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The significance of enzymes in the biogeochemical functioning of constructed wetlands

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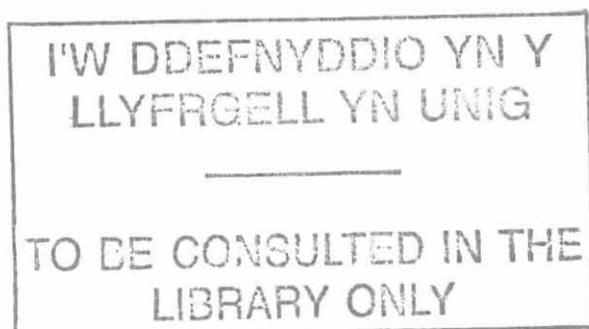
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The significance of enzymes in the biogeochemical functioning of constructed wetlands

A thesis submitted to the University of Wales by
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in candidature for the degree of
PHILOSOPHIAE DOCTOR

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Summary

Artificial wetlands, constructed specifically to treat wastewaters, are increasingly used in addition to, or instead of, conventional water treatment methods. The treatment efficiency of these may be improved if nutrient cycling could be manipulated via soil processes. Extracellular enzyme activities in the soils of constructed wetlands were therefore examined to investigate the biogeochemistry of these treatment wetlands. Fluorescent model substrates were used to determine the relative activities of cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase and xylosidase; a colorimetric substrate was used to examine phenol oxidase.

Field studies of constructed wetlands built for different purposes provided initial information about the levels and regulators of enzyme activity in these artificial soils. Enzyme activities and gas fluxes were lower than in natural wetlands; however there was an unexpected similarity in nutrient cycling between constructed wetlands and natural soil and sediment systems, which was shown by the relative activities of extracellular enzymes. Laboratory experiments clarified the relationships between enzyme activity and pH, redox potential, temperature, oxygenation, phenolic concentration and dissolved organic carbon supply, and showed that considerable potential for enzyme manipulation, and therefore improved treatment efficiency, exists.

Soil temperature correlated with the activity of several enzymes, suggesting that extracellular decomposition may be faster at higher temperatures. Soil temperature was the strongest determinant of nitrous oxide and carbon dioxide fluxes; methane flux did not show any seasonal variation, and was not influenced by pH. Dissolved organic carbon concentration increased or decreased the activity of different enzymes. The microbial population appeared to be essential for denitrification, and compounds which may act as microbial inhibitors could therefore be detrimental to wetland treatment. The existing, 'natural' carbon-cycling enzyme activity of soil was enhanced by adding commercial enzymes, although the capacity of wetland soil to immobilize additional enzymes may depend on the organic material available.

Extracellular enzymes are useful indicators of nutrient cycling, and represent a potential means of manipulating the treatment processes occurring in constructed wetlands.

Thanks to...

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...Therefore go forth companion; when you find
No highway more, no track, all being blind,
The way to go shall glimmer in the mind.

Though you have conquered Earth and chartered Sea
And planned the courses of all stars that be
Adventure on, more wonders are in Thee.

Adventure on, for from the littlest clue
Has come whatever worth man ever knew;
The next to lighten all men may be you...

John Masefield, 'The Wanderer'

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List of abbreviations

AMD	acid mine drainage
BOD ₅	5-day Biological Oxygen Demand
CAT	Centre for Alternative Technology
CH ₄	methane
CO ₂	carbon dioxide
COD	chemical oxygen demand
Diqc	2-carboxy-2,3-dihydroindole-5,6-quinone
DO	dissolved oxygen
DOC	dissolved organic carbon
MAD	mean absolute deviation
MD	mean difference
MUF	methylumbelliferyl
n.d.	not determined
NH ₄	ammonia
NO ₃	nitrate
N ₂ O	nitrous oxide
PO ₄	phosphate
s.e.	standard error
SO ₄	sulphate
Temp	temperature

Chapter I

Introduction

Introduction

1.1 The origin of wetlands as treatment systems

Purifying polluted water using wetlands has developed from something of a provident coincidence between humanity and nature, an efficient but unheeded filtering system, to a specialized, widely applied and highly successful science. If we were to trace this development back to its very root, we would doubtless arrive at the riparian wetlands which accompany freshwater courses to the sea, at the mangrove swamps lining tropical watercourses, and at the sparse, salty, flat wetland which frequently covers coastal flood zones. All these wetlands are uniquely productive (Boyt *et al* 1977, Brix & Schierup 1989) because their constant water exchange carries rich supplies of nutrients to them, yet many are also nutrient sinks because the flooded soil does not allow complete decomposition of the organic matter in them (Urban *et al* 1988, Freeman *et al* 1995a).

Whilst natural wetlands have filtered and cleaned waste waters discharged to them for centuries, it is only relatively recently, in the last fifty years, that the great potential of wetlands as passive biological treatment systems has begun to be realised, developed and exploited. In the case of natural riparian wetlands, nutrients from the surrounding catchment are transformed as the watershed drains through the wetland; coastal salt marshes moderate the impact of domestic sewage discharged over them to the sea (Boyt *et al* 1977, Soukup *et al* 1994); wherever wastewater creates newly saturated ground, wetland vegetation colonizes and thrives on the available nutrients.

History records wetlands as indisputably valued resources for food, fuel and materials (to the Marsh Arabs (Moss 1988) and Somerset Levels people (Coles 1989), for example) but in the twentieth century the value of wetlands has more often come from the potential of the land they occupy than from their intrinsic

properties; the fenland of Cambridgeshire has been extensively drained for horticulture in the last hundred years, the flow country of Perthshire drained for timber production, the Florida Everglades used for extensive luxury development, Malaysian mangrove swamps cleared to make way for aquaculture (the so-called "blue revolution") and countless riparian wetlands removed during river channelization work (Brix & Schierup 1989). The signing of the Ramsar convention (Convention on Wetlands of International Importance) has ensured the preservation of valuable natural wetland sites, and the development of constructed wetlands technology for wastewater treatment has begun to reverse the unpopularity of wetlands, with a new realisation of the benefits and possibilities they offer.

1.2 Treatment of wastewaters

The type of treatment conventionally applied to wastewaters largely depends on the nature of their contamination, and on the volume to be disposed of. In the United Kingdom the government-funded Environment Agency controls the quality and quantity of wastewater discharges by issuing discharge consents under the Water Resources Act (1991) in England and Wales, the Control of Pollution Act (1974) in Scotland and the Water Act (1972) in Northern Ireland. The exception to this is discharges from abandoned mines. Other countries have similar, or no, legislation. In England and Wales the Environmental Pollution Act (1990) also makes it an offence to deliberately or accidentally cause pollution of controlled waters.

Wastewaters which require treatment before discharge to a watercourse may contain inorganic, organic and/or toxic contaminants, depending on their origin, which are potentially polluting in different ways. Inorganic pollutants may colour or cloud the receiving water, lowering photosynthesis rates; in nutrient-limited systems the addition of some inorganic forms (e.g. phosphate) can create eutrophication problems; organic pollutants invariably add oxygen demand to the

system receiving them, reducing the oxygen available to higher forms of aquatic life as micro-organisms sequester it; organic pollutants may also lower photosynthesis rates if they are in the form of suspended solids; toxic contaminants may directly harm aquatic life and are frequently recalcitrant, so persist in the aquatic environment where they may bioaccumulate or biomagnify. The removal of all these forms of pollutants from discharged wastewater is highly desirable.

Conventionally, contaminated waters have either been discharged to water bodies without treatment (but with attendant problems of toxic, nutrient or thermal pollution), or been treated using biological, chemical and/or mechanical methods. Using wetland systems to treat waste waters needs the same energy input per kilogram of waste, but a much greater proportion of the energy is renewable: passive solar radiation, microbial metabolic energy, and gravity.

1.3 Constructed wetlands for water treatment

Wetlands can provide cheap, effective wastewater treatment in most temperate and sub-tropical climates (e.g. Maehlum & Stalnacke 1999, Norway; Kantawanichkul *et al* 1999, Thailand; Billore *et al* 1999, India). Processes such as settling and Biological Oxygen Demand (BOD₅) removal are relatively unaffected by temperature (Kadlec & Knight 1996), although the area of wetland required will be determined by its operating temperature, which depends on the prevailing climate (Kadlec & Knight 1996). They are especially suited to upgrading existing sewage treatment systems, or for complete treatment on a small scale, but are not a panacea for the problem of contaminated waters. The advantages over conventional types of wastewater treatment include low operating costs, low energy requirements, suitability for on-site water treatment (i.e. at the site of wastewater production), and flexible operation which is less susceptible to shock loadings (Brix & Schierup 1989).

Some types of waste water are inherently more suitable for wetland treatment than others, and suitability tends to depend upon the molecular transformations which are required before the water can be considered safe to discharge. In turn, the definition of "safe to discharge" will depend upon the nutrient status and habitat value of the receiving water body. Wetland substrates with a high organic matter and clay content have raised cation exchange capacities, so during times of excessive loading pollutants can be temporarily adsorbed and then slowly eluted as the concentration in the influent falls to normal levels again; this buffers microorganisms from shock loadings, and is crucial for their survival (Cobban *et al* 1998).

a. Mechanisms of amelioration in wetlands

The area of constructed wetland required for the effective treatment of any waste water depends on the degree of contamination, and the volume of water to be treated. Area is proportional to both of these, and so for heavily polluted or large-volume wastewaters, wetland treatment may be inappropriate because it is land extensive. The Kickuth equation is commonly used to calculate the area of treatment wetland required for a wastewater with a given BOD₅:

$$A_h = \frac{Q_d (\ln C_o - \ln C_t)}{K_{BOD}}$$

where A_h = bed surface area

Q_d = daily average flow rate, m³ d⁻¹

C_o = daily average BOD₅ of inflow, mg l⁻¹

C_t = required daily average BOD₅ of outflow, mg l⁻¹

K_{BOD} = a rate constant, m/d

In the United Kingdom, A_h is usually approximately 5 m² per person equivalent for sewage treatment (Cooper & Green 1995). Other wastewaters may require different areas depending on their BOD₅ loadings, a selection of which are shown in Table 1.1.

Table 1.1

Average BOD₅ loadings of wastewaters (data calculated from Kadlec & Knight 1996)

Industry	Average BOD₅, mg l⁻¹
Petroleum and coal	90
Domestic	300
Chemicals	580
Pulp and paper	700
Landfill leachate	42 - 10,900

The requirements for installation of a treatment wetland are governed by the nature of the wastewater, but in general there is a need for a suitable area of land, a receiving water course or soakaway, a gradient across the system (between inflow and outflow) and consent from the relevant authority to discharge the outflow to a watercourse or to groundwater.

Mineralization

Mineralization refers to the complete breakdown of pollutants by the action of bacteria, fungi, plants and soil enzymes to simple molecules utilizable for metabolism, and ultimately (and ideally) to carbon dioxide, water and ions. Mineralization removes the biological oxygen demand of waste, so it may safely be discharged to a watercourse. Mineralization is the ultimate goal in the effective treatment of organic pollution such as sewage and wood/paper mill wastes.

Adsorption

During adsorption, pollutants are retained within a treatment system by binding to organic matter, clay colloids, etc. A wetland may have a finite limit for such storage. Adsorption is effective for inorganic pollution, such as some mine drainage, and metal contaminated water.

b. Wetland degradation of complex organic molecules

The decomposition of complex synthetic molecules in soil has been previously investigated, and found to be achievable. For example Kiss *et al* (1975) report the biodegradation in ordinary soil of diethylstilbestrol, a very active synthetic estrogen, and of sodium dioctylsulfosuccinate, a synthetic anionic detergent. Although the products and pathways of decomposition were not investigated, in both cases the process was shown to be at least partly extracellular by the use of γ -irradiated and autoclaved controls. Another ubiquitous xenobiotic, polychlorinated biphenyls (PCBs), can be biotransformed and metabolised by microorganisms in water, sediment and activated sludge (Clark *et al* 1979, Tucker *et al* 1975). PCBs do not have a significant effect on anaerobic or aerobic bacterial carbon cycling or phosphate mineralization in the water column or sediments of freshwater environments, but they do affect nitrogen mineralization (Sayler *et al* 1982).

Constructed wetlands can effectively remove pollutants such as phenols and cresols in textile dye plant effluent which are usually difficult to treat, and at ICI Billingham (north east England), 95-97% removal of contaminants such as di, tetra and penta- chlorophenols is achieved by a large constructed wetland (Cobban *et al* 1998). McKinlay & Kasperek (1996) studied the disposal of atrazine (a farm pesticide) using marsh plants, and found that the mechanism of decline of pesticide was microbiological rather than physical.

Xenobiotics are anthropogenic compounds, often introduced into the environment at concentrations which cause undesirable or fatal effects to biota (Bollag 1992). Ideally, xenobiotics are transformed to carbon dioxide, water and mineral elements, but many are highly persistent, resistant to natural transformation or degradation or are converted to intermediates which are as or more toxic than the parent compounds (Bollag 1992). Binding of phenolic and anilinic chemicals to humic material may decrease the amount of xenobiotic available to interact with

the biota, therefore reducing toxicity. Xenobiotic wastewaters require enriched communities of specific bacteria for the complete or partial mineralization of the compounds they contain, and these may best be obtained by laboratory culture of micro-organisms for use in bioreactors. Although some soils may develop the ability to break down recalcitrant compounds if left in contact with them for long enough, a treatment wetland does not normally provide this length of contact; in addition, micro-organisms may not be able to use the compounds as a sole source of carbon and nitrogen, and would require additional nutrients to enable their mineralization. Fogel *et al* (1982) found that the insecticide methoxychlor was degraded in soil under anaerobic conditions after three months, probably by a co-metabolic mechanism. A wetland which initially acted as a closed circuit until it developed the specific ability to mineralize a pollutant would be an interesting solution.

Many man-made compounds resemble naturally occurring humic acid precursors, so can be incorporated into humus during its formation (Bollag 1992). The incorporation of xenobiotics into humus can be enhanced by adding extracellular enzymes, abiotic catalysts, or by altering the physicochemical conditions of the soil (Bollag 1992).

c. Sewage treatment

Domestic sewage is a mixture of all household wastewaters, and as such contains a broad range of molecules and particulates. Approximately 0.1 % of domestic sewage is screenable solids such as paper and plastics, which are removed by coarse filtration, and the remaining 99.9 % is liquid, of which 70 % is organic (7 % fats, 18 % carbohydrates, 45 % proteins) and 30 % is inorganic (grit, salt and metals) (Grant *et al* 1996). The liquid component contains a variety of non-screenable solids such as bacteria, faeces, food particles, fats, oils, detergents and sediment; it also contains dissolved material such as organic matter, proteins, urea, carbohydrates, fatty acids, DNA and ions (Grant *et al* 1996). If discharged

untreated, sewage causes serious pollution of the receiving water course because its high organic content produces a high Biological Oxygen Demand (BOD₅), its suspended solids obscure light, and it may contain pathogenic bacteria, viruses, worms and chemicals (Grant *et al* 1996).

