, investigations were undertaken to ascertain whether the valuable properties of mushroom

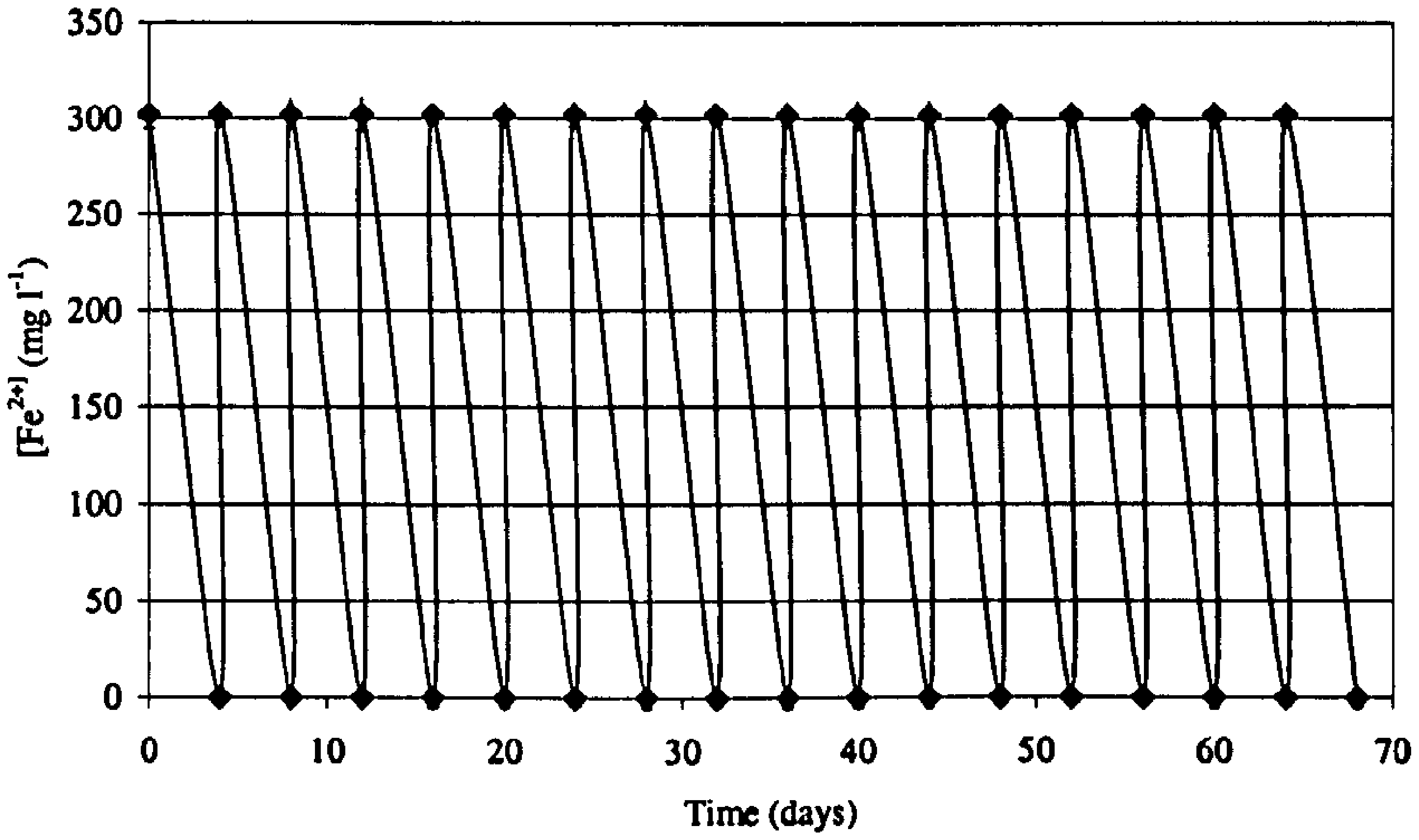


Figure 6.9: The longevity of iron removal in mushroom compost based microcosms when treated with synthetic acid mine drainage added every four days. Mean±s.e., n=4.

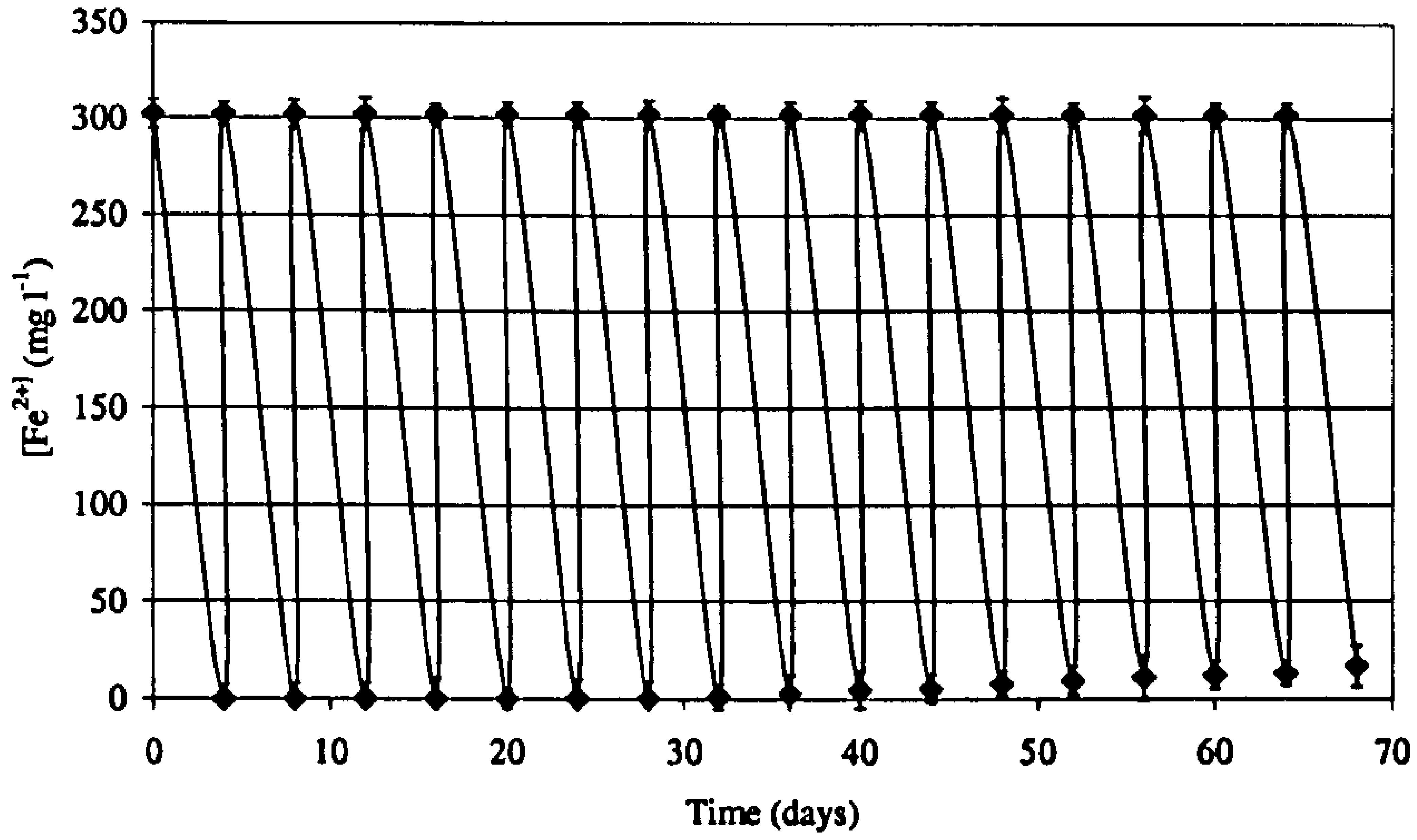


Figure 6.10: The longevity of iron removal in gravel based microcosms when treated with synthetic acid mine drainage added every four days. Mean±s.e., n=4.

compost to remove metals, in particular iron and produce a more alkaline pH, and peat to ameliorate sulphate, were short lived or viable in the long-term as a remediation strategy i.e. the longevity of these soils in treating mine waters. Investigations as to the longevity of iron remediation in microcosms containing mushroom compost, showed no reduction in remediation ability over the experimental period. This was not the case in the gravel microcosms, where a decrease in remediation capabilities were observed after 30 days. Naturally, this investigation requires a longer trial to allow comparison with other studies such as (Kolbash and Romanoski, 1989).

6.4 Summary

Irrespective of mine water origin, mushroom compost consistently decreases the iron concentration within the forecasted retention time of four days. It was also capable of successfully decreasing the concentration of other metals. Mushroom compost is therefore, the most beneficial soil for metal removal. Planting did not have the same detrimental effect, as previously observed, when treating other metals such as aluminium, cadmium, copper and zinc, but re-mobilisation of manganese and cobalt did occur. During the four day experimental period, waters treated with peat soils, showed a decrease in sulphate concentrations. Longevity studies have shown that with a mine water residency time of four days and over the 10 week experimental period, mushroom compost can consistently decrease the iron concentration without decreasing efficiency.

Chapter 7

Complexation and Competition

Earlier research, detailed in this thesis, has shown the successful treatment of ferruginous mine drainage waters using mushroom compost, to concentrations below that set out by Environmental Quality Standards. However, the efficiency is hampered by the presence of plants. During plant decomposition substantial quantities of DOC are released into the environment. According to Pinney *et al.* (2000) dissolved organic carbon derived from plants, present in wetlands can not only act as a bacterial energy source, but also complex with metals. For this reason the relationship, if any, between iron and dissolved organic carbon (DOC) was investigated to ascertain why plants have been shown to have a negative effect in the remediation of the waters utilised in this research. It may be that the plants release dissolved organic carbon (DOC) which can then form complexes with the aqueous iron. This investigation was undertaken using high-performance liquid chromatography (HPLC), an extremely useful analytical technique, allowing small volumes to be injected.

In the natural environment, phenolics in the dissolved organic carbon (DOC) are important in nutrient cycling and have been reported to bind to metals such as aluminium, thus reducing the metal's toxicity (McColl and Pohlman, 1986). Phenolics are naturally occurring substances and are characterised by a hydroxyl group (one or more) bearing benzene ring. They can occur in two forms; polymeric (e.g. tannins) or monomeric (Appel, 1993). In this chapter, experimental microcosms were used to investigate the complexation of iron with natural phenolics.

Moreover, as planting was shown (chapter 4) to have a negative effect on the remediation of mine drainage waters, in terms of metal removal, we hypothesise that the response may have been caused by competition between plant and microbe. If this did occur, microbes would be starved of nutrients and so be less able to participate in the remediation process. For this purpose, plants were supplied with Long-Ashtons solution (an inorganic fertilizer/nutrient media) to overcome the microbial competition. Likewise, the microbial community was stimulated using lactate. Lactate has been shown to be utilised by microorganisms especially sulphate reducing bacteria, as a carbon source (for example Elliott *et al.* (1998) and

Tsukamoto and Miller (1999)), therefore, by stimulation, an increased biomass and rate of activity may be observed.

It is anticipated that the Long-Ashtons medium will increase the treatment efficiency by allowing the microbes better access to inorganic nutrients. In contrast, microbial stimulation with lactate, is expected to promote treatment efficiency by directly supporting microbial metabolism.

7.1 Complexation

7.1.1 Complexation of Iron with Plant Derived Dissolved Organic Carbon

Materials and Methods

Determination of the molecular weights of the dissolved organic carbon found in the interstitial water of microcosms treating Mona mine drainage water was undertaken using high performance liquid chromatography (HPLC - Cecil 1100), with a flow rate of 1ml min^{-1} and an injection volume of 150μl. Readings were taken at a detection level of 254.0nm. A mobile phase of tris-HCl buffer (pH

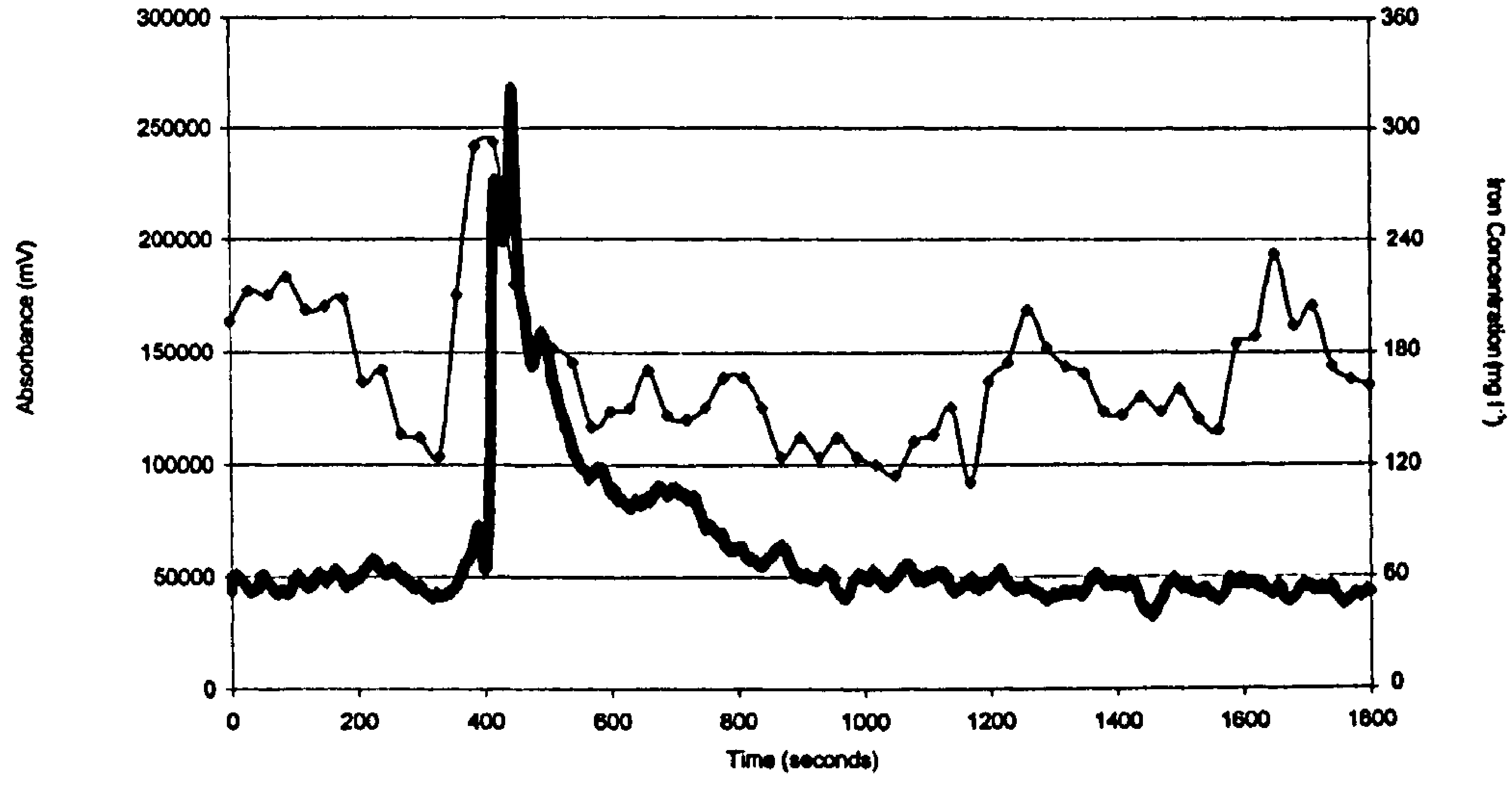


Figure 7.1: A typical AMW spectrum of DOC and its association with dissolved iron, obtained from interstitial water samples taken from planted peat microcosms after 15 days. Note: heavy line = dissolved organic carbon and fine line = dissolved iron concentration.