Conventional sewage treatment involves preliminary treatment, to remove large solids, followed by primary treatment in which suspended solids are settled or floated out to reduce BOD₅; this is largely a physical and/or chemical process. The liquid then receives (biological) secondary treatment, in which micro-organisms further reduce BOD₅ by mineralizing organic matter. This may be followed by tertiary treatment (which may be biological, physical or chemical) to further eliminate BOD₅, nutrients and pathogens. Domestic sewage can be treated using constructed wetlands if the solids are first removed, or liquidated using a septic tank, and the liquid is passed through one or more wetlands. The organic components of sewage must be transformed to inorganic nutrients for uptake by micro-organisms, soil animals or plants.

In some areas of the world sewage sludge is discharged to areas of coastal salt marsh, which appears to have a remediating effect on the sludge (Soukup *et al* 1994), although the consequences for the salt marsh are not well known as sewage sludge contains heavy metals, chlorinated hydrocarbons and other pollutants (Valiela *et al* 1975). Nitrogen is a principal component of sewage wastes, and often limits plant growth in coastal waters (Ryther & Dunstan 1971); Valiela *et al* (1975) found that standing crops of salt marsh plants responded to nitrogen (but not phosphorus) enrichment by sewage addition, although the pattern of vegetation dominance was changed. Soukup *et al* (1994) found that nitrogen removal from sewage by a coastal wetland (New South Wales, Australia) was faster in summer.

d. Landfill leachate treatment

Landfilling is the cheapest method of disposal for solid urban waste, and also minimizes adverse environmental effects, allowing waste to decompose under controlled conditions until it is transformed to relatively inert, stable material (Lema *et al* 1988). In the UK over 90% of all domestic and commercial solid waste is now landfilled (Lema *et al* 1988). The high BOD₅, high acid content, excessive amounts of ammonium (values of 800-1000 mg l⁻¹ ammoniacal nitrogen are typical in raw leachate (Kowalik *et al* 1996)) and high heavy metal content make leachate a serious pollution problem (Kowalik *et al* 1996), and in most countries landfill leachate is considered to present a serious environmental pollution hazard, as even small landfills may impact groundwater (Britz 1995). The collection and treatment of leachate are the greatest problems associated with landfill sites, although pre-treatment of waste before landfilling can affect the quality of the leachate. For example incorporating lime-treated or anaerobic-digested sludge in landfill sites solves the problem of disposal of wastewater sludge, and aids landfill management by greatly reducing the COD of the leachate (Lema *et al* 1988).

Leachate formation

Landfill leachate is formed when precipitation percolates through the waste and takes up organic and inorganic products by physical extraction, and hydrolytic and fermentative processes; there is also some formation from moisture trapped in the refuse (Lema *et al* 1988). Generally, leachates have high concentrations of soluble organic matter and inorganic ions, and are a complex and highly variable mixture of soluble organic, inorganic and bacteriological components, and suspended solids, in liquid form. Organic materials are partially or totally mineralized, so leachates have intermediate products as well as toxic organics, heavy metals, and xenobiotics (Lema *et al* 1988). Climate can significantly influence leachate production via precipitation (Lema *et al* 1988).

Factors affecting composition

The exact composition of a leachate depends on the type and especially the age of the waste, and on precipitation; there is no "average" leachate (Britz 1995). Young landfills tend to produce leachate with a high Chemical Oxygen Demand (COD), because fatty acids are present, but this is easily degradable due to a high BOD₅:COD ratio. They also tend to have a higher pH, and lower heavy metal concentrations, than leachates from older landfills (Britz 1995). Older landfills produce leachate which contains ammonia (a potent fish toxin), iron compounds which precipitate and stain, and has an offensive odor (from reduced sulphur compounds). It may also contain pathogenic bacteria. These leachates have a lower proportion of labile organic compounds, and so biological treatment becomes less effective (Britz 1995). Leachate should be carefully characterized before treatment, as its strength can increase over time through evaporation (Britz 1995).

The goal of treatment is to reduce the concentration of pollutants to levels acceptable for surface water discharge, or to pretreat to acceptable levels for transfer to an off-site treatment facility (Britz 1995). Treatment will change from biological to a combination of chemical and biological as the landfill waste ages.

Treatment methods

Leachate may be transported off-site, e.g. to a municipal waste water treatment plant (the preferred option), which is advantageous because leachate usually has excess nitrogen, and sewage has excess phosphorus (Britz 1995). However, there may be toxic organic compounds which can not be degraded in a conventional sewage plant, and heavy metals.

Recirculation

Recirculation back through the waste mass can significantly reduce leachate contaminant concentration in a short time period; biological treatment is thus

accomplished within the landfill (Britz 1995). Recycling leachate reduces the time required for stabilization, optimizes methane production and reduces organic fractions (Britz 1995). pH control is necessary for partial neutralization and metal removal, and also to maintain an active methanogenic population.

Land irrigation

High concentrations of toxic elements in leachate may make land used for irrigation unfit for agriculture, and pollute surface and ground waters; this method is possibly more suitable for low-strength leachate, and cannot be used during colder months when it could lead to waterlogged conditions (Britz 1995). When vegetated land is irrigated, there is a further volume reduction by plant uptake and transpiration, although high-strength leachate could potentially damage vegetation. Leachate irrigation may decrease the microbial biomass in the soil, heavy metals in the leachate may be phytotoxic, it may produce odours, and a large area of land is required (Britz 1995).

Physical and chemical treatment

Physical and chemical treatments include chemical evaporation, oxidation or precipitation (Britz 1995). Chemical evaporation uses a two-stage distillation with high pH followed by an acid step; this results in low organic and metal concentrations, but is expensive. Chemical oxidation (by wet oxidation, ozonation, peroxide treatment or chemical reduction) either renders contaminants insoluble, gasifies them or stabilizes them as relatively innocuous substances (Britz 1995). Chemical precipitation includes the addition of lime (Ca(OH)_2), alum ($\text{Al}_2(\text{SO}_4)_3$), ferric chloride (FeCl_3), sodium sulfide (Na_2S) or ferrous sulphate (FeSO_4), which remove heavy metals colouration, suspended solids, ammonia, heavy cations and some organic matter, but not COD (Britz 1995).

Activated carbon adsorption effectively removes most organics, but not acetone and methanol. Enzminger *et al* (1987) report that this method is the most

extensively used physical-chemical means of removal of organics from leachate. However, frequent regeneration of carbon columns is required and the method is only cost effective for the removal of residual organics when total dissolved solids are less than 200 mg l^{-1} . Gamma irradiation can be used to decompose refractory substances under aerobic conditions. This method can decompose most organics without sludge production, but is very expensive at high organic concentrations. Reverse osmosis may be used, but suffers from membrane fouling; to prolong membrane life, suspended solids and colloids must first be eliminated. Leachate from older landfills may be weaker, but higher in ammonia; raising the pH with lime to form ammonia gas, and then bubbling with air to remove the ammonia is cost effective and simple.

Biological treatment

Biological treatment may be aerobic or anaerobic. Aerobic reactors such as activated sludge plants, aerobic filters, continuous-flow aerobic reactors, rotating biological contactors and sequencing batch reactors depend on microorganisms in an oxygen-rich environment, which oxidize organics to carbon dioxide, water and cellular material (biomass). Lagoons, ponds and reed beds are one of the least expensive biological treatments, if enough land is available; they utilize aerobic and anaerobic micro-organism metabolism, in addition to photosynthesis and sedimentation, but there is potential for surface and groundwater pollution, offensive smells and nuisance insects (Britz 1995). Older leachate is high in ammonia, so provides nutrients for eutrophication and exerts an oxygen demand through nitrification (Britz 1995). Poor removal of ammoniacal nitrogen limits the potential of reed beds for treatment of raw leachates, although they may have potential as secondary polishing systems for leachates already treated in aerobic biological plants (Robinson 1993).

Anaerobic biological treatment depends on microbial associations, in which micro-organisms growing under anaerobic conditions convert organics to

methane, carbon dioxide and other metabolites. This is generally seen as economical because there is not the high energy requirement of aerobic systems, the methane produced can be used for heat or power, less sludge is generated, the nutrient requirements (nitrogen and phosphorus) are lower than for aerobic systems, and pathogens are destroyed (Britz 1995). Hydrogen sulphide formed is a good precipitant for most metals. Anaerobic reactors include conventional digesters, anaerobic filters and fixed film reactors (Britz 1995).

In combined physical, chemical and biological systems a substantial part of the organic load is removed anaerobically, producing methane, while the remainder is removed aerobically, using aeration. This is one of the most economical carbon removal solutions. Keenan *et al* (1983) concluded that no one treatment method separately achieves a high enough removal efficiency for landfill leachate.

e. Acid mine drainage treatment

Water draining from abandoned mines is becoming one of the most prominent sources of freshwater pollution (Younger 1997), as a consequence of the increasing numbers of coal and metal mine workings now being abandoned. By definition, this form of pollution is concentrated in areas of the UK, United States and Scandinavia where the geology has made mining profitable.

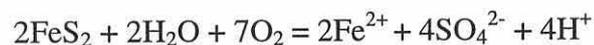
Contaminating mine drainage is one of the major hydrogeological and geochemical problems arising from mankind's intrusion into the geosphere (Banks *et al* 1997), but far from being a modern problem its historical presence is evident from names such as the north Cornish Red River, the Ochre Dykes of Derbyshire and South Yorkshire, the Yellow Stream of Lancashire, the Norwegian Raubekken and the Spanish Rio Tinto (Banks *et al* 1997).

Potentially polluting mine drainage is created when coal or metal mines are abandoned and the pumps used to dewater the mine (to allow access to strata

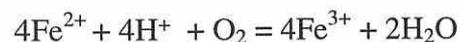
below the water table) are switched off. The water table rebounds to its natural level (Jarvis 1997), in the process leaching out minerals which have become oxidized while the strata were exposed to the atmosphere (Banks *et al* 1997). Very similar effects occur in mine spoil and tailings heaps, where minerals freshly removed from the mine become oxidized on being brought to the surface (Younger 1997).

In the UK and Scandinavia, mine drainage waters may be (1) saline, (2) acidic, heavy metal-containing, sulphate waters from pyrite oxidation, or (3) alkaline, hydrogen sulphide-containing waters which are heavy metal poor due to buffering reactions and/or sulphate reduction (Banks *et al* 1997). Acid mine drainage is thus characterized by high sulphate, high iron (ferric and ferrous), and a pH of less than 3 (Johnson 1995).

The mineral strata associated with coal measures very frequently contain iron sulphides (Jarvis 1997), and the oxidation of these is the cause of the acid in many mine drainages (Banks *et al* 1997); pyrite (iron sulphide, FeS₂), is oxidized to ferrous iron, sulphate and protons according to the equation:



The ferrous iron is then further partially oxidized to ferric iron, which consumes some protons:



However because the ferric iron may then accept electrons for further pyrite oxidation, or hydrolysis may occur, both of these reactions release further protons (Banks *et al* 1997). Overall, acid is produced, but this acidity will only reduce the pH of the drainage when it exceeds the natural alkalinity available from bicarbonate, calcite, dolomite etc in the system (Banks *et al* 1997). The acidic solution causes secondary reactions, which affect the acidity and concentrations of base and acidic cations; ultimately the hydrogeochemical conditions in the mine determine the final pH, ferric/ferrous iron, aluminium and manganese

concentrations of the discharging water (Hedin 1997).

The chemical and biological oxidation of sulphide ores is accelerated by acidophilic metal-mobilizing bacteria such as *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, which are involved in both direct and indirect oxidation of sulphide minerals (Johnson 1995). For the direct oxidation of sulphide minerals, there must be intimate contact between microbes and minerals. Cells of the "*Thiobacillus*-like" bacteria attached to iron sulphide directly oxidize it to Fe^{3+} , SO_4^{2-} and H^+ and the same bacteria indirectly oxidize the ferrous iron so produced to ferric iron.

Indirect oxidation, however, does not require contact between micro-organisms and minerals, because the sulphides are chemically oxidized by soluble ferric iron, which is reduced to ferrous iron; this has implications for constructed wetlands because ferric iron-laden acidic waters in the anoxic zone of a wetland may catalyse the dissolution of pyritic minerals, resulting in the production of soluble ferrous iron and generating further acidity (Johnson 1995).

The mine drainage problem

The Water Resources Act (1991) makes it an offence to knowingly discharge polluting substances to a water course without a specific "consent to discharge", which often sets an upper limit on the concentration of a pollutant which can be released. Sources of pollution such as sewage and industrial effluents have been required to conform to this legislation for some ten years now. However, prior to December 31st 1999, water draining from abandoned mines was exempt from the Water Resources Act. The owners of unworked mines had no legal liability whatsoever for the damage caused to receiving water courses (Younger 1997).

Jarvis (1997) estimates that in the United Kingdom the length of watercourse affected by acid mine drainage is 200 km, in which the main effects are the raised

concentrations of dissolved iron, which are directly toxic to aquatic life; blanketing of substrata with iron oxyhydroxides (ochre) causing chronic effects on invertebrate habitats and fish spawning gravels; and the gross aesthetic impact which constrains regeneration of the area (Edwards *et al* 1997). Emerging mine drainage may appear clear and relatively clean, and its potential for environmental damage is not apparent until it becomes oxidized. Then, ferrous iron is oxidized to ferric iron by contact with the atmosphere, and being less soluble at low pH than ferrous iron this is precipitated onto the stream bed.

Acid mine drainage (AMD) is hostile to most aquatic life - hydras, sponges, flatworms and vertebrates (Johnson 1995) - though several insect species (including beetles, caddis fly and mosquito larvae, and bloodworms) have been found in some locations (Lackey 1939). Although the dominant life forms in AMD are microbial, the majority of bacterial species in unpolluted streams are killed on exposure to AMD.

Treatment of acid mine drainage

Methods of treating acid mine drainage to render it harmless to life in the receiving water course can be broadly divided into active and passive strategies; active treatments further fall into chemical and biological groups. Choice of treatment depends on the volume of effluent to be dealt with, the type and concentration of contaminants, the size and ecological status of the receiving water, the ease of disposal of treatment by-products, treatment costs, ease of operation/maintenance, space available, and applicable legislation.