7.5) was used requiring two important steps to be undertaken in its preparation. Firstly, the solution was filtered so as to remove any particulate matter that may damage the column and secondly, it was degassed. This latter procedure was undertaken to minimise the chromatographic affects of air. The column was also maintained at 35°C using a column heater thus minimising changes in the baseline and the elution time of the analytes. For the purpose of this investigation, the eluate was collected every 30 seconds and retained for total iron analysis using atomic absorption spectrometry or AAS, details of which can be found in chapter 2 (section 2.3.1). Interstitial water samples taken from microcosms containing planted peat soils treating Mona mine drainage were used for this experiment.

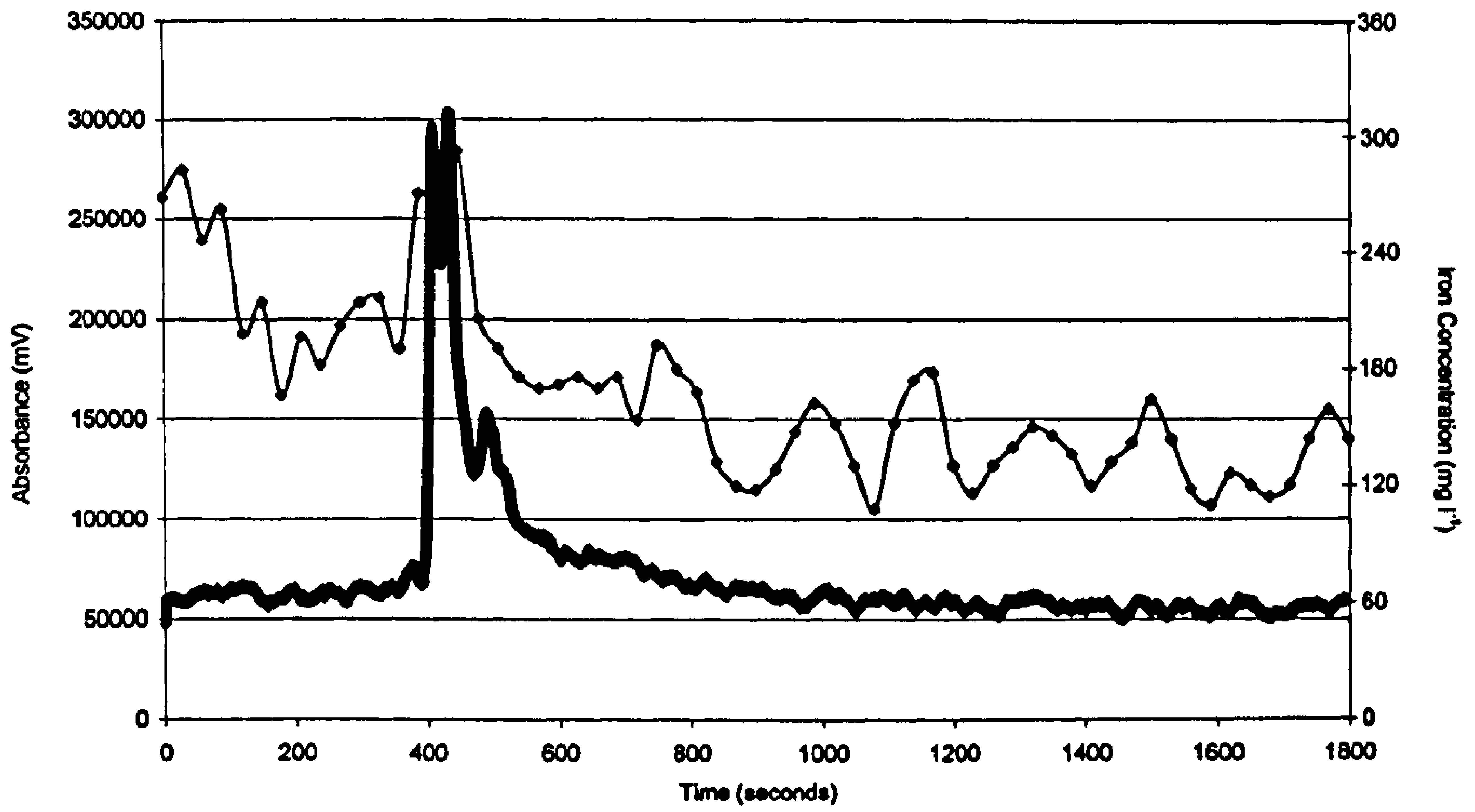


Figure 7.2: A typical AMW spectrum of DOC and its association with dissolved iron, obtained from interstitial water samples taken from planted peat microcosms after 23 days. Note: heavy line = dissolved organic carbon and fine line = dissolved iron concentration.

Results

Figures 7.1, 7.2 and 7.3 show the degree of association between dissolved organic carbon (DOC) and total iron in interstitial water from planted and unplanted peat microcosms treating Mona mine drainage, shown by HPLC determination of the apparent molecular weight (AMW) spectrum and the extent by which the iron is associated with the peaks. From figures 7.1 and 7.2 it can be seen that there is a relationship between the peaks and troughs displayed on the graphs representing total iron (thin line) and dissolved organic carbon (heavy line), obtained from the interstitial water of planted microcosms. In comparison, interstitial water from

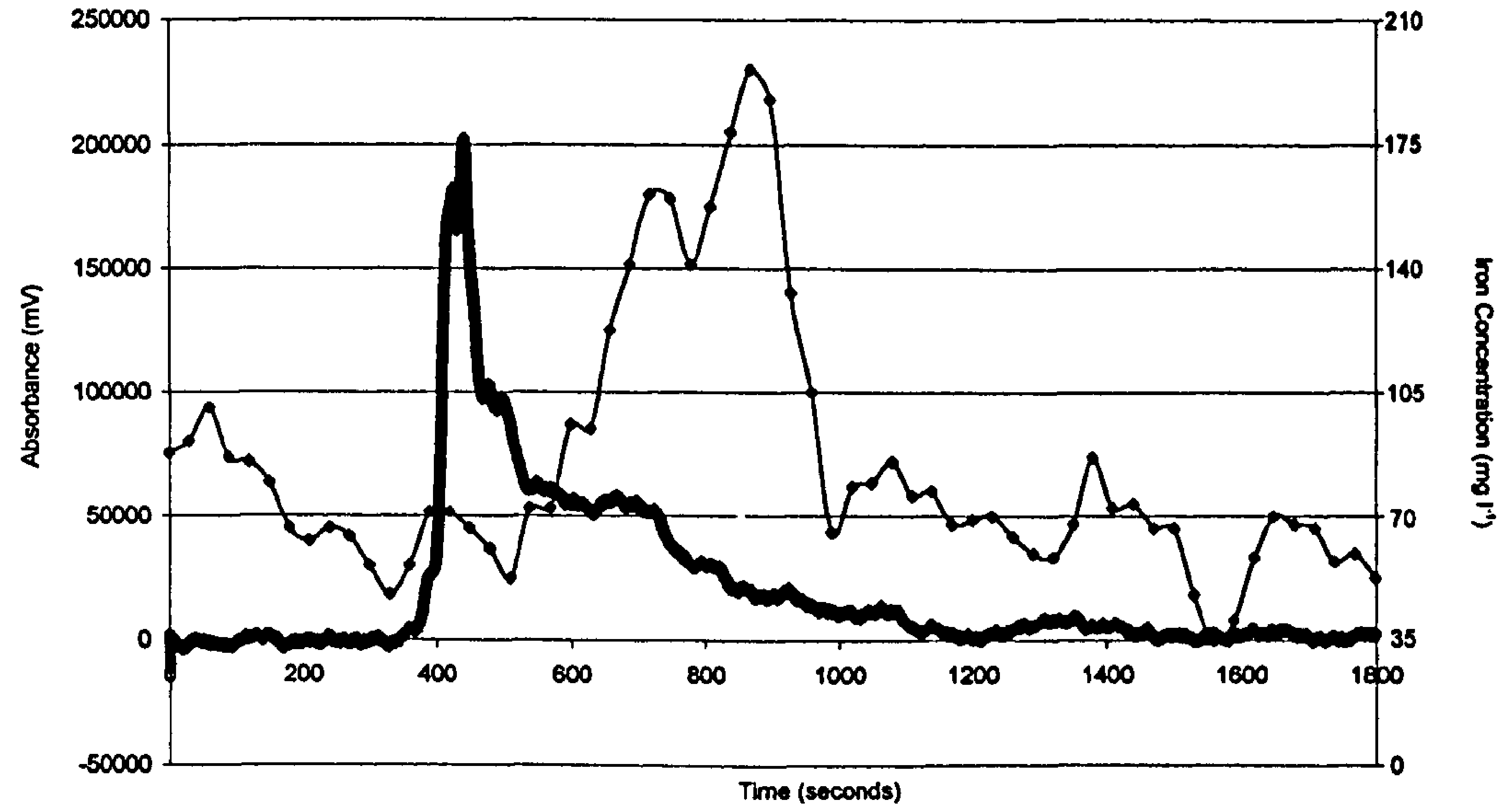


Figure 7.3: A typical AMW spectrum of DOC and its association with dissolved iron, obtained from interstitial water samples taken from unplanted peat microcosms after 19 days. Note: heavy line = dissolved organic carbon and fine line = dissolved iron concentration.

the unplanted soil (figure 7.3) does not appear to show this trend. The iron elutes at a different time to DOC.

7.1.2 Complexation of Iron with the Phenolic Component of Plant Derived Dissolved Organic Carbon

Materials and Methods

Initial investigations were undertaken to examine the hypothesis that metals, in this case iron, form complexes with phenolics in these microcosms treating mine drainage. For this purpose, interstitial water samples obtained from the microcosms used for the planting and soil experiments post addition of Ynysarwed or Mona mine drainage water, were used. The test for polyphenolic substances was undertaken using the protocol adapted from Box (1983) and folin-ciocalteau phenol reagent, the protocol for which was outlined in chapter 2 (section 2.6).

Planted mushroom compost microcosms were set up, in the described manner, treating water from Mona mine drainage. The following additions were made at a volume of 200ml, to each set (microcosms set up in quadruplicate) of microcosms, on day 10 – the point at which re-mobilisation of iron occurs in the planted soils:

- Phenolics – natural phenolic water was obtained from the wetland where the plants for this research were obtained (The phenolic concentration at start was 4.19mgl^{-1}). One set of three microcosms had phenolic additions that were pre-filtered through GF/C filters then 0.2μm, to remove any particulate matter and micro-organisms. The second, were added 'raw', thus also including the microbial community.
- Tannic acid – 1mM solution was made up using MilliQ water.

Controls were set up using additions of MilliQ water. Solutions and dilutions were made using MilliQ water.

Each microcosm was sampled on a regular basis using the sample port described in chapter 2 (section 2.2.2). These samples were handled, treated and analysed for dissolved iron as detailed in the same chapter.

Results

Figures 7.4 and 7.5 show the effect of soil and planting on the concentration of polyphenolic substances in microcosms treating Ynysarwed and Mona mine drainage waters respectively.