Active chemical treatment is the conventional method, and prior to the 1980s, minewater treatment was almost exclusively chemical, involving the addition of basic chemicals such as calcium hydroxide ($\text{Ca}(\text{OH})_2$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3) and ammonia (NH_3) to neutralize acidity and promote the precipitation of metal solids (primarily metal hydroxides). Heavy

metals are typically precipitated as hydroxides (Banks *et al* 1997). The resulting sludge was left for metal-rich solids to settle out. Aerators, mixers, chemical oxidizers and coagulants were also often used to increase the kinetics of chemical reactions and sludge settling (Hedin 1997). Chemical treatment of minewaters is reliable and easily available, but expensive, and the large amount of sludge produced presents a secondary disposal problem. Other chemical treatments include the addition of scrap iron to galvanically reduce and precipitate copper from solution, ion-exchange techniques, electrochemically assisted sorption onto dead biomass, reverse osmosis, and electrolysis to recover copper (Banks *et al* 1997).

Active biological treatment (bioremediation) involves the controlled use of neutrophilic or acidophilic micro-organisms to reduce sulphate and/or iron in bioreactors; under anoxic conditions this generates alkalinity which neutralises the acidity of mine drainage, and sulphide which can efficiently remove metals such as iron and manganese from solution by forming insoluble sulphides (Tarutis & Unz 1995). Bioremediation produces a relatively stable, dense sludge which has no major environmental effects. Although they are initially expensive, the long-term maintenance of bioreactors is inexpensive.

Passive treatment of minewaters uses aerobic surface flow wetlands, anaerobic sub-surface flow compost wetlands, or anoxic limestone drains (Ranson & Edwards 1997, Hedin *et al* 1994) to initiate iron and sulphate reduction, precipitate metal ions and neutralize acidity. For mine drainages which are not intensely polluting, chemical treatment is an expensive option, and in the last fifteen years passive treatment using wetlands, limestone drains and organic substrates has gained attention for remediation of these (Hedin 1997). Passive treatment originated with the observation of Tuttle *et al* (1969) of mine drainage amelioration by a sawdust pile, and subsequently from observations of water quality improvement associated with the flow of contaminated mine water

through natural, so-called "volunteer" wetlands (Younger 1997). Although it requires more land area, wetland treatment does not need costly reagents and extensive operational attention and maintenance (Hedin *et al* 1994). The most frequent cause of treatment failure in constructed wetlands is under-sizing (Banks *et al* 1997).

Technicalities of wetland treatment

Successful AMD wetland treatment systems neutralize acidity and precipitate metal contaminants (Hedin 1997); iron precipitates most readily as an oxyhydroxide under circum-neutral conditions, so this is an important requirement of treatment wetlands. Manganese also precipitates naturally from alkaline water.

Acid mine drainage treatment using wetlands is still, despite many studies and reports, "a black box technology, not entirely under control" (McGinness *et al* 1997) and consequently the potential of these systems must still be underestimated. On the whole, construction of mine water treatment wetlands is based on assumptions about water chemistry, engineering principles, and ecological function, with little regard for the flow of organic material through the wetland, whether the primary producers of a system are able to support the sulphate-reducing bacteria necessary to the system, or whether the wetland produces annually enough organic material to maintain the sorption of free metals (McGinness *et al* 1997). The provision of enough organic substrate for alkali-generating micro-organisms is an important factor affecting the efficiency of mine drainage treatment wetlands; either bulk materials must be added periodically, or plant life which continuously adds organic matter as exudates and dead biomass must be sustained. Similarly, acid mine drainage can be treated by adding sawdust, straw, hay, peat etc. directly to the drainage stream; these organic materials act as supports for microflora and as carbon sources (electron donors) for reduction.

Very little is known about the microbial ecology or mechanisms involved in acid mine drainage mitigation within wetlands (Webb *et al* 1998); it is generally believed that pH increase and metal removal occur by a combination of ion exchange on to organic matter and inorganic materials, and that metal uptake by plants and filtration of colloidal and suspended materials are less important processes than microbially mediated transformations (Johnson 1995). The complex anaerobic ecology within wetland sediments is considered central to the treatment processes, as is metal removal by sulphate reducing bacteria (SRB), which generate hydrogen sulphide and cause precipitation of metals from solution as insoluble metal sulphides (Webb *et al* 1998).

Wetlands effectively filter metals from contaminated water due to the high metal-binding affinity of their soils (Beining & Otte, 1996). Whilst some authors believe that the longevity of wetlands for filtering may be centuries rather than decades (Beining & Otte 1996) others stress that the long term viability of wetlands has not yet been adequately addressed (Banks *et al* 1997). The availability of dissolved organic carbon in the wetland may affect iron mobility (Beining & Otte 1996).

Aerobic wetlands

Aerobic wetlands promote mixed oxidation and hydrolysis reactions, and are most effective when mine drainage is net alkaline (Hedin *et al* 1994). Aerobic processes such as adsorption and ion exchange (Eger 1994) cause the oxidation of ferrous iron to ferric, and subsequent precipitation of iron oxyhydroxides (Webb *et al* 1998). Aerobic processes are limited by the available exchange sites in the substrate, and by transport of contaminants to these sites. Aerobic processes can decrease the pH, so are less effective at removing metals as the solution pH decreases (Eger 1994). Oxygen enrichment of wetland soil by plants is unlikely to significantly increase the precipitation of iron.

Anaerobic wetlands

Organic substrate wetlands promote anaerobic bacterial activity, resulting in the precipitation of metal sulphides and the generation of bicarbonate alkalinity (Hedin *et al* 1994). Anaerobic processes, e.g. sulphate reduction, occur in the saturated zone below the soil/water interface, and complex organics are reduced to simpler organics by bacteria, which can be used by sulphate reducing bacteria. Sulphate reduction removes metals as well as increasing the pH. To promote sulphate reduction in a wetland, the flow must be forced through the anaerobic zone (Eger 1994).

Controlling mine drainage pollution

Recommendations for the control of acid mine drainage pollution include (1) precluding formation by preventing oxidation of sulphide minerals, (2) preventing migration from the site of production, (3) collection and treatment before release to a watercourse (Johnson 1995). Banks *et al* (1997) also suggest continuing pumping after mine closure, plugging drainage adits and allowing mines to refill, and novel treatments such as sacrificial anodes to suppress oxidation of metal sulphide ores, applying a potential difference to the ore body to oppose the natural galvanic couple generated by ore oxidation, application of bactericides to hinder pyrite oxidation (demonstrated in spoil tips), and the use of bacterial bioreactors.

At present there has been no investigation of carbon flow within wetland treatment systems. It is important that the design of wetlands will promote the bacterial consortia necessary to remove metals in the most efficient manner (Banks *et al* 1997).

1.4 Types of constructed wetland

The range of designs for constructed wetlands is now comprehensive, with each having particular advantages in terms of specific treatment objectives, construction method, local material availability, location, etc. Pride *et al* (1990)

divide constructed wetlands into two broad categories of 'free surface water' and 'vegetated submerged bed', these having respectively water passing over the bottom sediments and through the above ground plant zone, or water passing through a permeable sediment in which the plants are rooted. The latter design may also be referred to as 'subsurface flow wetland' (Hammer & Bastian 1989). Constructed wetlands may also be described according to their hydraulic system, that is as 'horizontal flow', in which a water depth of some 30-50 cm is always maintained and the wastewater moves horizontally through the bed medium; or as 'vertical flow' in which wastewater inflow tends to be intermittent so that the water spreads out on the bed surface before percolating (by gravity) to the bottom of the bed and being released (Grant *et al* 1996). Horizontal flow systems are usually used for tertiary treatment, and vertical flow for primary or secondary treatment, due to the different oxygenation conditions which each supports.

'Floating macrophyte' systems are also used, for both secondary and tertiary water treatment, in which the treatment concept is based on harvesting the plant biomass (usually duckweed or water hyacinth) (Brix & Schierup 1989).

Parameters used in the design of constructed wetlands include hydraulic residence time, hydraulic loading rate, organic loading rate, total nitrogen loading rate, ammonia loading rate and water column depth (Pride *et al* 1990). Frequently a wetland treatment system will consist of several wetlands operating either in series or in parallel, in which the wastewater quality is progressively improved, for example by first removing BOD₅ in one wetland, and subsequently feeding the water to a second wetland for nitrogen removal (White 1995). The need for both aerobic and anaerobic zones for completely effective ammoniacal nitrogen removal requires careful design of wetlands intended to treat high-nitrogen wastewaters.

The variety of aquatic plants used in constructed wetlands is extensive, and includes *Phragmites australis*, *Typha* spp., *Juncus* spp., *Scripus* spp., *Schoenoplectus* and *Carex* spp. (Farahbakhshazad & Morrison 1998). Some systems employ a single species, others have mixed culture, but irrespective of species the functions of macrophytes in constructed wetlands are generally agreed to be the same.

1.5 Influence of plants in constructed wetland systems

Shallow, eutrophic, aquatic ecosystems stocked with macrophytes are among the most productive in the world (Brix & Schierup 1989). The presence of macrophytes is part of the definition of natural wetlands, but as the major remediation processes in treatment wetlands are physical and microbial, plants may at first appear superfluous.

Macrophytes use solar energy to assimilate inorganic carbon from the atmosphere to produce organic matter which subsequently provides energy for heterotrophs (Westlake 1963), and may influence heterotrophs by their physical presence and their metabolism. Vegetation in wetlands distributes and reduces the current velocities of water, aids sedimentation, reduces erosion and resuspension, increases contact between water and plant surfaces and has an insulating effect in winter (Brix 1994). Root growth in the bed helps to decompose organic matter, and disturbs and loosens the soil, leaving tubular macro pores which increase and stabilize the soil hydraulic conductivity (Kickuth 1981). Roots, rhizomes and dead material become colonized by dense communities of photosynthetic algae, bacteria and protozoa and these biofilms are responsible for the majority of microbial processing in wetlands (Brix 1994). Rooted macrophytes take up nutrients released by micro-organisms, mainly through their roots, but also through immersed stems and leaves; this uptake is estimated at 30-150 kg P ha⁻¹ year⁻¹ and 200-2500 kg N ha⁻¹ year⁻¹ (Brix 1994). If wetland plants are not harvested, the incorporated nutrients are returned to the soil on plant death and

decomposition; long-term nutrient storage in wetlands is by the undecomposed litter fraction, and the deposition of refractory nutrient-containing compounds (Kadlec & Knight 1996). Plants may also release antibiotics (Seidel 1964, 1966), compounds which affect the growth of other species, and organic compounds which may act as carbon sources for denitrifiers and thus increase nitrate removal (Brix 1994).

The zone of soil influenced by plant root exudates is the rhizosphere. Rhizome biofilms support higher densities of a range of bacteria (May *et al* 1990) than gravel biofilms do; this may be the result of exudation of soluble organics by rhizomes and the decay of dead plant material within the biofilm, which stimulates heterotrophic activity and increases the local rate of denitrification (Knowles 1982). Rovira (1969) described plant root exudates as "substances released into the surrounding medium by healthy and intact plant roots", including sugars, amino acids, peptides, enzymes, vitamins, organic acids, nucleotides, fungal stimulators, inhibitors and attractants. Amylase, deoxyribonuclease, invertase, peroxidase, phosphatase and ribonuclease were enzymes listed as root exudate compounds by Grayston *et al* (1996). Exudation by normally functioning roots is only approximately 0.4% of photosynthesized carbon; physical and chemical root damage increases this (Rovira 1969), and root exudation may also be affected by plant species, age and development, and pH, water supply, temperature, anoxia, light intensity, carbon dioxide concentration and micro-organisms (Grayston *et al* 1996).

In saturated soils the pore spaces are filled with water instead of air, leading to anaerobic conditions in all but a few millimetres at the surface of the soil. Plants growing in such soil must obtain oxygen for their roots by internal transport from their aerial organs, and such internal lacunal systems may occupy up to 60% of the total tissue volume (Brix 1994). The oxidation of the rhizosphere detoxifies hydrogen sulphide and reduced forms of iron and manganese produced under the

saturated conditions (Armstrong 1971, Gambrell & Patrick 1978, Ponnampereuma 1965), and activates biogeochemical processes including the oxidation of organic compounds and ammonia nitrification (Reddy *et al* 1989). In subsurface flow wetlands oxygen leakage from roots to the rhizosphere is important for the aerobic degradation of oxygen-demanding substances (Brix 1994). Oxygen release is greatest from the root apices, and there is little or no release from old roots and rhizomes; release rate estimates vary widely, from $0.02 \text{ g m}^{-2} \text{ day}^{-1}$ to $12 \text{ g m}^{-2} \text{ day}^{-1}$ (Brix 1994) (partly due to differences in technique, partly to seasonal variation). Release depends on the internal oxygen concentration, oxygen demand of the medium and the root wall permeability (Sorrell and Armstrong 1994). Gas transport may be by passive molecular diffusion (determined by concentration gradients) or convective flow (bulk flow), driven by temperature, water vapour pressure differences or the Venturi effect. Convective through-flow considerably increases the root length that can be aerated compared to diffusion alone, so such plants have more potential to release oxygen via their roots (Brix 1994). Differences between species in oxidizing the rhizosphere are attributed to differences in root respiratory activity, in lacuna and aerenchyma tissue, root wall permeability, root length, and root exudation of carbohydrates and enzymes into the rhizosphere (Reddy *et al* 1989).

The classification of aquatic macrophyte-based wastewater treatment systems may be according to hydraulic regime and the life-form of the dominating macrophyte (Kowalik *et al* 1995):

- 1 surface flow with an exposed free water surface
 - a) free floating macrophyte based systems
 - b) submerged macrophyte-based systems
 - c) rooted emergent macrophyte based systems
- 2 subsurface flow emergent macrophyte based systems
 - a) with horizontal subsurface flow
 - b) with vertical subsurface flow (percolation)

- 3 complex multi-stage systems, incorporating a combination of the above features and oxidation ponds, sand filtration, etc.

The advantages of macrophyte-based systems over conventional water treatment include the low operating costs, low energy requirements, siting at the point of wastewater production, a lower susceptibility to shock loading (Brix & Schierup 1989) and the potential for resource recovery by harvesting and utilizing the biomass produced (as an energy source, compost, animal feed). The increased land area required over conventional treatment, and a decreased performance during winter in temperate regions can be disadvantages.

1.6 Nutrient cycling in wetlands

Living tissue is sustained by nutrients, which provide the energy for maintenance, growth and reproduction. Nutrients are therefore the essential components from which living tissue is composed, and must be continuously available.

Consequently, when living tissue dies it is essential that the nutrients it contains become available for re-utilization in other tissues. The turnover of nutrients in this way is referred to as cycling, and is frequently considered in terms of models such as the carbon cycle, nitrogen cycle and phosphorus cycle.

The transformation of dead tissue to utilizable nutrients, usually called mineralization, is ultimately achieved by the action of enzymes; the digestive enzymes of soil or aquatic organisms begin the decomposition process, which is continued by extra- and intracellular enzymes of microorganisms.

a. Carbon cycling

The world soil carbon pool is some five times as big as the atmospheric carbon pool (Killham 1996), which serves to emphasize the importance of soil as both a source and sink of different carbon forms. Carbon enters the soil directly from primary producers, and indirectly via secondary producers and their wastes, and

anthropogenic processes which contribute polycyclic aromatic hydrocarbons, pesticides, etc. Decomposition and respiration return carbon to the atmosphere as carbon dioxide from aerobic environments, and as methane from anaerobic environments. Breakdown of polymeric organic carbon molecules such as cellulose, starch, hemicellulose, proteins and chitin begins with hydrolysis and fermentation by extracellular or membrane-bound hydrolytic enzymes, followed by metabolism to intermediates such as fatty acids, alcohols, carbon dioxide and dihydrogen gas under anaerobic conditions, or just carbon dioxide under aerobic conditions (Nedwell 1984).

In natural soils, soil organic carbon can be roughly divided into four forms (Killham 1996):

Aromatic	50%
Nitrogenous	20%
Carbohydrate	15%
Fatty acids/alkane carbon	15%

These forms are distributed between insoluble, soluble and biomass carbon pools, of which the most important is the microbial biomass pool, as this processes and redistributes all forms of carbon input (Killham 1996). The largest fraction of all organic carbon entering soil is derived from plant residues such as cellulose (15-60%), hemicellulose (10-30%), lignin (5-30%) and protein (2-15%) (Paul & Clark 1989).

In natural wetlands, most organic matter is also comprised of plant-derived complex polysaccharides such as cellulose and lignin which are broken down to simple monomers by the action of various extracellular enzymes (Sinsabaugh *et al* 1991). Carbonaceous organic matter can be extremely resistant to microbial degradation, requiring many different enzymes; for example six of the (extracellular) enzymes involved in the degradation of lignocellulose are β -1,4-glucosidase, β -1,4-endoglucanase (endocellulase), cellobiohydrolase, β -xylosidase,

phenol oxidase and phenol peroxidase (Jackson *et al* 1995).

The degradation of crystalline cellulose requires three classes of hydrolytic enzymes: exocellulase, endocellulase and β -glucosidase (Eriksson & Wood 1985, Marsden & Grey 1986). Many micro-organisms produce cellulases, but very few produce all three enzymes, resulting in a synergistic interaction between organisms which achieves the complete degradation of cellulose (Tyler 1974). In many organisms the cellulase enzyme complex includes endo-1,4- β -glucanase, β -glucosidase and cellobiohydrolase (Tarakanov & Lavlinskii 1999). Of these, cellobiohydrolase shows activity towards cellobiose, cellotriose, cellotetraose and cellopentaose in almost equal proportions (Herner *et al* 1999). Exocellulases bind to the crystalline cellulose and release celluloligosaccharides from it, and endocellulases cleave β -glucosidic bonds in the non-crystalline parts.

Beta-glucosidase catalyzes the hydrolysis of glucosides, releasing glucose molecules from dimers and trimers such as cellobiose and cellotriose (Chróst 1991, Alef & Nannipieri 1995), and its activity is reported to correlate significantly with soil organic matter (Eivazi & Tabatabai 1988). The enzyme is produced by heterotrophic micro-organisms, and its broad specificity will catalyze the hydrolysis of β -linked (1-2, 1-3, 1-4, 1-6) disaccharides of glucose, celluhexose and carboxymethylcellulose (Chróst 1991). The activity of this and other glucosidases and galactosidases play a major role in carbohydrate degradation in soils, and their hydrolysis products provide energy for soil micro-organisms.

The main component of hemicellulose in plants is xylan (Carmona *et al* 1997), and xylosidase is involved in hemicellulose breakdown (Jones 1990). As a group, xylanases include endoxylanases, which catalyse xylan hydrolysis to oligosaccharides, and exoxylanases, which release monomers from oligosaccharides (Alef & Nannipieri 1995).

Soil microorganisms produce extracellular oxidoreductases (either peroxidases or polyphenol oxidases), which are capable of catalyzing the oxidation of many aromatic compounds by reduction of hydrogen peroxide or other organic hydroperoxides (Al-Kassim *et al* 1994):



Phenol oxidase is one enzyme amongst several (e.g. ligninases, manganese peroxidases) thought to be involved in the biodegradation of lignin (McLatchey & Reddy 1998). Lignin is the second most abundant polymer on earth, and protects most cellulose and hemicellulose from enzymatic hydrolysis (Kirk & Farrell 1987); it thus represents a large resource of carbon, yet its structural features constrain its biodegradation, and it cannot be used by micro-organisms as a sole carbon source. Lignin molecules have no specific order, as they are formed by chemical polycondensation involving phenols and free radicals (Paul & Clark 1989). The structure is based on phenyl propanoid units, each of which contains an aromatic (six-carbon) ring and a three-carbon chain; the structure may include any or all of the functional groups aldehyde, phenol, methoxyl (side chain), hydroxyl, carbonyl, propanoid or phenyl, as well as ether linkages (Paul & Clark 1989).

In order to degrade lignin, enzymes must be extracellular (lignin molecules are between 600-100 kD), non-specific (lignin does not have a standard structure) and non-hydrolytic (lignin is insoluble). Peroxidases, from plants and microorganisms,

catalyze a range of reactions including the polymerization and depolymerization of lignin; polyphenol oxidases are divided into laccases and tyrosinases, both of which require bimolecular oxygen, but no coenzymes (Bollag 1992).

Constructed wetlands receive high carbon loadings not only as dissolved and particulate organic and inorganic carbon from effluent inflow (Biological Oxygen Demand), but also from root exudation, and from seasonal deposition of plant material on the bed surface (Reddy & D'Angelo 1997). Carbonaceous BOD₅ may be removed by settling of particulates, or breakdown of soluble matter by micro-organisms, while plant material is largely incorporated into the soil and provides long-term carbon and nutrient storage (Reddy & D'Angelo 1997). Breakdown of all forms of carbon is a stepwise process, beginning with abiotic leaching and fragmentation, then extracellular enzymatic hydrolysis, and aerobic and anaerobic catabolic metabolism of heterotrophic micro-organisms (Reddy & D'Angelo 1997).

Proteases have an important role in the soil carbon cycle as they release carbon-based amino acids for intracellular metabolism, and in the nitrogen cycle. Similarly the extracellular enzymes which break down chitin have roles which overlap both the carbon and nitrogen cycles; these are described below.

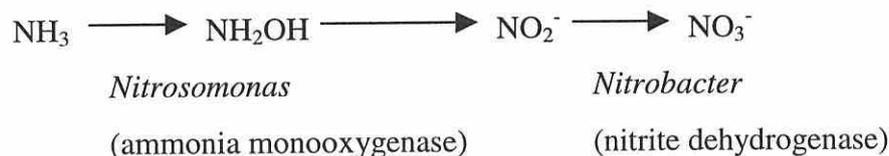
b. Nitrogen cycling

Nitrogen enters soil incorporated mainly in proteins, microbial cell wall constituents such as chitin and peptidoglycans, and nucleic acids (Paul & Clark 1989). The nitrogen atom exists in a number of different oxidation states, and changes between them are frequently mediated by soil organisms, resulting in the formation of inorganic forms which are easily lost from the ecosystem (e.g. nitrous oxide, dinitrogen); thus a nitrogen deficit often limits plant productivity (Paul & Clark 1989). The nitrogen cycle in soil involves the extracellular breakdown of these nitrogenous compounds by enzymes such as chitinases,

proteinases, peptidases and ureases, followed by the eventual (intracellular) conversion of ammonium in the surface aerobic layer to nitrate and then gaseous products by specific bacterial species (Reddy *et al* 1986). Uptake of ammonia by plant roots lowers the oxygen demand of the soil, increases the oxygenated zone and thus allows additional nitrification; the nitrate produced is effectively removed by a combination of plant uptake and denitrification (Farahbakhshazad & Morrison, 1997). All soil nitrogen transformations associated with microbial metabolism are affected by changes in redox potential (Reddy *et al* 1986).

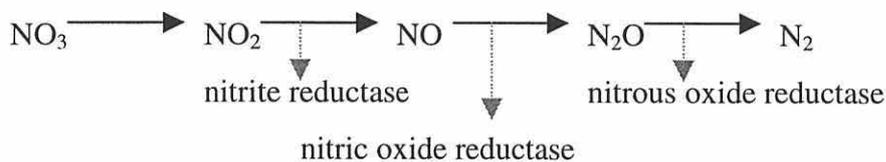
Nitrification

Nitrification results in the transformation of ammonia to nitrite and nitrate in soils. It occurs at a redox potential greater than +300 mV (Reddy *et al* 1986), and is slow below 5 °C (Robinson 1993) and above 40 °C (Paul & Clark 1989). The optimum pH for nitrification is 6.6 – 8.0. For each milligram of ammonia nitrified, 4.3 mg of oxygen are required (Reddy *et al* 1989). Removing the carbonaceous Biological Oxygen Demand of wastewater first is the key to enhancing nitrification (White 1995) as carbon utilizing bacteria then cannot thrive, so that nitrifiers dominate in the second stage. The oxidation of ammonia to nitrate during nitrification is mediated by autotrophic bacteria under aerobic conditions (Reddy & Patrick 1984). Where easily degradable organic matter is readily available oxygen levels are low and subsequently nitrifying activity is low (Williams *et al* 1994). Nitrification requires the presence of at least two genera of bacteria (Averill 1995) for the following transformations:



Denitrification

Denitrification is the anaerobic utilization by bacteria of nitrogen oxides as terminal electron acceptors in place of oxygen (Averill 1995). Microbial denitrification is the major means of nitrogen loss from wetlands, with molecular nitrogen gas being the dominant product:



Denitrifiers in soil are facultative anaerobes, mainly heterotrophic bacteria such as *Pseudomonad* and *Alcaligenes* species (Killham 1996), which use nitrate as an oxygen source in anaerobic conditions, and can grow aerobically without it (Hammer & Knight 1994). Denitrification occurs in the anaerobic soil layer, where the redox potential is less than +300 mV, requiring oxidised mineral nitrogen and readily available organic carbon (Knowles 1982). As most denitrifiers grow best at pH 6 – 8, the rate of denitrification is slow below pH 5 (Paul & Clark 1989). The rate of denitrification depends on the supply of nitrate, the energy source, microflora and temperature. All denitrification steps are carried out by a single organism (Knowles 1982), unlike nitrification which involves several species; some organisms lack the final enzyme in the sequence and produce nitrous oxide instead of nitrogen gas. Because of the requirement for both aerobic and anaerobic soil conditions for nitrogen mineralization, water level manipulation is important for good nitrogen removal (Sloey *et al* 1978).

Denitrification is controlled by factors including nitrogen oxide reductase, which is thought to be repressed by molecular oxygen; pH which is positively correlated with denitrification, the optimum being pH 7-8 although it may occur up to pH 11; temperature, the optimum being 25-65 °C (Hammer & Knight 1994, Knowles 1982). The “ideal” biomass carbon: nitrogen ratio for denitrification is 2:1 (van

Oostrom & Russell 1994). Inhibitors of denitrification include acetylene, azide, cyanide, nitrapyrin, pesticides and sulphur compounds; many have metabolic effects other than those on the specific reductases involved in denitrification (Knowles 1982).

The balance of denitrification and nitrification rates is influenced by the chemical environment and can be manipulated by design and operating procedures to produce effluents suitable for agriculture (high nitrate) or discharge (low nitrate) (Williams *et al* 1994). Alternate periods of sediment flooding and drying have been suggested to improve nitrogen removal efficiency by facilitating the sequential coupling of nitrification and denitrification (Wijler & Delwiche 1954, Brix 1990).

Constructed wetlands receive nitrogen in particulate and dissolved organic and inorganic forms, although the exact ratios depend on the particular effluent (Reddy & D'Angelo 1997). Particulate forms will be removed by settlement, and dissolved forms by ammonia volatilization, nitrification, denitrification and mineralization via micro-organisms and extracellular enzymes. Extracellular enzymes involved in nitrogen cycling include urease, chitinase, N-acetylglucosaminidase and proteases. Proteases play an important part in the soil nitrogen cycle by releasing nitrogen-rich amino acids for microbial uptake (Skujinš 1976). Urease, which is found in many microorganisms (Skujinš 1976), transforms urea to ammonium and carbon dioxide, from where it may enter microbial intracellular metabolism.

Chitinase and N-acetylglucosaminidase together break down chitin to monomers of N-acetylglucosamine for intracellular transformation, and therefore have a significant role in both the nitrogen and carbon cycles (Ueno *et al* 1991). Chitin (poly- β -1,4-N-acetylglucosamine) is the most abundant natural amino polysaccharide, and is a major organic component of fungal cell walls, arthropod

exoskeletons and molluscs (Chróst 1991). It has been described as one of the most significant fractions of humus-bound nitrogen in soil (Aber & Melillo 1991), and its rate of decomposition in soil is therefore significant in soil nitrogen supply. The degradation of chitin is begun by N-acetylglucosaminase (chitinase), which degrades the polymer to N, N'-diacetylchitobiose; subsequently the activity of chitobiase on this oligosaccharide releases N-acetylglucosamine (Jeuniaux 1966, Ueno *et al* 1991). The two enzymes work synergistically, and are considered to play significant roles in the soil carbon and nitrogen cycles. Veon *et al* (1990) found a significant correlation between N-acetylglucosaminase activity and nitrogen content in soil. N-acetylglucosaminase is synthesized by a variety of chitin-containing organisms, and also by some plants as an inducible defence against pathogenic fungi (Ueno *et al* 1991).

c. Phosphorus cycling

A conceptual phosphorus cycle was described by Stewart & Tiessen (1987). In nature, phosphorus exists largely in insoluble or very poorly soluble inorganic forms; soil organisms are very important in solubilizing inorganic phosphorus, mineralizing organic phosphorus, and immobilizing available soil phosphorus (Paul & Clark 1989). There is little seasonal change in total soil organic phosphorus because mineralization and immobilization occur simultaneously (Stewart & Tiessen 1987). The immobilization, mineralization and re-distribution of phosphorus in soil depend equally on physico-chemical properties (e.g. phosphorus sorption on colloidal surfaces) and uptake by microorganisms, mycorrhizas and plants (Stewart & Tiessen 1987).