In microcosms treating Ynysarwed mine water, a generally lower concentration

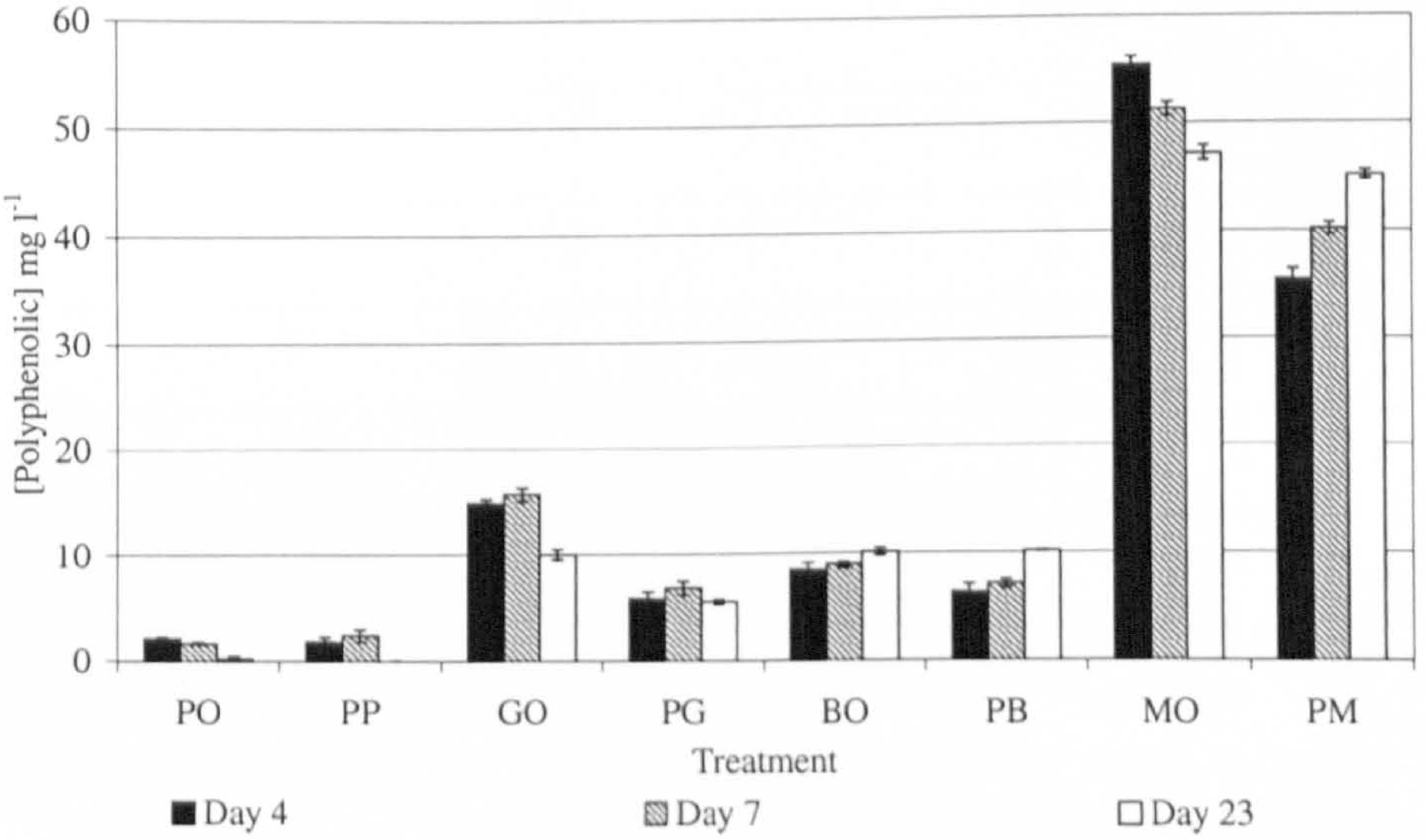


Figure 7.4: The effect of soil type and planting on the concentration of polyphenolic substances in the interstitial water from microcosms treating water obtained from Ynysarwed. Key: PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost. Mean±s.e., n=4.

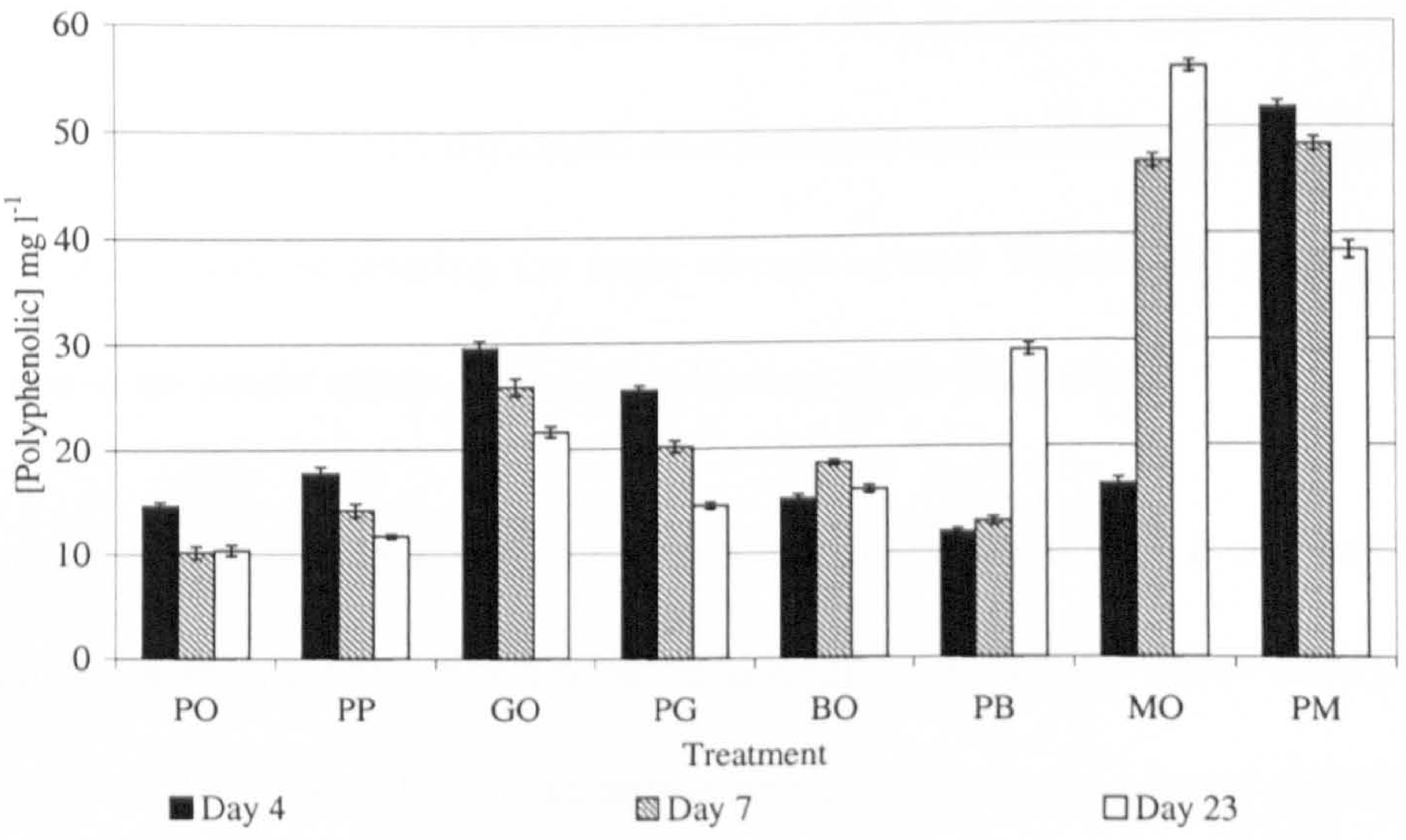
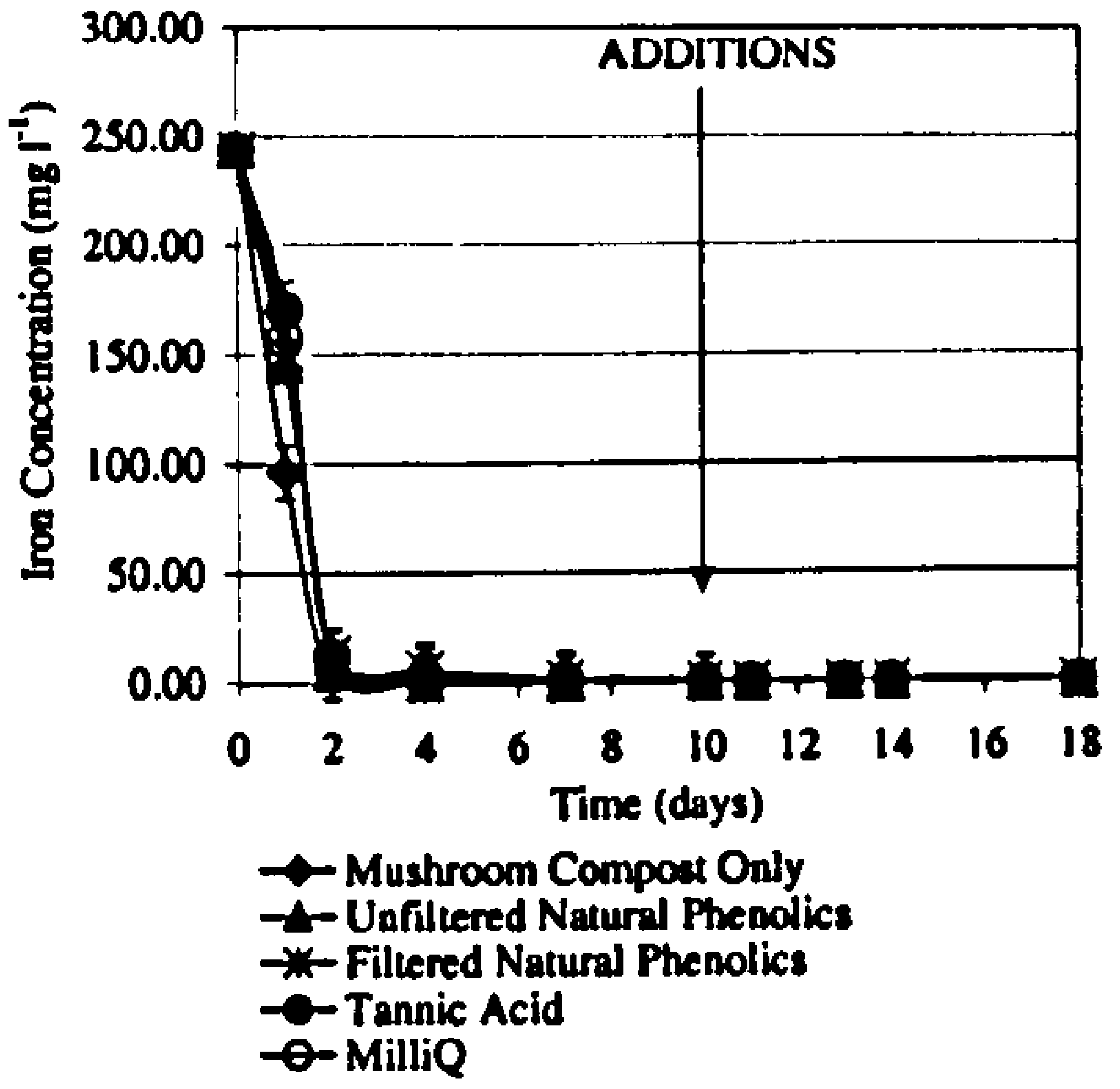


Figure 7.5: The effect on concentrations of polyphenolic substances in the interstitial water from microcosms treating water obtained from Mona adit, when soil type is altered and in the presence and absence of plants. Key: PO = peat only, PP = planted peat, GO = gravel only, PG = planted gravel, BO = bark-chippings only, PB = planted bark-chippings, MO = mushroom compost only and PM = planted mushroom compost. Mean±s.e., n=4.

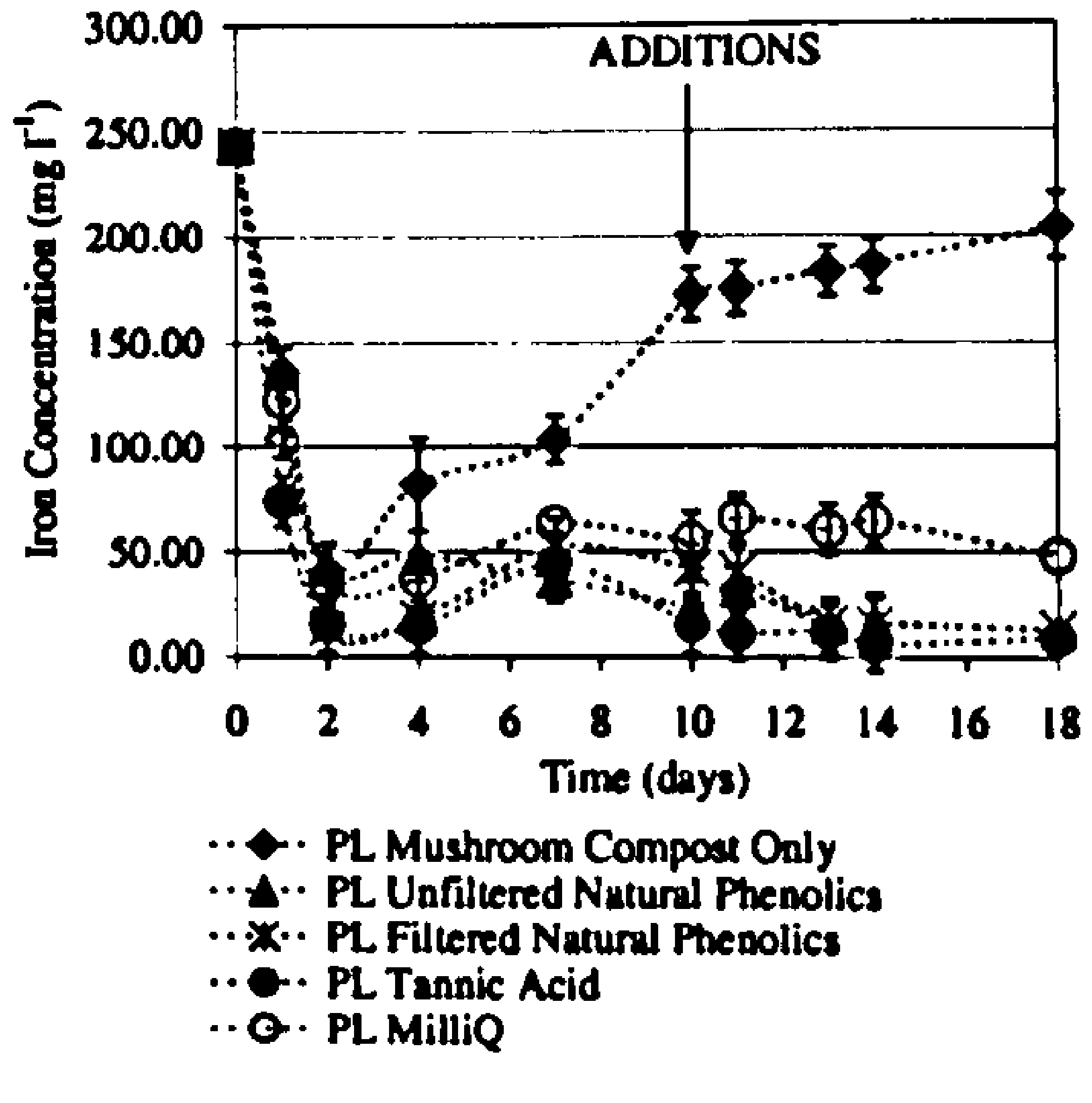
of polyphenolic substances was observed in the interstitial water from planted microcosms compared to their unplanted counterparts (up-to 48% $P<0.1$; figure 7.4) This was not observed in microcosms treating Mona mine water, where similar or higher concentrations were observed in the interstitial water from planted microcosms (figure 7.5). Those microcosms treating the more acidic Mona water, also displayed a generally higher (on average 65%, $P<0.0001$ in peat, gravel and bark-chipping planted and unplanted microcosms) concentration of polyphenolic substances than those treating the more circum-neutral Ynysarwed water. Therefore, the more acidic water (Mona) is releasing more phenolics.