The central component of the phosphorus cycle according to Stewart & Tiessen is biomass phosphorus, which is taken up directly by predators or saprophytes and incorporated into new consumer biomass, accompanied by rapid rates of carbon and nitrogen transformation. Organic phosphorus in soils is mainly incorporated in phytates, nucleic acids and their derivatives, and phospholipids (Paul & Clark

1989). Stable organic phosphorus accumulates in chemically resistant and aggregate protected forms, and its immobilization depends on the availability of inorganic phosphorus and organic substrates for growth and maintenance of the soil biomass (Stewart & Tiessen 1987).

Phosphorus entering constructed wetlands is removed biotically by vegetation, periphyton and microbial uptake, and mineralization; abiotic processes include settling, adsorption, precipitation and exchange between the soil and overlying water (Reddy & D'Angelo 1997). The removal of phosphorus by constructed wetlands is strongly dependent on the bed medium (Wood & McAtamney 1996), and bed materials rich in calcium, iron and/or aluminium have been recommended. The phosphate concentration in saturated wetland soils is usually much higher than in the overlying water, creating a vertical diffusion gradient; the diffusion of phosphate out of soil is decreased by biological fixation and phosphate sorption onto oxidized iron (Chambers & Odum 1990). Soil pH has a considerable effect on solubilization of phosphorus from sediments into overlying water; at pH 5-7, phosphorus is less likely to go into solution than at higher or lower values (Sloey *et al* 1978). Individual soil phosphorus compounds include several specific phosphate esters such as phospholipids, glycerol phosphates, phosphatidyl choline and nucleic acids, but these account for no more than 1-2% of total soil organic phosphorus (P_o) (Stewart & Tiessen 1987). Inositol phosphorus in various combinations with humic and fulvic acids, or as metal complexes, may account for up to one third of soil P_o (Stewart & Tiessen 1987).

The sorption and release of soil phosphate is governed by redox potential, oxygen supply, microbiological activity, temperature and soil characteristics, and soil pH, amount and type of clay minerals, organic matter and extractable iron and aluminium oxides affect phosphorus dynamics (Khalid *et al* 1977). In flooded, anaerobic soils phosphorus is released and iron is oxidized (Patrick & Khalid 1974, Chambers & Odum 1990), due to changes in ferric oxyhydroxides caused

by soil reduction; extractable iron was found to be the most important contributor to phosphorus sorption by flooded soils. Organic soil phosphorus dynamics are greatly influenced by the interactions of biological, chemical and physical properties of soil, and microbes, fauna and plants are all involved in the rapid cycling of phosphorus compounds (Stewart & Tiessen 1987).

Extracellular enzymes which are involved in the transformation of organic phosphorus to microbially available forms are broadly labelled phosphatases. These enzymes catalyze the hydrolysis of phosphate esters (Killham 1996), have broad specificity, and can act on many different substrates at different rates (Alef *et al* 1995). Phosphomonoesterases, which include phytase, nucleotidases, sugar phosphatases and glycerophosphatases, release inorganic phosphorus from organic compounds and have been extensively studied in soil. These enzymes may be classified as acid, neutral or alkaline phosphatases according to their optimum pH; acid and alkaline phosphatases have been found in soil, with acid phosphatase predominating in acid soil, and alkaline phosphatase in alkaline soil (Eivazi & Tabatabai 1977). Other phosphatases include nucleases and phospholipases (diester hydrolases), and triester hydrolases such as phosphoamidases (Alef *et al* 1995). It has been suggested that phosphatase activity only becomes important after the initial breakdown of soil organic matter by other microbial enzymes, this initial step being rate-limiting to organic phosphorus mineralization (Appiah & Thompson 1974, cited in Spier & Ross 1978).

Phosphatase activity is influenced particularly by pH and temperature, organic matter content, soil moisture and anaerobiosis (Alef & Nannipieri 1995), so varies seasonally (Spier & Ross 1978). Trace elements such as mercury, arsenic, silver and cadmium significantly inhibit soil phosphatase activity (Juma & Tabatabai 1977), as does orthophosphate (Spier & Ross 1978). Glenn (1976) reported that the synthesis of extracellular phosphatase was governed by inorganic phosphorus

the synthesis of extracellular phosphatase was governed by inorganic phosphorous levels in several species of microorganism, and may be co-regulated with other metabolic events.

d. Sulphur cycling

Sulphur is of equal importance to nitrogen for the maintenance of life, as it is essential for protein formation (Paul & Clark 1989). In soil, much more than 90 % of sulphur (in non-calcareous, non-tropical surface soils) is in organic forms (Killham 1996) such as sulphate esters, carbon-bonded sulphur and inert forms.

Soil sulphur may originate from mineral weathering, atmospheric inputs such as sulphur dioxide, or organic inputs such as fertilizer and manure. Sulphur is transferred between the inorganic and organic pools by soil biota, in particular the microbial biomass. Organic sulphur mineralization may be biological or biochemical. For carbon-bonded sulphur the process is biological, as carbon is oxidized for energy; for other, non-carbon sulphur forms, the process is biochemical and takes place extracellularly via enzymes such as sulphatase (Killham 1996). The sulphur thus released into the inorganic pool may have oxidation states between -2 (sulphide) and $+6$ (sulphate), but oxidation usually rapidly converts all inorganic sulphur to sulphate. The rate of mineralization is influenced by factors such as water potential, temperature, pH, plants, and form and quantity of organic sulphur, as these are variables which affect microbial growth and production/activity of enzymes.

The sulphur cycle in soil consists of atmospheric, anthropogenic and natural organic inputs, which enter the sulphate, reduced sulphur or organic sulphur pools. In addition, soil contains pools of stable sulphate, sulphur minerals, sulphide, stable reduced sulphur and biomass sulphur, with transformations between all these pools being immobilization, mineralization, plant uptake, root exudation, oxidation and reduction (Killham 1996). Most sulphur taken up by

plants and microbes is in the form of sulphate, and as the inorganic sulphur pool in soil is usually very small, biological uptake is limited by the rate of mineralization. The hydrolysis of organic sulphate esters is catalysed by sulphatases, which release inorganic sulphate from their substrates, according to the equation:



This results in inorganic sulphate being available for plants (Klose *et al* 1999).

Sulphate esters typically account for about half of the organic sulphur present in freshwater sediments (Wieder *et al* 1988). Sulphatase activity has been found to correlate significantly with soil organic carbon (Tabatabai & Bremner 1970), total nitrogen and cation exchange capacity (Alef & Nannipieri 1995) and soil microbial biomass (Klose *et al* 1999). The enzyme is inhibited by inorganic sulphur, phosphate and arsenic and molybdenum oxides. Jarvis *et al* (1987) found no correlation between arylsulphatase activity and sulphur mineralization, even though the majority of sulphur in surface soils is present as organic sulphates (Tabatabai & Bremner 1970). According to Sinsabaugh & Linkins (1990) sulphatase activity is inversely correlated with particle size and is unrelated to the activity of other enzymes. This enzyme may be synthesized by sulphate-reducing bacteria to supply sulphate for respiration. Klose *et al* (1999) reported that approximately 45% of arylsulphatase was extracellular, and 55% associated with the soil microbial biomass, which is considered to be the primary source of soil enzymes.

In soil organic matter the carbon:sulphur ratio is less consistent than the carbon:nitrogen ratio (Paul & Clark 1989), which results from differences in parent material, leaching and sulphur inputs. Although it is an essential nutrient, sulphur can become damaging if present in excess. Acidic rain, formed downwind of heavily industrialized areas, has a high sulphate concentration which damages

lakes and forests; sulphide can have major effects on denitrification, because it inhibits the reduction of nitrous oxide to molecular nitrogen.

1.7 Biogeochemistry in constructed wetlands

In natural wetlands the suspension of normal decomposition processes, due to the flooded (and therefore anaerobic) soil, allows organic matter to accumulate and unique biogeochemical processes to dominate. Organic matter accumulation in constructed wetlands would generally be considered unwelcome, as mineralization and conversion to plant biomass are preferable. Oxygen leakage from plant roots creates a mosaic of aerobic and anaerobic microsites, which allows the complete or partial transformation of pollutants in wastewater to minerals, gases and water; a notable exception is in the treatment of acid mine drainage, where sulphate reduction in anaerobic zones is desirable (Johnson 1995). Water table fluctuations also create alternating aerobic and anaerobic conditions which stimulate organic matter decomposition and nutrient release depending on the availability of electron acceptors (Reddy & Patrick 1975, D'Angelo & Reddy 1994). Organic matter decomposition may be detrimental in metal removal wetlands as the removal mechanisms tend to be physical processes such as binding to soils, sediments and soluble organics, precipitation as insoluble salts and plant uptake (Kadlec & Knight 1996), therefore zero, or slow, decomposition is desirable.

The general fate of organic and inorganic pollutants entering constructed wetlands is known, but exactly how the processes of amelioration are affected, enhanced or retarded by the specific conditions of a particular wetland is unclear (Reddy & D'Angelo 1997). Not only the biogeochemical conditions such as pH, oxygenation and redox potential of wetland soil affect the nutrient transformations in it, but also the water retention time, bed size, hydrology, bed material and microbial population. Much has already been published on these latter issues, and in this thesis the biogeochemistry of constructed wetlands is focussed upon.

a. Enzymes

Enzymes are "amphoteric (able to act as base and acid) globular proteins composed of many acidic and basic groups of amino acid side-chains" (Wharton & Eisenthal, 1981) which are able to act as biological catalysts; they are able to speed up reactions which would otherwise either proceed extremely slowly (too slowly to support life in cells), or would require conditions not compatible with life (high temperature, extreme pH). The intrinsic *modus operandi* of enzymes is to lower the activation energy of a specific reaction, thus speeding it up. An enzyme combines with its substrate(s) in a temporary enzyme/substrate complex which quickly breaks down to release the unchanged enzyme, and the reaction product(s).

Enzymatic reactions may be anabolic or catabolic, but all enzymes have common characteristics; enzymes remain unchanged at the end of the reactions they catalyse, they are specific to particular substrates, and they do not change the equilibrium point of the reaction they take part in. Similarly, all enzymes are affected by temperature, pH, pressure and product/substrate concentrations. Because they remain unchanged after reacting, a small amount of enzyme is able to act on a large amount of substrate. These characteristics apply equally to active soil enzymes as to metabolic (intracellular) enzymes. Certain groups of enzymes work more efficiently in the presence of cofactors such as simple inorganic ions, prosthetic groups or coenzymes (Frankenberger & Johanson 1982). Enzyme activity may be reduced by inactivation, denaturation or inhibition. In inactivation (which is always reversible), unfavourable conditions such as too low a temperature prevent the enzyme from catalysing the reaction. Inhibition occurs when molecules other than the specific substrate molecule combine with the active site of the enzyme (competitive reversible inhibition) or with another part of the enzyme molecule (non-competitive reversible inhibition), preventing binding by specific substrate molecules. In irreversible inhibition, ions such as heavy metals cause protein precipitation in the active site.

b. Soil enzymes

Microorganisms are considered to be the most important source of soil enzymes (Spier & Ross 1978, Mawdsley & Burns 1994). Microbial enzymes play a crucial role in a variety of fixation, oxidation and reduction reactions, in converting organic matter to inorganic forms for plant growth (Burns 1977), in the initial phases of decomposition of organic residues and in the transformation of some mineral compounds under unfavourable conditions for microbial proliferation (Kiss *et al* 1975). Enzymes present in soil originate from soil animals, plants and micro-organisms (Ladd 1978) and thus may potentially encompass the entire range of enzymes produced by these organisms. However the relative contributions of enzymes from different sources are generally unknown, and difficult to quantify, and the persistence of different enzymes in soil is likely to vary (Burns 1983). Enzymes may be released from live cells, exuded to facilitate extracellular metabolism, or released after cell or organism death. They may be synthesised continuously (constitutive) or only in the presence of so-called inducer compounds (inducible) (Cohen 1980). Similarly, enzyme synthesis may be repressed in the presence of certain compounds, notably the enzyme reaction product.

Enzyme activity is generally reported to be greater in the rhizosphere than in the bulk soil, due to the greater substrate availability and increased numbers of microorganisms there (Skujinš 1976). To complete the cycling of organic matter, large polymers must be broken down into units which microorganisms can metabolise, generally less than 1000 MW. This requires initial extracellular processes to release small molecules. Low molecular weight organic material such as simple sugars, amino acids and urea may be hydrolysed both intra- or extracellularly (Burns 1977).

The study of soil enzyme activity was first reported in 1899, so has been in progress for one hundred years, in a large variety of soil types (Burns 1982). Now

more than 50 enzymes are known to be active in soil; different enzymes correlate with different soil factors such as the microflora, organic carbon, pH, plant roots, soil fauna, lichens and soil surface algae (Burns 1977).

The most frequently studied soil enzymes are oxidoreductases and hydrolases, but the range of soil enzymes covers oxidoreductases (including dehydrogenases), transferases (including dextranase and aminotransferase), hydrolases (including lipases, phosphatases, sulphatase, amylase, cellulase, α - and β -glucosidase, proteinases, urease) and lyases (including glutamate decarboxylase and aromatic amino acid decarboxylases) (Ladd 1978).

Ladd (1978) categorized soil enzymes according to their location in the soil matrix:

- 1 Extracellular enzymes free in soil solution or bound to inorganic or organic soil constituents
- 2 Enzymes present in particulate soil debris
- 3 Enzymes present in dead cells or in non-proliferating cells

and Burns (1982), further divided them into ten categories:

- 1 Within the cytoplasm of proliferating microbial, plant and animal cells, of which many are associated with essential metabolic processes such as glycolysis; many cannot function extracellularly because they depend on co-factors, cell physiology, etc.
- 2 Restricted to the periplasm of proliferating Gram-negative bacteria. If these enzymes leak from the cells (through cell damage or increased membrane permeability) they join a different category.
- 3 Attached to the outer surface of viable cells, with active sites in the surrounding environment (medium), and so described as extracellular; many

bacterial polysaccharases are in this category, as are enzymes in the mucigel of plant roots and micro-organisms.