Figure 7.6 shows the dissolved iron concentration in planted (PL) and unplanted experimental microcosms filled with mushroom compost, to which Mona mine drainage has been added. It also shows the effect of the aqueous additions of phenolics to these microcosms (after 10 days) on the iron concentrations.

In all of the experimental microcosms, there was an extremely significant drop (up-to 61%, $P<0.01$) in dissolved iron over the first forty-eight hours and it can be seen that all of the unplanted microcosms maintained approximately zero total iron concentrations. It can also be seen from figure 7.6 that each of the planted microcosms, except those containing planted mushroom compost, followed a similar decline in iron concentrations, followed by a marked increase of a magnitude of up-to four times the concentration observed on day 2 (2-7 days) and then a grad-



(a) Unplanted soils



(b) Planted soils

Figure 7.6: The effect of tannic acid and phenolic additions to planted and unplanted microcosms ameliorating water obtained from Mona adit in microcosms containing mushroom compost. Note: after the water sample was taken on the 10th day, aqueous additions of phenolics and tannic acid were made to the microcosms and their affect can be observed. See figure 7.7 for detailed responses post additions. Where 'PL' denotes planting. Mean±s.e., n=4.

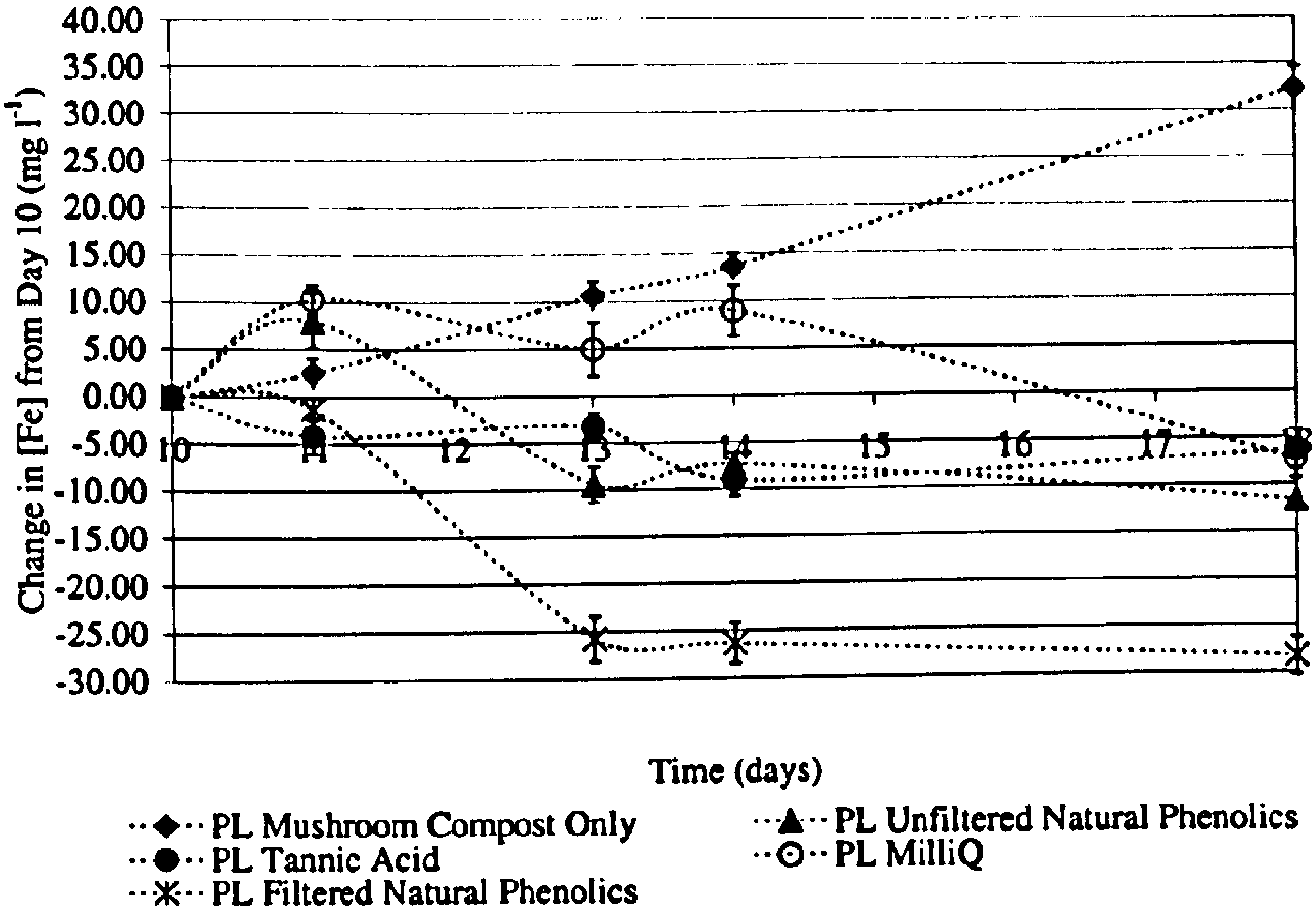


Figure 7.7: The relative change in total iron in planted microcosms following the additions of phenolics, acid mine drainage (AMD), tannic acid and MilliQ water. Mean±s.e., n=4. Where 'PL' denotes planting.

ual decline. Microcosms containing planted mushroom compost do not follow this trend but continues to display an increase in dissolved iron concentration.

The presence of plants (and their associated microflora) consistently causes an increase in iron after day 2 as can be seen in figure 7.6. The degree of increase is variable from batch to batch, presumably because of unavoidable variation between batches. From figure 7.6, it was difficult to discern the relative effect of each of the additions, therefore, figure 7.7 facilitates a comparison of effects.

Figure 7.7 shows that following the addition of MilliQ water, there was an increase in the amount of dissolved iron. This increase was only seen in one other case and that was following the addition of unfiltered natural phenolics, although in this instance, it was followed by a more rapid decrease. The most marked decrease in dissolved iron was observed in those planted peat microcosms after the addition of filtered natural phenolics. From this figure, the re-mobilisation of iron can also be clearly seen.

7.1.3 Overall Discussion

Investigations were undertaken to study the mechanisms that were imparing the ability of planted mushroom compost to efficiently remove metals, in particular iron, from metal-rich mine waters such as Mona. There were two mechanisms proposed. Firstly, that iron was forming complexes with the naturally occurring DOC. Secondly, that the iron was forming complexes with the plant derived polyphenolic substances present in the DOC. The results of the HPLC dissolved organic carbon analysis showed that complexation is occurring between iron and plant derived DOC (shown by the association of peaks in figures 7.1 and 7.2). It would seem that this phenomena may be preventing the removal of iron from these microcosms. Fenner *et al.* (2001) also observed this effect when studying peat pore water samples obtained from a natural wetland in Plynlimon, Mid Wales, following a period of drought then re-wetting. Seasonal variations in the apparent molecular weight (AMW) of the DOC was also observed (Fenner *et al.* (2001)). Further research is needed into this complexation phenomena in wetlands treating (acid) mine drainage to precisely determine which molecular weight is responsible.

From the results outlined in this section, it can be seen that a substantial decrease in iron concentration was observed in all unplanted microcosms over the first forty-eight hours. Indeed, each of the planted microcosms followed a similar decline in iron concentrations, followed by a marked increase (2-7 days) and then a gradual decline. Again, this phenomenon was observed in previous investigations undertaken over 23 days using planted mushroom compost (figure 4.3(d)). It can also be seen that by augmenting the natural concentrations of polyphenolic substances within these planted microcosms, with aqueous additions of natural phenolics and tannic acid on day ten (when the characteristic re-mobilisation occurs), a decrease iron concentration is observed (figure 7.6). Therefore, when natural phenolics and tannins are added to planted microcosms, they reduce the availability of soluble iron.

McColl and Pohlman (1986) found that the toxicity of aluminium could be reduced by phenolics binding to it. It is possible that some iron–organic matter complexes are occurring within these microcosms, which maintains this iron in soluble form. However, the decrease in iron after the phenolic addition, suggests that this is not due to phenolics. Indeed, analysis of pre-experimental polyphenolic compounds in a variety of soils (mushroom compost, peat. bark-chipping and gravel) are shown in figures 7.4 and 7.5. These figures show that generally planted soils do not have concentrations of polyphenolic substances that are higher than their unplanted counterparts, with a few exceptions such as day four mushroom compost treating mine water from Mona (figure 7.5).

It has already been shown throughout this research that mushroom compost is extremely effective in facilitating the process of metal removal. Once again the results shown in this section show the negative effect that planting has on the remediation of iron. The presence of plants and associated microflora produces, as seen in the other planted soils, re-mobilisation. If the experimental period is continued post 18 days, to the 23 days as seen in figure 4.3(d) (chapter 4), iron concentration decreases. This may be because the complexing agent is being metabolised/broken down by the microflora.

7.2 Competition for Nutrients

7.2.1 Materials and Methods

Mona mine drainage water was added to mushroom compost-based microcosms and the following additions were added at the pre-described point (day 10, when re-mobilisation of iron occured), at a volume of 200 ml to quadruplicate groups of microcosms:

- Long-Ashtons solution obtained courtesy of the Plant Physiology and Biochemistry Research Group at Bangor University, derived from protocols outlined by Hewitt (1966) and Smith *et al.* (1983). Prior to the solution's addition to the test microcosms, the stock solution was diluted by half and then added in the appropriate volume.
- Lactate - 1mM solution of L-(+)-Lactic Acid (2-Hydroxypropionic Acid) sodium salt (Sigma L-7022, Lot. 98H2605) was made. The lactate concentration used in this experiment was similar to that used by Tsukamoto and Miller (1999), although higher concentrations have been used (Elliott *et al.*, 1998).

Once again, all solutions and dilutions were made using MilliQ water and controls were set up using MilliQ water.

Interstitial water samples were taken using the pre-described method (chapter 2, section 2.2.2) and analysed for dissolved iron.

7.2.2 Results

Iron concentration in planted (PL) and unplanted mushroom compost microcosms treating water obtained from Mona adit are presented in figure 7.8. The most important feature of this figure is that it also shows the effect of the addition of Long-Ashtons solution, lactate and MilliQ water after ten days on these concentrations, at a time when iron re-mobilisation is occurring in these samples.

Figure 7.8 shows that over the first 48 hours there is a substantial decrease in total iron and that the unplanted microcosms, once again, maintain approximately zero total iron concentrations. The planted microcosms on the other hand, exhibited a similar initial decline in iron concentrations but this was then followed by a small significant increase (2-7 days. $P<0.05$) by a magnitude of up-to 2.15 times the observed iron concentration on day 2. It should be noted that the re-mobilisation of iron, as observed in these planted microcosms, is less marked in those treating

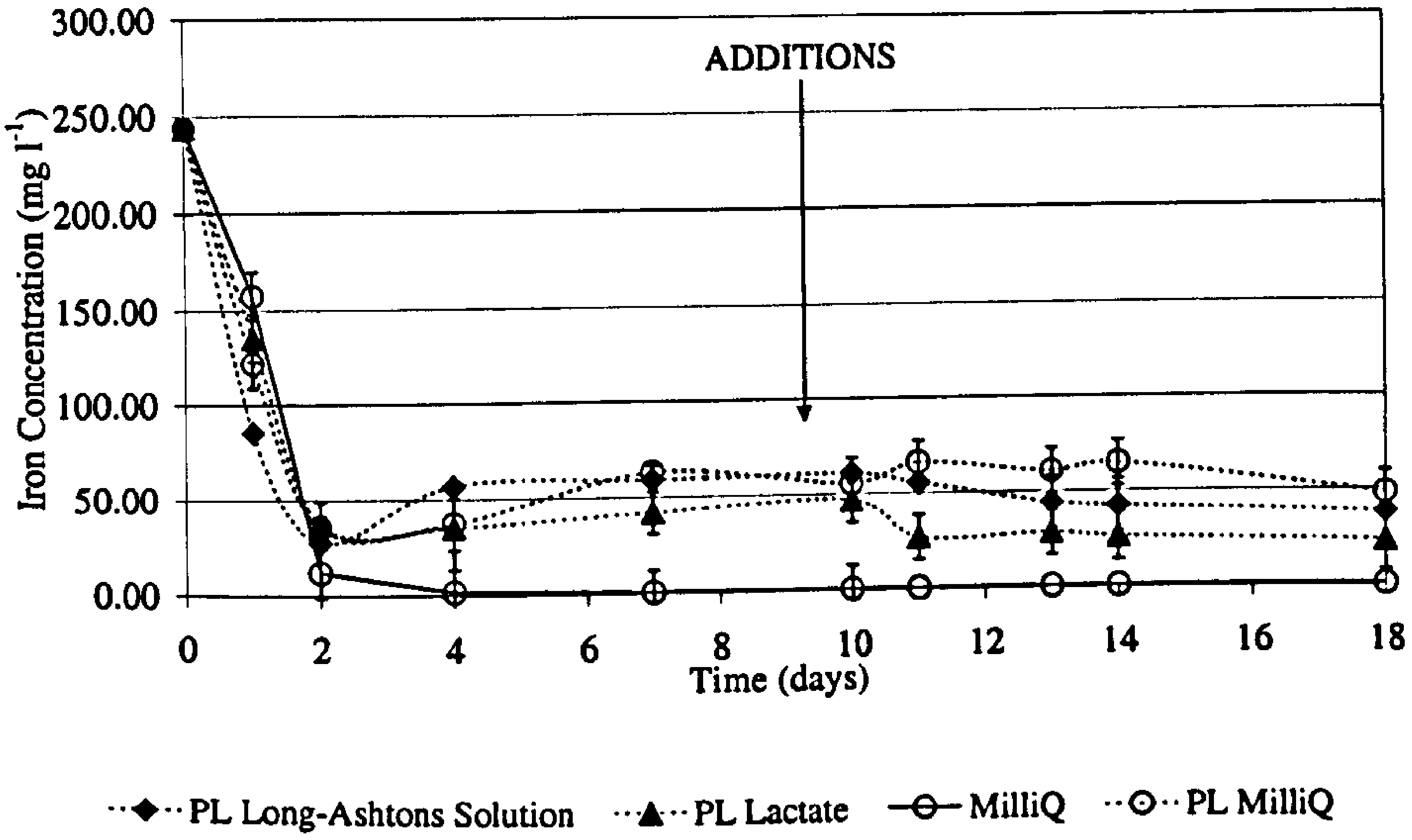


Figure 7.8: The effect of aqueous additions of lactate and Long-Ashtons solution on the amelioration of Mona mine drainage water in microcosms containing mushroom compost. Note: after the water sample was taken on the tenth day, aqueous additions of Long-Ashtons solution and lactate were made to the microcosms and their effect can be observed. Mean±s.e., n=4. Where 'PL' denotes planting.

Mona mine water as shown in figure 4.3 (chapter 4), than those treating Ynysarwed mine water (figure 4.2, chapter 4). Again these results are consistent with previous data shown in this chapter and those prior (for example chapter 4).

Following the aqueous additions (day 10), a significant decrease in iron concentration was observed on day 18 compared to that when the additions were made (day 10), in those microcosms with the addition of lactate and Long-Ashtons solution ($t=4.99$, 6df, $P<0.0001$ and $t=8.045$, 6df, $P=0.0002$, respectively). The addition of lactate proving to be the more effective at decreasing the iron concentration in these microcosms.

Again, it was difficult to discern the relative effect of the Long-Ashtons solution and lactate additions due to the similarity in trends. Therefore, figure 7.9 was composed to facilitate comparison.

Figure 7.9 shows more clearly the decrease in iron concentration in samples receiving additions of Long-Ashtons solution and lactate, with the latter producing the most instantaneous decrease in iron concentration.

Statistical analysis using a t-test, showed there to be a significant difference in mean iron concentration in microcosms following the addition of lactate or Long-Ashtons solution (day 10) to the end of the experimental period ($t=3.019$, 8df, $P=0.0166$).

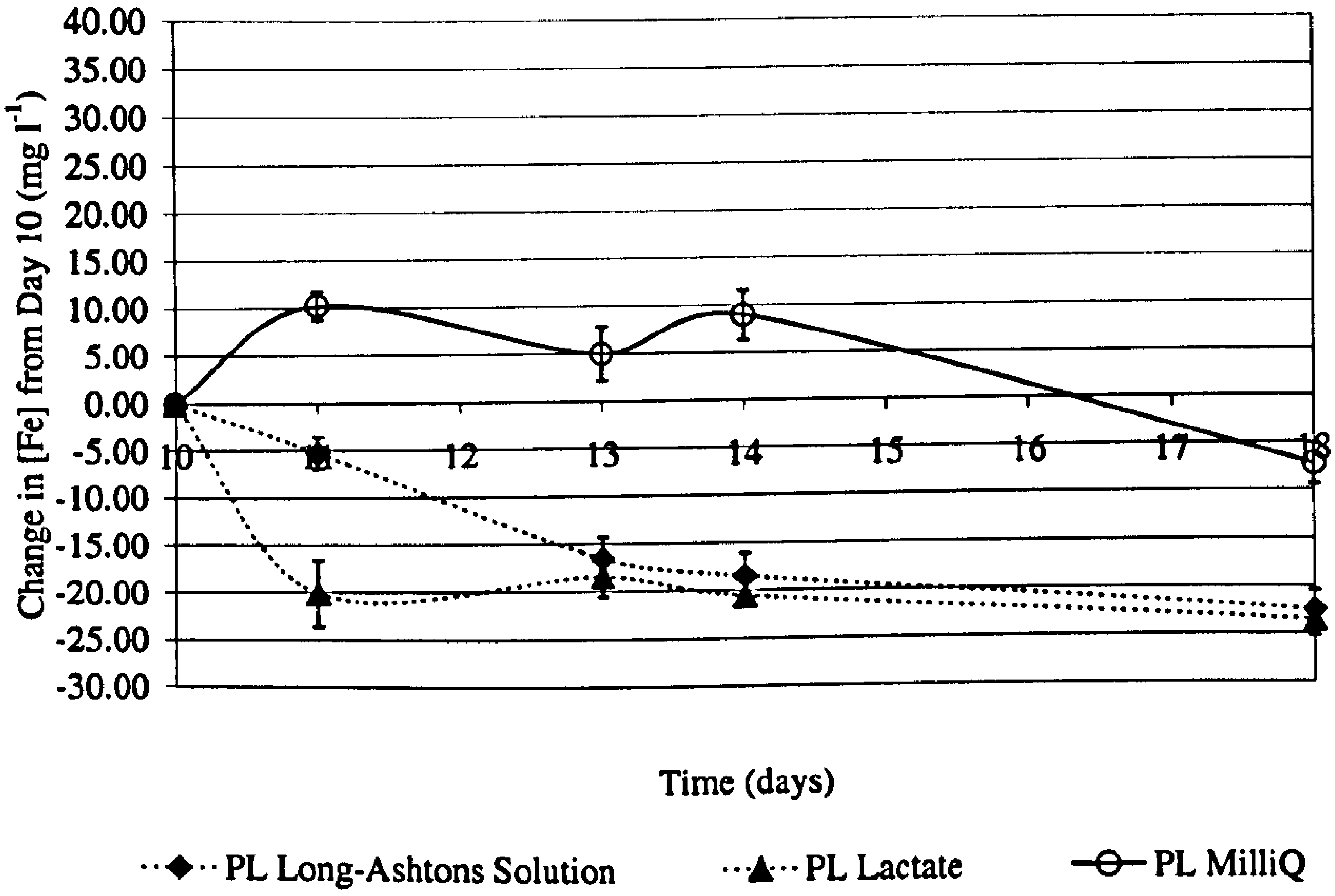


Figure 7.9: The relative change in total iron in planted microcosms following the additions of Long-Ashtons solution, lactate and MilliQ water. Mean±s.e., n=4. Where 'PL' denotes planting.

7.2.3 Discussion

Previous research detailed in this thesis (chapters 4 and 5) showed that the presence of plants and their associated microflora had a detrimental effect on the removal of metals, in particular iron, from (acid) mine drainage waters. It was shown that after an initial decrease in iron concentration over the first twenty-four to forty-eight hours, re-mobilisation of metals was observed. Therefore, the roles of plants and microbes within these microcosms were investigated. Competition from plants was overcome by using Long-Ashtons solution (an inorganic nutrient media) and microbes, in particular sulphate reducing bacteria (SRBs) with the addition of lactate.

The results show that all microcosms exhibit an initial decrease in iron concentration and re-mobilisation is occurring in the planted soils (figure 7.8). In this experiment, the lactate and Long-Ashtons solutions were all made with MilliQ water and not surprisingly their effect is greater than MilliQ water alone (figure 7.8). These results reinforce the suggestions made by, for example Lindsay (1991), and discussions within this thesis (chapter 4), that the re-mobilisation and solubilisation of iron is not an abiotic phenomenon but indeed biotic. Whilst atmospheric oxygen may be responsible in part for the oxidation of ferrous iron, the situation seems far more complex due to the shapes of the graphs displayed above.

If a bacterial stimulus in the form of lactate is added to the microcosms, or indeed any of the other additions, there will possibly be a lag-time before the effect of the microorganisms is seen (although in this instance the effect of lactate addition was instantaneous). As a consequence, iron removal occurs at a slow rate before the microorganisms have an affect, leading to a more rapid iron remediation being observed (figure 7.8). These results suggest that some competition is occurring between the plants and the microbial population for resources. Thus competition from plants may add to the detrimental effects caused by the complexation highlighted in section 7.1.

7.3 Summary

Research detailed previously in this thesis (chapters 4 and 5) found mushroom compost to be the most beneficial soil type for the remediation of iron from mine drainage waters. These studies also showed that the presence of plants had a detrimental effect on a soils ability to remediate iron, leading to its re-mobilisation. Therefore, microcosms studies were used to research the mechanisms occurring within planted and unplanted soils, facilitating the aforementioned effects. The results have shown that some competition is occurring between the indigenous plants and microbes for resources, for example inorganic nutrients, and that this may impare the microbes ability to ameliorate the available iron. Secondly, the available iron appears to be forming complexes with the plant derived dissolved organic carbon present in the system, thus preventing the removal of iron from these pilot wetlands. However, these complexes do not appear to be due to the phenolic constituents.

Chapter 8

Conclusions

The main objective of this research was to investigate:

1. Iron oxidation in mine drainage waters as a mechanism for iron removal.
2. To elucidate the biogeochemical processes occurring within wetlands constructed for the treatment of these waters.
3. Allow the design of a practical treatment system.

Laboratory based investigations were undertaken to fulfill the aims of this thesis (Chapter 1, section 1.4).

8.1 Factors Affecting Iron Oxidation Rates

In this study, shake flask experiments were set up to determine the factors affecting the rate of iron oxidation in Ynysarwed and Wheal Jane waters, a method utilised by researchers, for example to investigate the effects of temperature on the microbial leaching of sulphide minerals (Ahonen and Tuovinen, 1991). Many researchers consider iron oxidation to be the rate limiting step in the treatment of mine drainage waters (Kirby *et al.*, 1999). Therefore, this oxidative process, should be included in any remediation strategy design (Singer and Stumm, 1970; Maree *et al.*, 1998). The experimental investigations, detailed in this thesis, on waters obtained from Ynysarwed and Wheal Jane mine drainage sites, showed that the rate limiting factor for iron oxidation in Ynysarwed and Wheal Jane waters, is primarily biological. The number of indigenous iron-oxidising bacteria at both of the aforementioned sites has been shown to be low, especially in those waters obtained from Ynysarwed, where no *T. ferrooxidans*- and *L. ferrooxidans*-like bacteria were found, although, two novel iron oxidising species were isolated. In contrast, the microbial counts on Wheal Jane water showed that this water body possessed higher numbers of iron oxidising organisms.

This research showed experimentally, that the growth of the iron oxidation, accelerating microorganisms found in these waters, are limited by the availability of inorganic nutrients, in particular phosphate. The concentration by which these waters appear to be limited is in the region of 0.3mM, although, more detailed investigations would be required to provide a precise result. Indeed at 10°C, a temperature akin to the mean environmental temperature of British water, the addition of inorganic nutrients, in particular phosphate, consistently increased oxidation rates. In terms of Ynysarwed, it increased from 0.18 ± 0.02 mg l^{-1} hr^{-1} to 0.59 ± 0.01 mg l^{-1} hr^{-1} following the addition of nutrients. These results support previous findings where increasing the numbers of iron oxidising bacteria have been shown to increase the rate of iron oxidation (Strömberg and Banwart, 1995).

Phosphate is important in microbial nutrition and is required by cells for the primary synthesis of nucleic acids and phospholipids. It is found in nature in the form of organic and inorganic phosphates, but clearly, the results from this research show that there is insufficient inorganic phosphate available in these mine drainage waters to enhance microbial biomass. Although, some organisms such as *Escherichia coli* synthesise new proteins, facilitating the organisms adaptation to a phosphate-deficient environment (Madigan *et al.*, 1997). Further work is required to investigate whether the ability of the organisms found in these mine drainage waters, exhibit this response due to the stressed conditions.

Application of these findings to the natural environment poses problems in terms of upsetting the balance of the natural ecosystem by increasing microbial numbers. Augmentation of phosphate concentration to these waters is not a problem, but again has environmental implications such as excess can lead to the eutrophication of waterways (Mason, 1995). Thomas *et al.* (1997) (also Thomas *et al.* (1998) and Stephen and Macnaughton (1999)) showed the potential use of natural microbial isolates which can degrade tributyl phosphate (TBP), which can be added in the treatment of mine drainage waters. These organisms utilise TBP as their sole source of carbon and phosphorus, releasing inorganic phosphate. Further studies are warranted to investigate the long-term implications of this research (detailed in chapter three of this thesis), on the natural environment. Although, it is possible, that the findings of this research have a value, and could be applied, as a pre-treatment system to a wetland.

8.2 The Role of Plants in Wetlands Constructed for the Treatment of (Acid) Mine Drainage

Previous research has emphasised the importance of wetlands and their associated vegetation in constructed systems, for the treatment of mine drainage waters (Eger and Lapakko, 1989; Skousen *et al.*, 1994). Indeed, research by Frostman (1993), has shown that metals, such as iron, have been successfully removed from experimental wetlands for a number of years. The type of vegetation used varies and is largely dependent upon climate and geographical location. The most commonly used plants in constructed wetlands are *Typha* spp. (cattails) and *Scirpus* spp. (bullrushes) (Kadlec, 1995), although, these constructed systems may be subject to floral changes with time, altering the mosaic of vegetation in these systems (Webster *et al.*, 1994).

Bench-scale micocosms were set up to investigate the effect of planting on the remediation of Ynysarwed and Mona mine drainage waters (chapter 5) using indigenous tolerant flora, from a natural wetland, North Wales (details chapter 2). Previous research (Macfie and Crowder, 1987; Emerson *et al.*, 1999) has shown the formation of iron plaques in the plants' rhizosphere and its affect on metal speciation. Although, for this to occur the pH must be low, without which metal precipitation will occur throughout the soil. The results of this research showed that planting had a negligible effect in terms of metal removal, in particular iron and to a lesser extent alkalinity generation, when compared to their unplanted counterparts. In some cases, e.g. planted peat, acidity was generated when treating Ynysarwed mine drainage water, but generally, planted soils were found to be more useful at ameliorating the acid of mine drainage waters, maintaining pH values between 5 and 7. Plants also have a positive effect on sulphate removal and this was found to be of greatest evidence in the microcosms containing planted peat.

However, planted soils were also shown to be responsible for the re-mobilisation of metals, reducing the treatment efficiency for metals.