4 Secreted by living cells as part of cell growth and division, into the aqueous soil phase; mostly of 20,000-40,000 molecular weight and largely produced by Gram-positive bacteria, fungi and plant roots. The extracellular location of these enzymes is usually inferred from their function, e.g. as hydrolases for high molecular weight substrates

5 Within non-proliferating (but live) cells, for example fungal spores, protozoan cysts, plant seeds and bacterial endospores.

6 Attached to entire dead cells and to cell debris. Enzymes in this category are rapidly broken down by proteases, but there is a constant turnover in soil of such cells and cell particles, and hence always some level of enzyme activity due to them.

7 Leaking from living cells or released from lysed cells, and surviving in the aqueous soil phase for a short period. Hydrolases such as urease and β -glucosidase fall into this category.

8 Temporarily associated in soluble or insoluble enzyme-substrate complexes.

9 Adsorbed to clay minerals (externally or internally), where enzymes may or may not retain their catalytic capacity (Stotzky & Burns 1982).

10 Associated with humic colloids by adsorption, entrapment, or co-polymerization as humic matter is formed. Enzymes in this category tend to have a longer half-life than those in the aqueous phase, and therefore (with those in

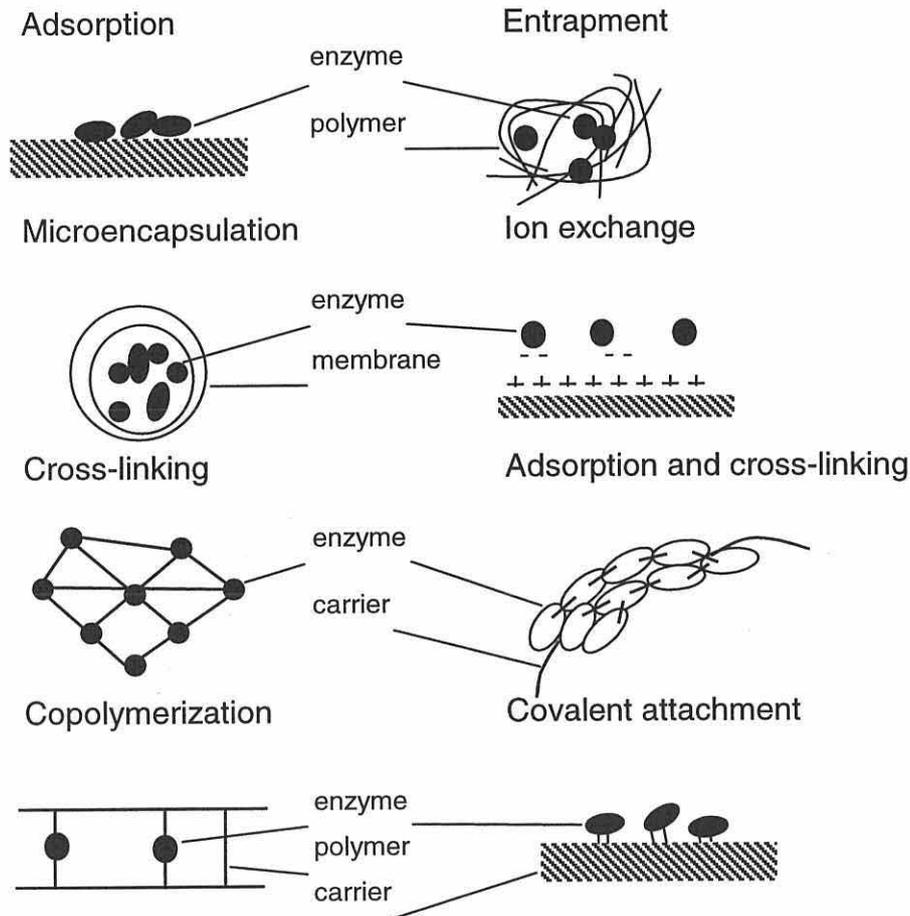
category 9), may remain in soils, in sediments and on suspended particulates in water for long periods.

Since free enzymes in a soil matrix would provide a ready source of degradable protein to microorganisms, yet soil retains enzymatic activity which is not associated with microbial metabolism (Skujinš 1976), it is clear that there are protective mechanisms which allow enzymes to survive in soil.

Weetall (1975) described the dominant mechanisms of enzyme immobilization and stabilization as microencapsulation, cross-linking, copolymer formation, adsorption, entrapment, ion-exchange, and covalent attachment (Figure 1.1).

Figure 1.1

Representation of methods of immobilizing enzymes (after Weetall 1975).



Ladd & Butler (1975) suggested hydrogen, ionic or covalent bonding of enzymes to soil humus, although the extent of each is difficult to determine, and Albert & Harter (1973) suggested that binding of proteins to clay minerals may be by a cation-exchange adsorption mechanism. Clay minerals are composed of silicon oxide and aluminium oxide or hydroxide sheets which carry an overall negative charge that adsorbs enzymes on and within the lattices; this may actually enhance enzyme function in some cases (Burns 1977). No clear hypothesis for enzyme stabilization has yet been proposed, but it seems extremely likely that clay and humus are involved together (McLaren *et al* 1975, Burns *et al* 1972).

The most universally supported mechanism of enzyme protection in soil is that of immobilization by adsorption to humic or colloidal material in the soil (Burns *et al* 1972, Ladd & Butler 1975, McLaren *et al* 1975, Weetall 1975, Burns 1982, Frankenberger & Johanson 1982). Immobilized enzymes have an important role in substrate degradation, and will survive or recover from treatments which inhibit or eliminate indigenous microflora (Burns 1983). Pettit *et al* (1977) noted that "remarkable stability is a general feature of soil enzymes".

Adsorption of enzymes in soil is generally enhanced with increasing clay and organic matter content because of the greater surface area and number of active sites of these materials compared to silt and sand (Burns 1983, Frankenberger & Johanson 1982). Many studies have shown that soil enzyme activities are significantly correlated with organic matter content, indicating that organic soil constituents protect enzymes from microbial degradation and other processes (Dick & Tabatabai 1993).

The enzymes immobilized on or in soil clays and humates are predominantly hydrolases (Burns 1983); many also function within the microbial cell (Glenn 1976). In some instances there is a direct relationship between the activity of an enzyme and the microbial population, indicating that intracellular microbial activity is a dominant contributor; but in other cases, large fluctuations in microbial numbers are not reflected in enzyme activities (Burns 1977). Exoenzyme activity can account for 50-90% of total activity (Burns 1977).

The enzyme activity of a soil depends on the abiotic factor, which includes extracellular enzymes, enzymes in dead cells, those associated with dead cell fragments and activity associated with living microbial cells (Skujinš 1976). The total activity of any particular enzyme in soil may be related to the available "active" or "biological" space rather than the total physical space (Nannipieri *et al* 1983), and total soil enzyme activity is composed of bound exoenzyme

(extracellular), free exoenzyme (extracellular, stabilized by colloid association), and intracellular enzyme (Burns 1977). Enzymes such as urease, arylsulphatase and phosphatases have been immobilized either after release from leaking or dead cells, or possibly by incorporation into resistant cell constituents before release. Other hydrolases were possibly or definitely extracellular before immobilization.

c. Extracellular enzymes

Burns (1983) suggests that “to warrant being called extracellular, an enzyme must be actively secreted by viable microorganisms, and not arise from damaged or lysed cells”, and concludes that “extracellular” be used to describe only enzymes which are external to the cell wall and in contact with the surrounding medium. Bacterial extracellular proteins are mostly in the range 20,000-40,000 molecular weight, but may be as low as 12,000 or as high as 500,000 (Glenn 1976).

Cohen (1980) identified four types of mechanism by which micro-organisms release extracellular enzymes, based on constitutive and inducible synthesis and secretion:

- 1 Constitutive synthesis/induced or de-repressed secretion
- 2 Constitutive synthesis/constitutive secretion
- 3 Induced synthesis/constitutive secretion
- 4 De-repressed synthesis/constitutive secretion

Induction and repression are related to concentration, period of exposure and temperature, pH and oxygen. Inducers may become repressors under different environmental conditions or at different concentrations (Cohen 1980).

The location of soil enzymes influences their persistence in a soil; those associated with the outer membranes of live cells (e.g. for extracellular digestion), or released from dead cells or cell debris may be rapidly, but continuously, turned over (Ladd 1978), whereas enzymes stabilized by association with clay or organic polymer complexes may be present for much longer.

The interaction of extracellular enzymes with humic substances has been widely investigated in terrestrial soil systems (Ladd & Butler 1970, Sinsabaugh & Linkins 1987), and to a lesser extent in aquatic systems (Stewart & Wetzel 1981). Polyphenolics and humics can be a major component of the dissolved organic acids of fresh waters, so can potentially interact with many enzymes (Dudley & Churchill 1995). However humic acid-enzyme binding can result in precipitation and immobilization, and humic-enzyme complexes partially or completely inactivate extracellular enzymes (Wetzel 1993). The association of humic acids and proteins is believed to be a surface phenomenon, in particular to general areas of the outer surface of the protein rather than to binding sites (Spencer *et al* 1988). Polyphenol binding to proteins is reversible because it is due to ionic interactions, hydrogen bonding or hydrophobic interactions. The importance of each of these may vary depending on the protein involved (Dudley & Churchill 1995). Formation of humic-serine protease complexes can increase the half-life of an enzyme's activity in dilute solutions, and such an increase in half-life could be an adaptive mechanism resulting in energy conservation due to decreased requirement for protein synthesis (Dudley & Churchill 1995).

Extracellular enzymes are subject to the same types of inhibition, repression/derepression and activation/deactivation as cytoplasmic enzymes (Burns 1983). As well as end-product inhibition, some exoenzymes are subject to catabolite repression, for example α -amylase is repressed by glucose in several species, and cellulase by glucose in *Pseudomonas* spp., and some exoenzymes are also inducible (Burns 1983). Whilst some enzymes are only able to function outside the cell, either because their substrates are insoluble or because they only assume their active configuration after export from the cell (Burns 1983), several enzymes found in soil originated as functional cytoplasmic enzymes, for example β -glucosidase.

1.8 Aims of the research

Extracellular enzymes are an essential component in the breakdown of all particulate organic matter, because they enable its transformation to molecules more suitable for cellular metabolism. Extracellular enzymes from different micro-organisms act synergistically to result in the total conversion of complex organic compounds, such as cellulose, which could not be achieved by one species alone. The aim of studying the enzyme activity of constructed wetlands' substrate is to discover more about the nutrient cycles which occur there; if there are similarities between these wetlands and natural wetlands and soils, then extrapolating from natural soils may provide valuable information about how to modify nutrient transformations in constructed wetlands. Similarly, studies of gas emissions from these artificial, or novel, soils could indicate how closely constructed wetlands resemble natural wetlands with respect to their nutrient cycles, and lead to better management which minimizes release of radiatively active gases.

The specific aims of the research were to investigate:

- 1 What levels of enzyme activity are found in treatment wetlands, and how these compare with those of natural wetlands
- 2 How long extracellular enzymes survive in constructed wetlands
- 3 Whether extracellular enzyme activity can be modified or influenced to improve wastewater treatment

To fulfill these aims, this thesis is constructed as follows: 1) a twelve month field survey of constructed wetlands, encompassing a variety of treatment functions and substrate types, was carried out to establish the range and seasonal variation in enzyme activity and water chemistry factors; 2) laboratory experiments were used to clarify some of the relationships discovered during the field work; 3) laboratory experiments were carried out to further elucidate the effects of pH, temperature, redox potential, oxygenation and phenolic concentration on soil enzyme activity,

and a larger experiment investigating the influence of carbon supply on soil extracellular enzyme activity was conducted; 4) enzyme addition experiments were used to investigate the longevity of soil enzyme activity in both static and continuous-flow experiments; 5) regression modeling was used as a means to predict potential enzyme activity.

To investigate nutrient cycling in constructed wetlands, the activity of six hydrolases and one oxidase were studied in soil from constructed wetlands, using fluorescent substrates. These enzymes were chosen to indicate the relative enzyme activities of the carbon cycle (cellobiohydrolase, β -glucosidase, xylosidase and phenol oxidase for aromatic biodegradation), nitrogen cycle (N-acetylglucosaminase), phosphorus cycle (phosphatase) and sulphur cycle (sulphatase). Cellobiohydrolase, β -glucosidase and xylosidase are important enzymes in different stages of cellulose and hemicellulose breakdown, with β -glucosidase representing the final transformation of glucose oligomers to microbially available substrates. Similarly N-acetylglucosaminase represents the final transformation of chitin polymers to monomers small enough for microbial uptake; this enzyme is also significant in the carbon cycle, since its substrate is carbonaceous as well as nitrogenous. Phosphatase and sulphatase both represent enzymic transformations which release inorganic microbial nutrients from organic matter; however the substrates of these enzymes frequently require release from more complex forms of organic matter before phosphatase and sulphatase can mineralize them. Phenol oxidase activity represents the breakdown of lignin-rich organic matter and also therefore indicates the rate of humic matter formation in soil.

Chapter II

Methodology and enzyme kinetics

Methodology development and enzyme kinetics

2.1 Assay of extracellular enzymes

The study of extracellular enzymes is limited by the availability of artificial substrates, if one wishes to avoid the problems associated with using natural substrates (quantitative analysis of enzyme reaction product or substrate, interference from background material in the sample). Measurement of enzyme activity has classically been by measuring the disappearance of a substrate, or the appearance of an enzyme product (Tabatabai 1982); brief incubations eliminate contributions from turnover in growing cells, while longer assays may require metabolic inhibitors (Burns 1977), and can be hampered by assimilation of enzyme reaction products by soil microorganisms (Ueno *et al* 1991).

In this thesis hydrolase activities are assayed using methylumbelliferyl (MUF) analogue substrates, and phenol oxidase activity using L-dihydroxyphenylalanine. MUF substrates have often been used for soil and sediment enzyme assays (Hoppe 1983, Freeman *et al* 1995b, Kang & Freeman 1997) as the low level of activity in such materials requires sensitive assay methods (Freeman *et al* 1995b). Methylumbelliferyl substrates contain the same bond between the 4-methylumbelliferone molecule and, for example, the glucose molecule (in the case of 4-methylumbelliferyl β -D-glucose), as the bond in the natural substrate (in this case cellulose) (Marxsen & Witzel 1991). Fluorescent substrates such as methylumbelliferyl analogues can provide approximately one hundred times greater sensitivity than conventional *p*-nitrophenyl substrates (Ueno *et al* 1991). However methylumbelliferyl substrates cannot be used to determine *in situ* rates of polymer hydrolysis (Jones 1990) as they are dimeric, in contrast to most natural substrates which are oligomeric or polymeric. Nevertheless, they provide useful quantitative and comparative data about extracellular enzyme activity by means of sensitive, simple and rapid measurements (Marxsen & Witzel 1991).