Investigations were conducted into the reasons for the poor performance of the planted soils in terms of metal (iron) removal. This was undertaken by investigating the possibilities of competition and complexation (chapter 7) occurring within these experimental bench-scale wetlands. An attempt was made to give the microbes associated with remediation a competitive advantage by eliminating the competition from plants for inorganic nutrients and by also adding an organic substrate. Plants were selectively stimulated by an inorganic nutrient solution and the microbial community by lactate addition. Lactate or whey addition has been shown to increase metal removal, by stimulating the microbial community in particular the sulphate reducing bacteria (Elliott *et al.*, 1998; Borek *et al.*, 1995). These organisms produce hydrogen sulphide which reacts with the metals forming insoluble metals sulphides, resulting in the precipitation of metals (Webb *et al.*, 1998). The results, detailed in chapter 7, have shown that some competition for inorganic nutrients is occurring between the indigenous plants and microbes, thus imparing the microbial remediation of iron. High performance liquid chromatography (HPLC) was used to address the hypothesis that iron was forming complexes with plant-derived dissolved organic carbon (DOC) and microcosm studies were used to investigate whether the phenolic component was responsible. Results from these investigations have shown that the plant derived dissolved organic carbon (DOC) is forming complexes with the available iron preventing iron removal from the pilot wetlands. However, further research showed that this complexation does not appear to be forming between iron and the phenolic constituents.

Potential for the application of these results in full scale wetland treatment systems will be discussed in a subsequent section (8.4).

8.3 The Importance of Soil Type in Constructed Wetlands for (A)MD Treatment

For the purpose of this research, microcosms were set up using varying types of soil, namely mushroom compost, bark-chippings, peat and gravel. The study attempts to evaluate whether the different soil types would enhance the rate of metal immobilisation according to the ability of the soil to support the actions of sulphate reducing bacteria. The results showed (detailed in chapters 4 and 6) that mushroom compost was the most beneficial soil of those tested, in terms of metal removal and alkalinity generation, with a required water residence time of only four days. This was shown to have a high sulphate concentration, which is of clear value to sulphate reducing bacteria (SRB). This was associated with high sulphatase activity and the presence of gypsum (calcium sulphate), both of which ensure an abundance of sulphate for sulphate reducing bacteria. By increasing sulphatase concentration in peat (a soil with low sulphatase activity and sulphate concentration), it was shown that an improvement in metal immobilisation was possible, similarly by adding calcium sulphate, confirming the hypothesis that gypsum and sulphatase are important in mushroom compost in terms of its remediative benefits.

Longevity experiments showed that mushroom compost could continue to remediate iron from synthetic mine drainage over a ten week test, without decreasing performance. The universality of the soils (i.e. potential value at other sites) outlined above and detailed in chapter 6, were investigated using a wide variety of highly contrasted mine waters obtained from throughout the British Isles. Mushroom compost and peat were consistently capable of satisfactorily remediating British (acid) mine drainage waters, irrespective of their origin, in terms of metal removal, alkalinity generation and sulphate reduction respectively. Finally, the universality in terms of the potential value of mushroom compost for treating other metals was evaluated in a comparison of removal rates for cadmium, aluminium, copper, zinc, cobalt and manganese. Mushroom compost was confirmed as the most favourable soil type for the treatment of metals. The applications of these results will be discussed in the following section.

8.4 Proposed Pilot System for the Treatment of (Acid) Mine Drainage

From the results obtained from investigations detailed in this thesis, it is possible to propose a universal remediation strategy for the amelioration of British waters affected by (acid) mine drainage and possibly those further afield. This remediation strategy is ostensibly a three-fold mechanism, each requiring a residence time of four days, involving (in the order in which treatment would occur);

1. iron oxidation;
2. metal removal and alkalinity generation in a unplanted wetland containing mushroom compost;
3. sulphate reduction and pH maintenance in a planted peat wetland.

Due to time constraints, it was necessary for a preliminary investigation to be undertaken by an undergraduate student, Niall Gilmartin, using synthetic mine drainage in the proposed pilot system. The pilot system was built of bench-scale proportions, using guttering at a gradient allowing continuous flow through the wetland and a residence time of four days within each treatment wetland. Results have shown this proposed pilot system to be highly effective in the treatment of (acid) mine drainage. Studies showed that successful iron removal occurred to levels that were undetectable, pH was successfully raised from an acidic 2.5 to near neutral and the high levels of sulphate were decreased in the planted pilot wetland, although further, more detailed work, is required.

Many hypotheses have been put forward as to the optimal size of these constructed treatment wetlands such as Hedin *et al.* (1994), taking into account factors such as flow rates, the physicochemical characteristics of the water and soils being utilised. Personal opinion is that many of the wetlands constructed today for the treatment of the waters are, in the first instance, possibly too small, not allowing an appropriate residence time, resulting in their varying success rates. The other being that the treatment wetlands are too juvenile using young non-tolerant ecotypes, not allowing for the benthic community to be well established and the presence of vast quantities of decaying organic matter from the aerial flora. This is suggested by the observation that when digging up the plants for this research, there was vast quantities of decaying organic matter associated with the root system. My opinion is that these constructed wetland systems should be of an area greater than that of a football pitch proportions (typically 120 metres × 90 metres) especially if the flow rate is equivalent or greater than that of Ynysarwed – 3 l^{-1} sec^{-1}. Using Kikuths equation (retention time = active volume ÷ flow rate) it was calculated that for the wetlands at Ynysarwed (3200 m^2 and 5660 m^2) the residence times would be twenty-one minutes twenty seconds and one hour six minutes. If the aforementioned football pitch dimensions are used with a similar depth of soil as found at Ynysarwed, this would then be increased to one hour twelve minutes. This research found that a residence time of four days is needed for the amelioration of mine waters. Therefore, the active volume of the wetland needs to be increased to 1036.8 m^3. This may be achieved by increasing the depth of soil in a football pitch size wetland to 24cm or by increasing the area of the wetland by a similar factor (80).

8.5 Further Study

In the first part of this thesis, the factors affecting the rate of iron oxidation in mine drainage waters, were investigated. Further investigations are needed to ascertain the effect of increasing the inorganic nutrient content (namely phosphate) on the immobilisation of metals within these systems (in-stream/river and wetland) and the long-term implications of enhancing these microbial communities. Further research is also needed to investigate the microbial community structure of wetlands such as those detailed above and ascertain if there exists correlation between the species types. Little is also known about the new isolates, isolated during this research and thus further work is required.

In this research it was highlighted that the complexation of iron with plant derived dissolved organic carbon was possibly preventing the removal of iron from these microcosms. Further investigations are required to ascertain the molecular weight of carbon responsible. Moreover, the dynamics of the interactions occurring within the organic soils must be addressed and monitored over the growing season. Long-term studies are needed to investigate the rate and quantities of labile organic carbon introduced into the system, which has environmental implications in terms of pollution control, in association with enzymatic studies, especially those involved in the degradation of organic matter.

During this research preliminary investigations, using HPLC, were undertaken into the organic acid component of these systems (data not shown - too little data, varying sucess). These substances are of importance as a carbon source to the microbial community in particular the heterotrophs and more research is needed.

Long-term experimental wetlands are required to investigate the longevity of the proposed pilot system detailed above and more detailed investigations are required as to the optimal size for wetlands used to ameliorate (acid) mine drainage waters.

Appendix A

Appendix B

Basal Salt Formulation

1.5 gl^{-1} $(NH_4)_2 SO_4$

0.5 gl^{-1} KCl

5.0 gl^{-1} $MgSO_4.7H_2O$

0.5 gl^{-1} NaH_2PO_4

0.1 gl^{-1} $Ca(NO_3)_2$

Adjust to pH 2.5 with sulphuric acid.

Note: 'ANALAR' grade chemicals and ultra pure water (MilliQ) to be used throughout.

References

Ahonen, L. and Tuovinen, O. H. (1991). Temperature effects on bacterial leaching of sulfide minerals in shake flask experiments. *Appl. Env. Microbiol.*, **57**, 138–145.

Amero, A. M., Hallberg, K. B., Lindström, E. B., and Jerez, C. A. (1994). An immunological assay for the detection and enumeration of thermophylic biomining microorganisms. *Appl. Env. Microbiol.*, **60**, 3470–3473.

Appel, H. M. (1993). Phenolics in ecological interactions - the importance of oxidation. *J. Chem. Ecol.*, **19**, 1521–1552.

Atlas, R. M. and Bartha, R. (1981). *Microbial ecology: Fundamentals and applications*. Addison-Wesley, Mass.

Banks, D., Younger, P. L., Arnesen, R. T., Iversen, E. R., and Banks, S. B. (1997). Mine-water chemistry: the good, the bad and the ugly. *Env. Geol.*, **32**, 157–174.

Bavor, H. J., Roser, D. J., and Adcock, P. W. (1995). Challenges for the development of advanced constructed wetland technology. *Wat. Sci. Tech.*, **32**, 13–20.

Béchard, G., Yamazaki, H., Gould, W. D., and Bédard, P. (1994). Use of cellulosic substrates for the microbial treatment of acid mine drainage. *J. Env. Qual.*, **23**, 111–116.

Benner, S. G., Blowes, D. W., and Ptacek, C. J. (1997). A full scale porous reactive wall for the prevention of acid mine drainage. *Ground Wat. Monit. Remed.*, **17**, 99–107.

Bloomfield, C., Kelso, W. I., and Pruden, G. (1976). Reaction between metals and humified organic matter. *J. Soil Sci.*, **27**, 16–31.

Borek, S. L., Dvorak, D. H., and Edenborn, H. M. (1995). Ability of cheese whey to support bacterial sulfate reduction in mine water treatment systems. In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 879–885.

Boulegue, J. and Michard, G. (1979). Sulphur speciations and redox processes in reducing environments. In E. A. Jenne, editor, *Chemical modeling in aqueous systems. ACS Symposium Series, vol 93*, pages 25–49. ACS, Washington.

Box, J. D. (1983). Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Wat. Res.*, **17**, 511–525.

Brenner, F. J. (1995). Reclaiming mine land for habitat diversity. In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 1231–1237.

Brierly, C. L. (1990). Bioremediation of metal-contaminated surface and groundwaters. *Geomicrobiol. J*, **8**, 201–223.

Brix, H. (1994). Functions of macrophytes in constructed wetlands. *Wat. Sci. Tech.*, **29**, 71–78.

Brix, H. (1997). Do macrophytes play a role in constructed treatment wetlands? *Wat. Sci. Tech.*, **35**, 11–17.

Brodie, G. A., Britt, C., Tomaszewski, T. M., and Taylor, H. N. (1993). Anoxic limestone drains to enforce performance of aerobic acid drainage treatment wetlands: experience of the tennessee valley authority. In G. A. Moshiri, editor, *Constructed Wetlands for Water Quality*, pages 129–138. Lewis Publishers, London.

Brookins, D. G. (1988). *Eh-pH Diagrams for Chemistry*. Springer-Verlag, Berlin.

Cambridge, M. (1997). Wheal Jane: The longterm treatment of acid mine drainage. In *Fourth International Conference on Acid Rock Drainage*, pages 1127–1144.

Christensen, B., Lake, M., and Lien, T. (1996). Treatment of acid mine water by sulphate-reducing bacteria: results from a bench scale experiment. *Wat. Res.*, **30**, 1617–1624.

Chrost, R. J. (1992). Significance of bacterial ectoenzymes in aquatic environments. *Hydrobiologia*, **245**, 61–70.

Clarke, L. B. (1995). *Coal mining and water quality*. IEA Coal Research, London.

Coleman, M. L., Hedrick, D. B., Lovley, D. R., White, D. C., and Pye, K. (1993). Reduction of Fe(III) in sediments by sulphate reducing bacteria. *Nature*, **361**, 436–438.

Cooper, P. F. and Hobson, J. A. (1989). Sewage treatment by reed bed systems: The present situation in the United Kingdom. In D. A. Hammer, editor, *Constructed wetlands for wastewater treatment - municipal, industrial and agricultural*, pages 153–171. Lewis publishers Inc., USA.

Corbitt, R. A. and Bowen, P. (1994). Constructed wetlands for wastewater treatment. In D. M. Kent, editor, *Applied Wetlands Science and Technology*, chapter 10, pages 221–241. Lewis Publishers, USA.

Das, A., Mishra, A. K., and Roy, P. (1992). Anaerobic growth on elemental sulfur using dissimilar iron reduction by autotrophic *thiobacillus ferrooxidans*. *FEMS Microbiol. Letters*, **97**, 167–172.

Davies, T. H. and Cottingham, P. D. (1994). The use of constructed wetlands for treating industrial effluent (textile dyes). *Wat. Sci. Tech.*, **29**, 227–232.

Dennison, F., Sen, A. M., Hallberg, K. B., and Johnson, D. B. (2001). Biological versus abiotic oxidation of iron in acid mine drainage waters: An important role for moderately acidophilic, iron -oxidizing bacteria. In V. S. T. Ciminelli and O. Garcia Jr., editors, *Biohydrometallurgy: Fundamentals, Technology and Sustainable Development*, pages 493–501. Elsevier, Amsterdam.

Denny, P. (1997). Implementation of constructed wetlands in developing countries. *Wat. Sci. Tech.*, **35**, 27–34.

Dinesh, R., Ramanathan, G., and Singh, H. (1999). Influence of chloride and sulfate-ions on soil enzymes. *Journal of agronomy and crop science – Zeitschrift fur acker und pflanzenbau*, **175**, 129–133.

Dojlido, J. R. and Best, G. A. (1993). *Chemistry of water and water pollution*. Ellis Horwood Ltd, England.

Dougan, P. R. (1987). Prevention of formation of acid drainage from high-sulfur coal refuse by inhibition of iron- and sulfur-oxidizing microorganisms. I. Preliminary experiments in controlled shaken flasks. *Env. Poll.*, **96**, 1–11.

Dougan, P. R. and Apel, W. A. (1983). Bacteria and acidic drainage from coal refuse: Inhibition by sodium lauryl sulphate and sodium benzoate. *Appl. Env. Microbiol.*, **46**, 279–282.

Dougan, P. R., Macmillan, C. B., and Pfister, R. M. (1970). Aerobic heterotrophic bacteria indigenous to pH 2.8 acid mine water: predominant slime-producing bacteria in acid streamers. *J. Bacteriol.*, **101**, 282–288.