Free methylumbelliferone (the product of enzymic cleavage of MUF substrates), but not the uncleaved substrates, is fluorescent (Hoppe 1983). Evidence for the extracellular hydrolysis of methylumbelliferyl substrates was described by Jones (1990); cultures of bacteria incubated in nutrient broth with methylumbelliferyl substrates were examined for solution and cellular fluorescence over a period of 24 h. Whilst the external solution developed increasing fluorescence, the bacterial cells never fluoresced, demonstrating that the MUF free acid product was not produced in or transported into the cells. The hydrolysis of fluorogenic substrates in the periplasmic, as opposed to cytoplasmic, space was also confirmed by Martinez & Azam (1993).

The aromatic substrate L-3,4-dihydroxyphenylalanine (L-dopa) has been used successfully to measure the activity of phenol oxidase and phenolase in the past (Pind *et al* 1994, Ostle 1994). Upon oxidation by phenol oxidase (or similar enzyme), the colourless L-dopa forms a red pigment, 2-carboxy-5,6-dihydroxyindole-5,6-quinone (abbreviated to diqc), or 2-carboxy-2,3-dihydro-6-hydroxyindole-1,5-quinonimine (a tautomer), which is stable (Mason 1948). The concentration of this pigment can be measured spectrophotometrically, and thus the rate of its production can be used to gauge the level of phenol oxidase activity.

2.2 Enzyme kinetics

Kinetic studies of enzyme activities provide substrate affinity and reaction velocity parameters, which usefully describe enzyme characteristics, and from which the turnover time of organic matter cycling can be calculated.

The kinetic parameters K_m and V_{max} of six hydrolases (cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase and xylosidase) and phenol oxidase, were determined by assaying soil from each wetland at a range of substrate concentrations, and using the resulting data to plot reaction velocity (v) against substrate concentration ($[s]$) (Figures 2.1-2.4, a-f, and Figure 2.5 a-d). The method, substrates and wetland sites are described in Chapter III.

Figure 2.1

Substrate concentration versus reaction velocity for (a) cellobiohydrolase, (b) β -glucosidase, (c) N-acetylglucosaminidase, (d) phosphatase, (e) sulphatase, (f) xylosidase in soil from the Centre for Alternative Technology wetland.

($v = \mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$, $[s] = \mu\text{mol l}^{-1}$) (Mean, $n = 5$, \pm s.e).

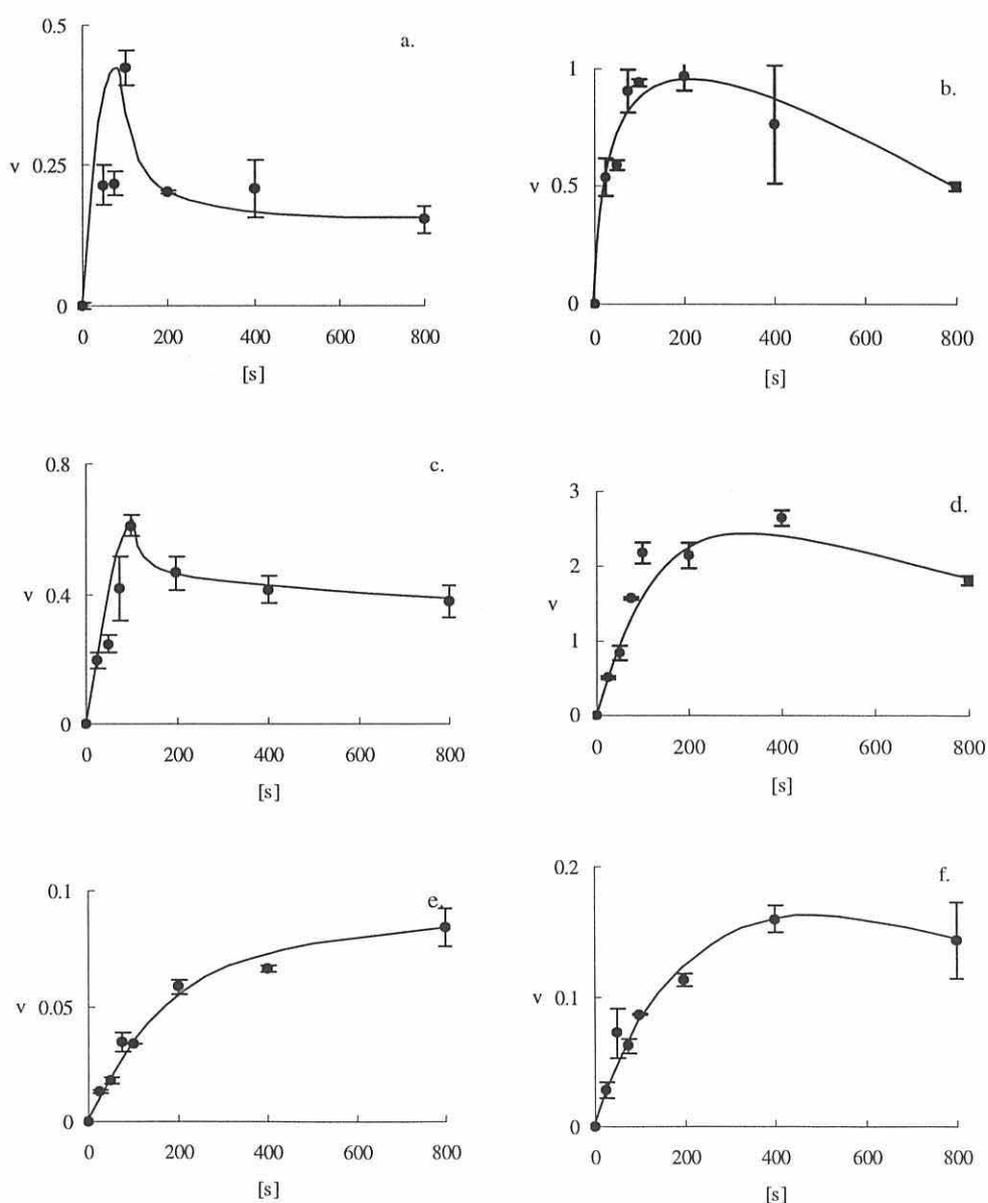


Figure 2.2

Substrate concentration versus reaction velocity for (a) cellobiohydrolase, (b) β -glucosidase, (c) N-acetylglucosaminidase, (d) phosphatase, (e) sulphatase, (f) xylosidase in soil from the Waun-y-Cwrt wetland.

($v = \mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$, $[s] = \mu\text{mol l}^{-1}$) (Mean, $n = 5$, \pm s.e.).

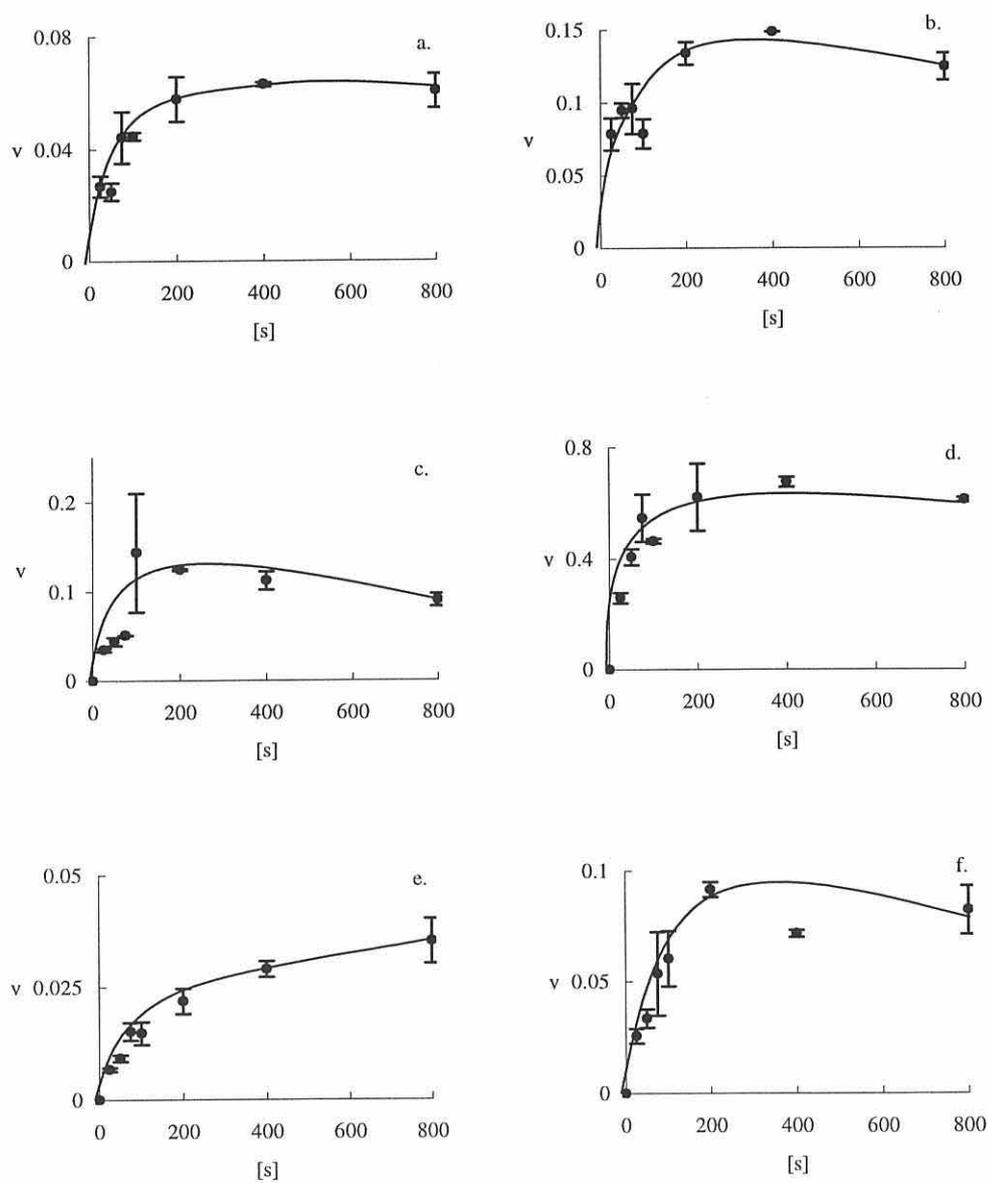


Figure 2.3

Substrate concentration versus reaction velocity for (a) cellobiohydrolase, (b) β -glucosidase, (c) N-acetylglucosaminidase, (d) phosphatase, (e) sulphatase, (f) xylosidase in soil from the Pelenna wetland.

($v = \mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$, $[S] = \mu\text{mol l}^{-1}$) (Mean, $n = 5$, \pm s.e.).

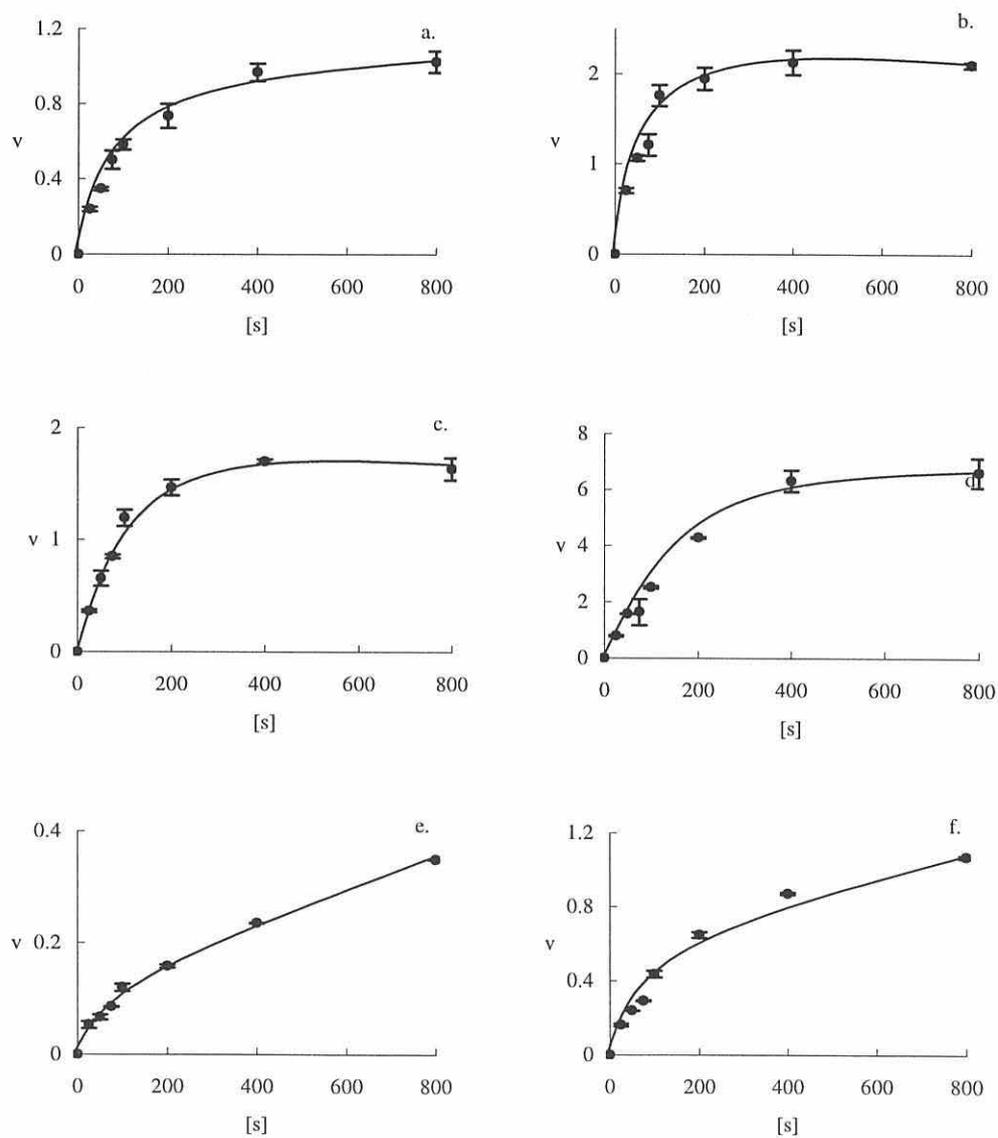


Figure 2.4

Substrate concentration versus reaction velocity for (a) cellobiohydrolase, (b) β -glucosidase, (c) N-acetylglucosaminidase, (d) phosphatase, (e) sulphatase, (f) xylosidase in soil from the Tollesbury wetland.