Dunbabin, J. S. and Bowmer, K. H. (1992). Potential use of constructed wetlands for treatment of industrial waste waters containing metals. *Sci. of the Tot. Env.*, **111**, 151–168.

Dvorak, D. A., Hedin, R. S., Edenborn, H. M., and McIntire, P. E. (1992). Treatment of metal contaminated water using bacterial sulfate reduction: results from pilot-scale reactors. *Biotechnol. Bioeng*, **40**, 606–616.

Dytham, C. (1990). *Choosing and using statistics: A biologist's guide*. Blackwell Science Ltd, London.

E.A. and Hyder (1991). Lime dosing to restore the upper Tywi. Technical report, EA.

Edwards, K. J., Gihring, T. M., and Banfield, J. F. (1999). Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. *Appl. Env. Microbiol.*, **65**, 3627–3632.

Eger, P. and Lapakko, K. (1989). *Use of wetlands to remove nickel and copper from mine drainage*, chapter 42e, pages 780–787. Lewis Publishers Inc., USA.

Eger, P. and Wagner, J. (1995). Sulfate reduction for the treatment of acid mine drainage; longterm solution or short term fix? In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 515–524.

Ehrenreich, A. and Widdel, F. (1994). Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Appl. Env. Microbiol.*, **60**, 4784–4792.

Eivazi, F. and Tabatabai, M. A. (1988). Glucosidases and galactosidases in soils. *Soil Biol. Biochem.*, **20**, 601–606.

Elliott, P., Ragusa, S., and Catcheside, D. (1998). Growth of sulfate-reducing bacteria under acidic conditions in an upflow anaerobic bioreactor as a treatment system for acid mine drainage. *Wat. Res.*, **32**, 3724–3730.

Emerson, D. and Moyer, C. (1997). Isolation and characterization of novel iron-oxidizing bacteria that can grow at circumneutral pH. *Appl. Env. Microbiol.*, **63**, 4784–4792.

Emerson, D., Weiss, J. V., and Megonigal, J. P. (1999). Iron-oxidizing bacteria are associated with ferric hydroxide precipitates (Fe-plaque) on the roots of wetland plants. *Appl. Env. Microbiol.*, **65**, 2758–2761.

Engleman Jr., C. J. and McDiffett, W. F. (1996). Accumulation of aluminium and iron by bryophytes in streams affected by acid-mine drainage. *Env. Poll.*, **94**, 67–74.

Evangelou, V. P. (1995). *Pyrite oxidation and its control.* CRC Press, New York.

Faulkner, S. P. and Richardson, C. J. (1989). Physical and chemical characteristics of freshwater wetland soils. In D. A. Hammer, editor, *Constructed wetlands for wastewater treatment – municipal, Industrial and agricultural*, chapter 4, pages 41–72. Lewis Publishers Inc., USA.

Fenner, N., Freeman, C., Hughes, S., and Reynolds, B. (2001). Molecular weight spectra of dissolved organic carbon in a rewetted welsh peatland and possible implications for water quality. *Soil Use and Management*, **17**, 106–112.

Ferris, F. G., Tazaki, K., and Fyfe, W. S. (1989a). Iron oxides in acid mine drainage environments and their association with bacteria. *Chem. Geol.*, **74**, 321–330.

Ferris, F. G., Schultze, S., Witten, T. C., Fyfe, W. S., and Beveridge, T. J. (1989b). Metal interactions with microbial biofilms in acidic and neutral pH environments. *Appl. Env. Microbiol.*, **55**, 1249–1257.

Fliermans, C. B. and Brock, T. D. (1972). Ecology of sulphur-oxidizing bacteria in hot acid soils. *J. Bacteriol.*, **111**, 343–350.

Fortin, D. and Beveridge, T. J. (1997). Microbial sulfate reduction within sulfidic mine tailings: Formation of diagenetic Fe sulfides. *Geomicrobiol. J.*, **14**, 1–21.

Fowler, J. and Cohen, L. (1990). *Practical statistics for field biology*. John Wiley and Sons, Chichester.

Freeman, C., Liska, G., Ostle, N. J., Jones, S. E., and Lock, M. A. (1995). The use of fluorogenic substrates for measuring enzyme activity in peatlands. *Plant and Soil*, **175**, 147–152.

Frostman, T. M. (1993). A peat/wetland treatment approach to acidic mine drainage abatement. In G. A. Moshiri, editor, *Constructed wetlands for water quality improvement*, pages 197–200. CRC Press, Inc., USA.

Gee, K. R., Sun, W. C., Bhalgat, M. K., Upson, R. H., Klaubert, D. H., Latham, K. A., and Haugland, R. P. (1999). Fluorogenic substrates based on fluorinated umbelliferones for continuous assays of phosphatase and β-galactosidases. *Analytical Biochem.*, **273**, 41–48.

Geiger, O., Röhrs, V., Weissenmayer, B., Finan, T. M., and Thomas-Oates, J. E. (1999). The regulator gene phoB mediates phosphate stress controlled synthesis of the membrane lipid diacylglyceryl-N,N,N-trimethylhomoserine in *rhizobium (sinorhizobium) meliloti*. *Molecular Microbiology*, **32**, 63–73.

Geller, W., Klapper, H., and Schultze, M. (1998). Natural and anthropogenic sulfuric acidification of lakes. In *Acidic Mining Lakes: Acid Mine Drainage, Limnology and Reclamation*, chapter 1, pages 3–14. Springer Press, New York.

Gersberg, R. M., Elkins, B. V., Lyon, S. R., and Goldsmith, C. R. (1986). Role of aquatic plants in wastewater treatment by artificial wetlands. *Wat. Res.*, **20**, 363–368.

Gray, N. F. (1997). Environmental impact and remediation of acid mine drainage: a management problem. *Env. Geol.*, **30**, 62–71.

Gray, N. F. and O'Neil, C. (1997). Acid-mine drainage toxicity testing. *Env. Geochem. and Health*, **19**, 165–171.

Hallberg, K. B. and Lindström, E. B. (1994). Characterization of *thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. *Microbiol.*, **140**, 3451–3456.

Hallberg, K. B. and Lindström, E. B. (1996). Multiple serotypes of the moderate thermophile *thiobacillus caldus*, a limitation of immunological assays for biomining microorganisms. *Appl. Env. Microbiol.*, **62**, 4243–4246.

Hamilton, W. A. (1994). Biocorrosion: The action of sulphate-reducing bacteria. In C. Ratledge, editor, *Biochemistry of microbial degradation*, chapter 17, pages 555–570. Kluwer Academic Publishers.

Head, I. M. (1998). Bioremediation: Towards a credible technology. *Microbiol.*, 144, 599–608.

Hedin, R. S. (1997). Passive minewater treatment in Eastern United States. In P. L. Younger, editor, *Minewater Treatment Using Wetlands.*

Hedin, R. S., Nairn, R. W., and Kleinmann, R. L. P. (1994). Passive treatment of coal mine drainage. Technical report, U.S. Bureau of Mines Information Circular IC-9389. Pittsburgh, U.S.A.: U.S. Bureau of mines.

Herr, C. and Gray, N. F. (1997). Metal contamination of riverine sediments below the Avoca mines, south east Ireland. *Env. Geochem. and Health.*, 19, 73–82.

Hewitt, E. J. (1966). Sand and water culture methods used in the study of plant nutrition. Technical report, Commonwealth Agric. Bureaux Tech. Comm. Number 2, second edition.

Hobbie, J. E., Daley, R. J., and Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluoresence microscopy. *App. Env. Microbiol.*, 33, 1225–1228.

Hoppe, H. G. (1983). The significance of exoenzymatic activities in the ecology of brackish water: Measurements by means of methylumbelliferyl substrates. *Marine Ecology and Progress Series*, 11, 299–308.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, P., and Xiaosu, D. (2001). Climate change 2001: The scientific basis. IPCC Third Assessment Report: Climate Change. Technical report, Intergovernmental Panel on Climate Change.

Howard, E. A., Emerick, J. C., and Wildeman, T. R. (1989). Design and construction of a research site for passive mine drainage treatment in Idaho Springs, Colorado. In D. A. Hammer, editor, *Constructed wetlands for wastewater treatment – municipal, Industrial and agricultural*, chapter 42b, pages 761–764. Lewis Publishers Inc., USA.

Hustwit, C. C., Ackman, T. E., and Erickson, P. E. (1992). The role of oxygen transfer in acid mine drainage (AMD) treatment. *Wat. Env. Res.*, **64**, 817–823.

Johnson, D. B. (1995a). Acidophilic microbial communities: Candidates for bioremediation of acidic mine effluents. *International Biodeterioration and Biodegradation*, pages 41–58.

Johnson, D. B. (1995b). Selective solid media for isolating and enumerating acidophilic bacteria. *J. Microbial. Methods*, **23**, 205–218.

Johnson, D. B. (1998). Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiol. Ecol.*, **27**, 307–317.

Johnson, D. B. and McGinness, S. (1991). Ferric iron reduction by acidophilic heterotrophic bacteria. *Appl. Env. Microbiol.*, **57**, 207–211.

Johnson, D. B. and Rang, L. (1993). Effects of acidophilic protozoa on populations of metal-mobilizing bacteria during the leaching of pyritic coal. *J. Gen. Microbiol.*, **139**, 1417–1423.

Johnson, D. B. and Roberto, F. F. (1997a). Biodiversity of acidophilic bacteria in mineral leaching and related environments. In Rawlings, editor, *Bioleaching of ores*.

Johnson, D. B. and Roberto, F. F. (1997b). Heterotrophic acidophiles and their roles in the bioleaching of sulphide minerals. In D. E. Rawlings, editor, *Biomining: Theory, Microbes and Industrial Processes*, chapter 13. Landes Bioscience, Austin, TX, USA.

Johnson, D. B., Ghauri, M. A., and Said, M. F. (1992). Isolation and characterization of an acidophilic, heterotrophic bacterium capable of oxidizing ferrous iron. *Appl. Env. Microbiol.*, 58, 1423–1428.

Johnson, D. B., Ghauri, M. A., and McGinness, S. (1993). Biogeochemical cycling of iron and sulphur in leaching environments. *FEMS Microbiol. Rev.*, **11**, 63–70.

Jørgensen, B. B. and Bak, F. (1991). Pathways and microbiology of thiosulphate transformations and sulphate reduction in a marine sediment Kattegat, Denmark. *Appl. Env. Microbiol.*, **57**, 847–856.

Kadlec, R. H. (1995). Overview: Surface flow constructed wetlands. *Wat. Sci. Tech*, 32, 1–2.

Kalin, M., Cairns, J., and McCready, R. (1991). Ecological engineering methods for acid mine drainage treatment of coal wastes. *Resources, Conservation and Recycling*, 5, 265–275.

Kelley, B. C., McAllister, P. J., and Rhodes, S. H. (1993). Bioremediation - application to waste processing in the mining industry. In *Biome 1993 International Conference and workshop. Applications of biotechnology to the minerals industry.*

Kelly, D. P., Chambers, L. A., and Trudinger, P. A. (1969). Cyanolysis and spectrophotometric estimation of trithionate in a mixture with thiosulfate and tetrathionate. *Analy. Chem.*, 41, 898–901.

Kent, D. M. (1994). *Applied Wetlands Science and Technology*. Lewis Publishers, USA.

Kinnear, P. R. and Gray, C. D. (2001). *SPSS for Windows Made Simple.* Psychology Press Ltd, UK.

Kirby, C. S., Thomas, H. M., Southam, G., and Donald, R. (1999). Relative contricutions of abiotic and biological factors in Fe(II) oxidation in mine drainage. *Appl. Geochem.*, **14**, 511–530.

Kleinmann, R. K. P., Crerar, D. A., and Pacelli, R. R. (1981). Biogeochemistry of acid mine drainage and a method to control acid formation. *Min. Eng.*, **33**, 300–305.

Knight, R. L. (1997). Wildlife habitat and public use benefits of treatment wetlands. *Wat. Sci. Tech.*, **35**, 35–43.

Kolbash, R. L. and Romanoski, T. L. (1989). Winsor Coal Company wetland: An overview. In D. A. Hammer, editor, *Constructed wetlands for wastewater treatment - municipal, industrial and agricultural*, chapter 42f, pages 788–792. Lewis publishers Inc., USA.

Kowalik, P., Mierzejeweski, M., Obarska-Pempkowiak, H., and Toczylowska, I. (1995). *Constructed wetlands for wastewater treatment from small communities.* Gsańsk.

Laboratories, B.-R. (1994). *Guide to Aminex HPLC columns for food and beverage, biotechnology and bio-organic analysis.* Life Sciences Group, USA.

Lackley, J. B. (1938). Aquatic life in waters polluted by acid mine waste. *Public Health Report*, **54**, 740–746.

Lamb, H. M., Dodds-Smith, M., and Gusek, J. (1998). Development of a long term strategy for the treatment of acid mine drainage at wheal jane. In W. Geller, H. Klapper, and W. Solomons, editors, *Acidic Mining Lakes: Acid Mine*

Drainage, Limnology and Reclamation, chapter 18, pages 335–346. Springer Press, New York.

Lindsay, W. L. (1991). Iron oxide solubilization by organic matter and its effects on iron availability. *Plant and Soil*, **130**, 27–34.

Lovley, D. R. and Phillips, E. J. P. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Appl. Env. Microbiol.*, **51**, 683–689.

Lovley, D. R. and Phillips, E. J. P. (1987). Rapid assay for microbially reducible ferric iron in aquatic sediments. *Appl. Env. Microbiol.*, **53**, 1536–1540.

Lundgren, D. G., Vestal, J. R., and Tabita, F. R. (1972). The microbiology of mine drainage pollution. In R. Mitchell, editor, *Water Pollution Microbiology*, pages 69–88. Wiley-Interscience, NY.

Macfie, S. M. and Crowder, A. A. (1987). Soil factors influencing ferric hydroxide plaque formation on roots of *typha latifolia* l. *Plant and Soil*, **102**, 177–184.

Machemer, S. D. and Wildeman, T. R. (1992). Adsorption compared with sulfide precipitation as metal removal processes from acid mine drainage in a constructed wetland. *J. Contam. Hydrol.*, **9**, 115–131.

Madigan, M. T., Martinko, J. M., and Parker, J. (1997). *Brock – Biology of Microorganisms. Eighth Edition*. Prentice Hall Internat. Inc., New Jersey.

Maree, J. P., Dingemans, D., Tonder, G. J., and Mtimkulu, S. (1998). Biological iron (II) oxidation as pre-treatment to limestone neutralisation of acid water. *Wat. Sci. Tech.*, **38**, 331–337.

Mason, C. F. (1995). *Biology of Freshwater Pollution, Second Edition*. Longman Scientific and technical, London.

McCleary, E. C. and Kepler, D. A. (1994). Ecological benefits of passive wetland treatment systems designed for acid mine drainage: With emphasis on watershed restoration. In *International Land Reclamation and Mine Drainage Conference, and Third International Conference on the Abatement of Acidic Drainage*, pages 111–119.

McColl, J. G. and Pohlman, A. A. (1986). Soluble organic acids and their chelating influence on Al and other metal dissolution in forst soils. *Wat. Air. and Soil Poll.*, **31**, 917–927.

McGinness, S. and Johnson, D. B. (1993). Seasonal variations in the microbiology and chemistry of an acid mine drainage stream. *Sci. Tot. Env.*, **132**, 27–41.

Mills, A. L. and Mallory, L. M. (1987). The community structure of sessile heterotrophic bacteria stressed by acid mine drainage. *Microb. Ecol.*, **14**, 219–232.

Mitsch, W. J. and Gosselink, J. A. (1993). *Wetlands*. Van Nostrand Reinhold, NY.

Mustin, C., DeDonata, P., and Berthelin, J. (1992). Qualification of the intergranular porosity formed in the bioleaching of pyrite by *thiobacillus ferrooxidans*. *Biotech. Bioeng.*, **39**, 1121–1127.

Nairn, R. W., Hedin, R. S., and Watzlaf, G. R. (1992). Generation of alkalinity in an anoxic limestone drain. In *Achieving Land Use Potential Through Reclamation*, chapter 77, pages 206–219. American Soc. Surface Mining and Reclamation.

Nemati, M., Harrison, S. T. L., Hansford, G. S., and Webb, C. (1998). Biological oxidation of ferrous sulphate by *thiobacillus ferrooxidans*: A review of the kinetic aspects. *Biochem. Eng. J.*, **1**, 171–190.

Nicholson, R. V., Elberling, B., and Williams, G. (1995). A new oxygen consumption technique to provide rapid assessment of tailings reactivity in the field and in the laboratory. In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 999–1006.

Nordstrom, D. K., Jenne, E. A., and Ball, J. W. (1979). Redox equilibria of iron in acid mine waters. In E. A. Jenne, editor, *Chemical modeling in aqueous systems*, chapter 3, pages 51–79. ACS, Washington.

Norris, P. R. and Johnson, D. B. (1996). Acidophilic microorganisms. In K. Horikoshi and W. D. Grant, editors, *Extremophiles: Microbial life in extreme environments*. John Wiley, NY.

Perkins, J. and Hunter, C. (2000). Removal of enteric bacteria in a surface flow constructed wetland in Yorkshire, England. *Wat. Res.*, 34, 1941–1947.

Phillips, P. and Bender, J. (1998). Bioremediation of metals in acid tailings by mixed microbial mats. In W. Geller, H. Klapper, and W. Solomons, editors, *Acidic Mining Lakes: Acid Mine Drainage, Limnology and Reclamation*, pages 347–363. Springer Press, New York.

Pinney, M. L., Westerhoff, P. K., and Baker, L. (2000). Transformations in dissolved organic carbon through constructed wetlands. *Wat. Res.*, 34, 1897–1911.

Pronk, J. T., Meijer, W. M., Hazeu, W., Dijken, J. P. V., Bos, P., and Kuenen, J. G. (1991). Growth of *thiobacillus ferrooxidans* on formic acid. *Appl. Env. Microbiol.*, 57, 2057–2062.

Pronk, J. T., DeBruyn, J. C., Bos, P., and Kuenen, J. G. (1992). Anaerobic growth of *thiobacillus ferrooxidans*. *Appl. Env. Microbiol.*, 58, 2227–2230.

Rabenhorst, M. C., James, B. R., and Shaw, J. N. (1992). Evaluation of potential wetland substrates for optimizing sulfate reduction. In *Achieving Land Use Potential Through Reclamation*, chapter 73/72, pages 90–97S. American Soc. Surface Mining and Reclamation.

Rackham, O. (1995). *The history of the countryside: The classic history of Britains landscape, flora and fauna.* Weidenfeld and Nicolson, London.

Rand, M. C., Greenburg, A. E., and Taros, M. J. (1975). Turbidimetric method. In *Standard methods for examination of waste waters*, chapter 42c, pages 496–798. USS American Public Health Association.

Reddy, K. R. and D'Angelo, E. M. (1997). Biogeochemical indicators to evaluate pollutant removal efficiency in constructed wetlands. *Wat. Sci. Tech.*, 35, 1–10.

Rowlands, J. (1966). *The Copper Mountain.*

Rowley, M. V., Warkentin, D. D., Yan, V. T., and Piroscho, B. M. (1994). The biosulphide process: Integrated biological/chemical acid mine drainage treatment – results of laboratory piloting. In *Proceedings of the Land Reclamation and Mine Drainage Conference and third international conference on the abatement of acidic drainage*, pages 205–213.

Sand, W., Gehrke, T., Hallmann, R., and Schippers, A. (1995). Sulfur chemistry, biofilm and the (in)direct mechanism - a critical evaluation of bacterial leaching. *Appl. Micro. Biotech.*, 43, 961–966.

Scheetz, B. E., Silsbee, M. R., and Schuek, J. (1995). Field applications of cementitious grouts to address the formation of acid mine drainage. In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 935–944.

Schrenk, M. O., Edwards, K. J. W., Goodman, R. M., Hamers, R. J., and Banfield, J. F. (1998). Distribution of *thiobacillus ferrooxidans and leptospirillum ferrooxidans*: Implications for generation of acid mine drainage. *Science*, **279**, 1519–1522.

Schwertmann, U. and Murad, E. (1983). The effects of pH on the formation of goethite and hematite from ferrihydrite. *Clays Clay Miner.*, **31**, 277–284.

Sen, A. M. and Johnson, B. (1999). Acidophilic sulphate-reducing bacteria: Candidates for bioremediation of acid mine drainage. In R. Amils and A. Ballester, editors, *Biohydrometallurgy and the Environment Towards the Mining of the 21st Century*, pages 709–718. Elsevier Press.

Shearer, R. E., Everson, W. A., Mausteller, J. W., and Zimmerer, R. P. (1970). Characteristics of viable anti-bacterial agents used to inhibit acid producing bacteria in mine waters. In *3rd symposium coal mine drainage research, Pittsburg*, pages 188–198.

Shuttleworth, K. L. and Unz, R. F. (1988). A growth inhibition model for *thiobacillus ferrooxidans*. In *Mine drainage and surface mine reclamation conference*, pages 97–103.

Silvola, J., Alm, J., Ahlholm, U., Nykänen, H., and Martikainen, P. J. (1996). CO_2 fluxes from peat in boreal mires under varying temperature and moisture conditions. *J. Ecol.*, **84**, 219–228.

Singer, P. C. and Stumm, W. (1970). Acid mine drainage; The rate limiting step. *Science*, **167**, 1121–1123.

Singh, R. K. and Chowdhury, S. (1999). Effect of mine discharge on the pattern of riverine habitat use of elephants *elephas maximus* and other mammals in Singhbhum Forests, Bihar, India. *J. Env. Manag.*, **57**, 177–192.

Skousen, J., Sexstone, A., Garbutt, K., and Sencindiver, J. (1994). Acid mine drainage treatment with wetlands and anoxic limestone drains. In D. M. Kent, editor, *Applied Wetlands Science and Technology*, chapter 12, pages 263–281. Lewis Publishers, USA.

Smith, G. S., Johnston, C. M., and Cornforth, I. S. (1983). Comparison of nutrient solutions for growth of plants in sand culture. *New Phytol*, **94**, 537–548.

Stephen, J. R. and Macnaughton, S. J. (1999). Developments in terrestrial bacterial remediation of metals. *Curr. Opinion Biotech.*, **10**, 230–233.

Stewart, B. R., Daniels, W. L., and Jackson, M. L. (1997). Evaluation of leachate quality from codisposed coal fly ash and coal refuse. *J. Env. Qual*, **26**, 1417–1424.

Straub, K. L., Benz, N., Schink, B., and Widdel, F. (1996). Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl. Env. Microbiol.*, **62**, 1458–1460.

Strömberg, B. and Banwart, S. (1995). Laboratory investigation of alkalinity producing reactions in sulphide containing waste rock. In *Mining and the environment. Sudbury 1995 Conference Proceedings*, pages 39–48.

Stumm, W. and Morgan, J. J. (1981). *Aquatic Chemistry*. Wiley, NY.

Sullivan, P. J. and Yelton, J. L. (1988). An evaluation of trace element release associated with acid mine drainage. *Env. Geol. Wat. Sci.*, **12**, 181–186.

Tarutis Jr., W. J. and Unz, R. F. (1995). Iron and manganese release in coal mine drainage wetland microcosms. *Wat. Sci. Tech.*, **32**, 187–192.

Taylor, H. N., Choate, K. D., and Brodie, G. A. (1993). Storm event effects on constructed wetlands discharges. In G. A. Moshiri, editor, *Constructed Wetlands for Water Quality*, pages 139–146. Lewis Publishers, London.

Tervet, D. J. and Harriman, R. (1988). Changes in pH and calcium after selective liming in the catchment of Loch Dee, a sensitive and rapid turnover loch in south-west Scotland. *Aquaculture and Fisheries Management*, **19**, 191–203.

Thomas, R. A. P., Morby, A. P., and Macaskie, L. E. (1997). The biodegradation of tributyl phosphatae by naturall occurring microbial isolates. *FEMS Microbiol. Letters*, **155**, 155–159.

Thomas, R. A. P., Morby, A. P., and Macaskie, L. E. (1998). The effect of growth conditions on the biodegradation of tributyl phosphate and the potential for the remedaition of acid mine drainage waters by a naturally-occurring mixed microbial culture. *Appl. Microbiol. and Biotech.*, **49**, 202–209.

Tsukamoto, T. K. and Miller, G. C. (1999). Methanol as a carbon source for microbiological treatment of acid mine drainage. *Wat. Res.*, **33**, 1365–1370.

Unz, R. F. and Dietz, J. M. (1986). Biological applications in the treatment of acid mine drainages. In *Biotech. and Bioeng. Symp. No 16*, pages 163–170. John Wiley and Sons, Inc.

Valiela, I. (1984). *Marine ecological processes*. Springer-Verlag, NY.

Vile, M. A. and Wieder, R. K. (1993). Alkalinity generation by Fe(III) reduction versus sulfate reduction in wetlands constructed for acid mine drainage treatment. *Water, Air and Soil Pollution*, **69**, 425–441.

Walton, K. C. and Johnson, D. B. (1992). Microbiological and chemical characteristics of an acidic stream draining an dissused copper mine. *Env. Poll.*, 76, 169–175.

Webb, J., McGinness, S., and Lappin-Scott, H. M. (1998). Metal removal by sulphate-reducing bacteria from natural and constructed wetlands. *J. Appl. Microbiol.*, 84, 240–248.

Webster, H. J., Lacki, M. J., and Hummer, J. W. (1994). Biotic development comparisons of a wetland constructed to treat mine water drainage with a natural wetland. In *Proceedings of the International Land Reclamation and Mine Drainage Conference and third international conference on the abatement of acidic drainage*, pages 102–110.

Wendt-Potthoff, K. and Neu, T. R. (1998). Microbial processes for potential in situ remediation of acid lakes. In W. Geller, H. Klapper, and W. Solomons, editors, *Acidic Mining Lakes: Acid Mine Drainage, Limnology and Reclamation*, pages 269–284. Springer Press, New York.

Wenerick, W. R., Stevens, S. E., Stark, H. J. W. L. R., and DeVeau, E. (1989). Tolerance of three wetland plant species to acid mine drainage: A greenhouse study. In D. A. Hammer, editor, *Constructed wetlands for wastewater treatment - municipal, industrial and agricultural*, pages 801–807. Lewis publishers Inc., USA.

White, C. and Gadd, G. M. (1998). Reduction of metal cations and oxyanions by anaerobic and metal-resistant microorganisms: Chemistry, physiology and potential for the control and bioremediation of toxic metal pollution. In R. Michell, editor, *Exremophiles – Microbial Life in Extreme Environments*, pages 233–254. Wiley-Liss.

Wichlacz, P. L. and Unz, R. F. (1986). Mineralogical and characteristics of residues produced in microbiological treatment of acid mine drainages. *Geomicrobiol. J.*, **4**, 407–422.

Wieder, R. K. (1994). Diel changes in iron (III)/iron(II) in effluent from constructed acid mine drainage treatment wetlands. *J. Env. Qual.*, **23**, 730–738.

Wildeman, T., Gusek, J., and Brodie, G. (1991). *Wetland design for mining operations.*

Witthar, S. R. (1993). Wetland water treatment systems. In G. A. Moshiri, editor, *Constructed Wetlands for Water Quality Improvement*, pages 147–155. CRC Press Inc.

Wood, A. (1995). Constructed wetlands in water pollution control: Fundamentals to their understanding. *Wat. Sci. Tech*, **32**, 21–29.

Ye, Z. H., Baker, A. J. M., Wong, M. H., and Willis, A. J. (1997). Zinc, lead and cadmium tolerance, uptake and accumulation by the common reed, *phragmites australis* (Cav.) Trin. ex Steudel. *Annals. of Botany*, **80**, 363–370.

Younger, P. L. (1997). The longevity of minewater pollution: A basis for decision making. *Sci. Tot. Env.*, **194**, 457–466.

Zhang, Y. L. and Evangelou, V. P. (1996). Influences of iron oxide forming conditions on pyrite oxidation. *Soil Sci.*, **161**, 852–864.

Ziemkiewicz, P. E., Skousen, J. G., and Brant, D. L. (1997). Acid mine drainage treatment with armored limestone in open limestone channels. *J. Env. Qual.*, **26**, 1017–1024.