($v = \mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$, $[s] = \mu\text{mol l}^{-1}$) (Mean, $n = 5$, \pm s.e.).

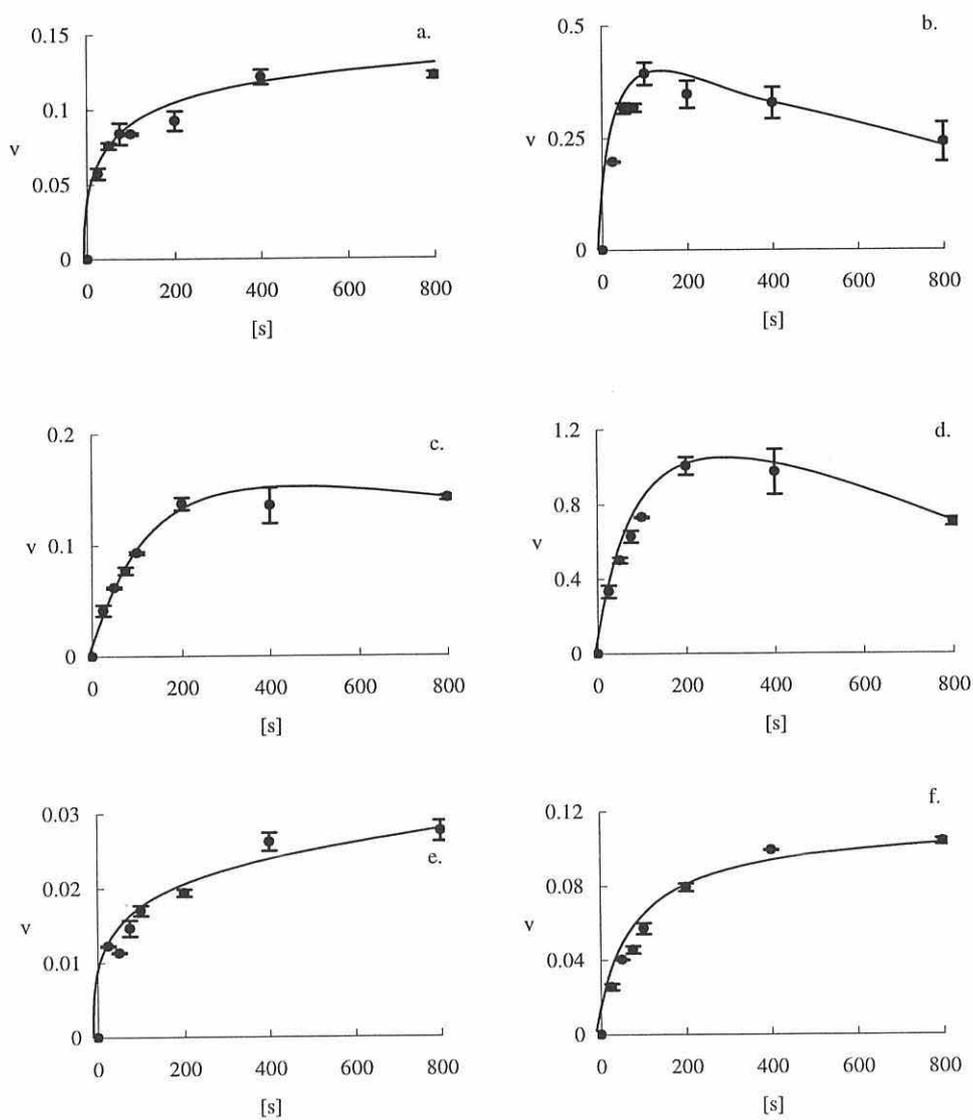
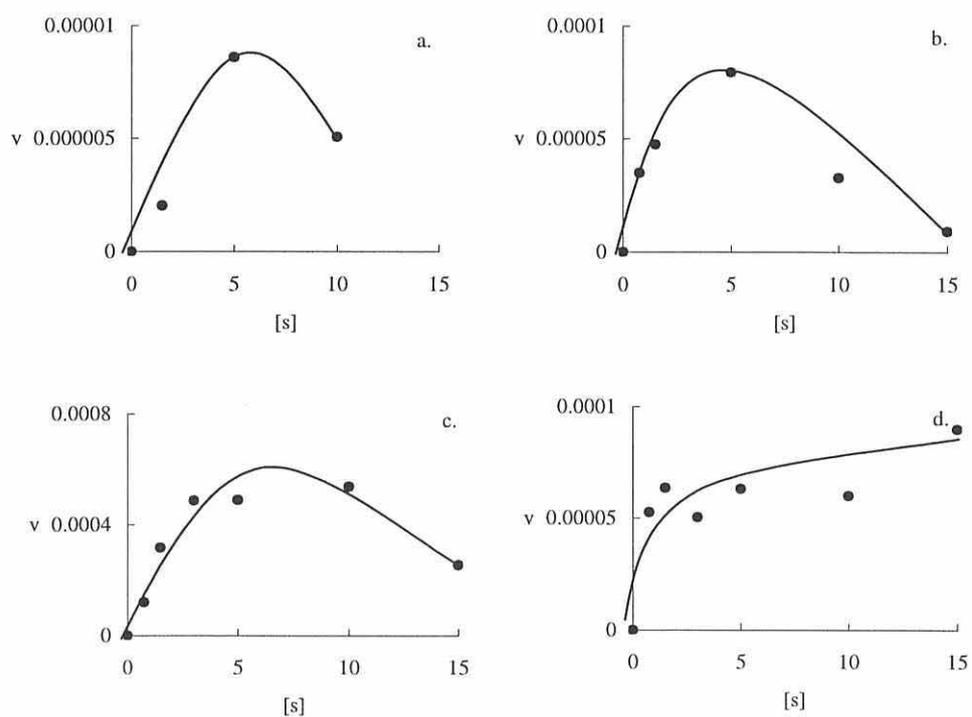


Figure 2.5

Substrate concentration versus reaction velocity for phenol oxidase in soil from (a) Centre for Alternative Technology, (b) Waun-y-Cwrt, (c) Pelenna and (d) Tollesbury wetlands.

($v = \text{mmol diqc g soil}^{-1} \text{ min}^{-1}$, $[s] = \text{mmol l}^{-1}$) (Mean, $n = 5$).



V_{\max} and K_m were determined from these data using the EnzPack programme (Version 3.1a, BIOSOFT) to produce Lineweaver-Burk, Eadie-Hoftsee and Hanes plots. These constants, and the correlation coefficient, r , for each plot for each enzyme, are given in Tables 2.1 – 2.4. For hydrolases, K_m units are $\mu\text{mol l}^{-1}$ and V_{\max} units are $\mu\text{mol MUF g soil}^{-1} \text{min}^{-1}$. For phenol oxidase, K_m units are mmol l^{-1} and V_{\max} units are $\text{mmol diqc g soil}^{-1} \text{min}^{-1}$.

Table 2.1

V_{\max} , K_m and r for Lineweaver-Burk, Eadie-Hoftsee and Hanes plots, Centre for Alternative Technology wetland.

	Lineweaver-Burk			Eadie-Hoftsee			Hanes		
	K_m	V_{\max}	r	K_m	V_{\max}	r	K_m	V_{\max}	r
Cel	77.17	0.55	0.93	29.02	0.37	0.25	166.7	0.92	0.51
β -glu	29.69	1.13	0.9	27.17	1.11	0.79	29.53	1.13	0.99
NAG	106.8	0.98	0.93	41.01	0.62	0.27	430.3	2.93	0.36
Pho	313.5	6.91	0.98	74.04	2.83	0.52	116.4	3.49	0.97
Sul	168.8	0.1	0.98	156.2	0.1	0.91	181.5	0.1	0.99
Xyl	186.1	0.26	0.97	94.16	0.18	0.82	138.3	0.21	0.98
Ph ox		n.d.			n.d.			n.d.	

Table 2.2

V_{\max} , K_m and r for Lineweaver-Burk, Eadie-Hoftsee and Hanes plots, Waun-y-Cwrt wetland.

	Lineweaver-Burk			Eadie-Hoftsee			Hanes		
	K_m	V_{\max}	r	K_m	V_{\max}	r	K_m	V_{\max}	r
Cel	36.55	0.07	0.99	38.61	0.07	0.98	45.46	0.07	1.00
β -glu	21.48	0.14	0.94	24.02	0.15	0.9	37.69	0.16	1.0
NAG	73.67	0.13	0.83		n.d.		851.2	0.91	0.1
Pho	53.05	0.83	0.99	43.94	0.77	0.94	38.85	0.74	1.0
Sul	101.4	0.03	0.97	118.7	0.04	0.94	152.2	0.04	1.0
Xyl	91.01	0.11	0.97	105.6	0.13	0.85	159.9	0.16	0.95
Ph ox	1.35	9.53	0.99	1.47	9.98	0.98	1.66	10.52	1.0

Table 2.3

V_{\max} , K_m and r for Lineweaver-Burk, Eadie-Hoftsee and Hanes plots, Pelenna wetland.

	Lineweaver-Burk			Eadie-Hoftsee			Hanes		
	K_m	V_{\max}	r	K_m	V_{\max}	r	K_m	V_{\max}	r
Cel	93.63	1.11	0.99	101.1	1.16	0.98	103.4	1.17	1.0
β -glu	65.73	2.53	0.99	63.63	2.51	0.92	62.08	2.49	1.0
NAG	160.2	2.71	1.0	122.5	2.31	0.93	113.2	2.22	0.99
Pho	254.2	8.83	0.99	255.8	9.09	0.91	257.6	9.1	0.98
Sul	97.34	0.24	0.95	156.6	0.32	0.84	293.8	0.45	0.97
Xyl	137.9	1.0	0.98	188.2	1.24	0.94	226.9	1.37	1.0
Ph ox	5.89	11.42	0.97	1.98	6.23	0.73	2.53	6.85	0.98

Table 2.4

V_{\max} , K_m and r for Lineweaver-Burk, Eadie-Hoftsee and Hanes plots, Tollesbury wetland.

	Lineweaver-Burk			Eadie-Hoftsee			Hanes		
	K_m	V_{\max}	r	K_m	V_{\max}	r	K_m	V_{\max}	r
Cel	27.07	0.12	0.98	29.08	0.12	0.94	41.53	0.13	1.0
β -glu	34.43	0.48	0.98	28.7	0.45	0.92	27.63	0.45	1.0
NAG	74.86	0.16	0.99	76.65	0.17	0.95	62.94	0.15	1.0
Pho	69.51	1.25	1.0	78.45	1.34	0.98	91.77	1.45	1.0
Sul	25.65	0.02	0.9	30.8	0.02	0.82	70.43	0.03	1.0
Xyl	82.22	0.11	0.99	94.16	0.12	0.97	99.07	0.12	1.0
Ph ox	0.57	9.08	0.99	0.65	9.38	1.0	0.59	9.20	0.99

The constants with the best fit (highest r value) in each case were used to plot Figures 2.6 a-g comparing the K_m and V_{\max} values for each enzyme between sites.

Figure 2.6

Comparison of K_m (■) and V_{max} (●) values between the four wetland field sites (Centre for Alternative Technology (C), Waun-y-Cwrt (W), Pelenna (P) and Tollesbury (T)), for cellobiohydrolase (a), β -glucosidase (b), N-acetylglucosaminidase (c) and phosphatase (d). K_m units are $\mu\text{mol l}^{-1}$ and V_{max} units are $\mu\text{mol MUF g soil}^{-1} \text{min}^{-1}$.

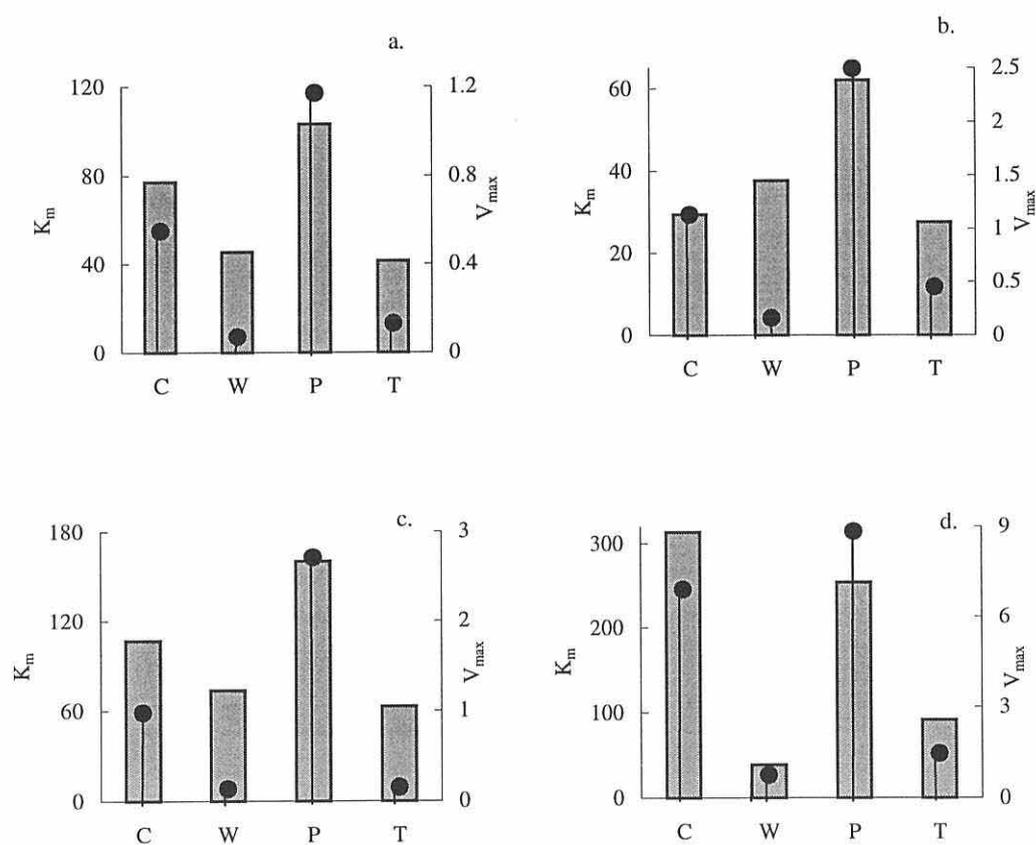
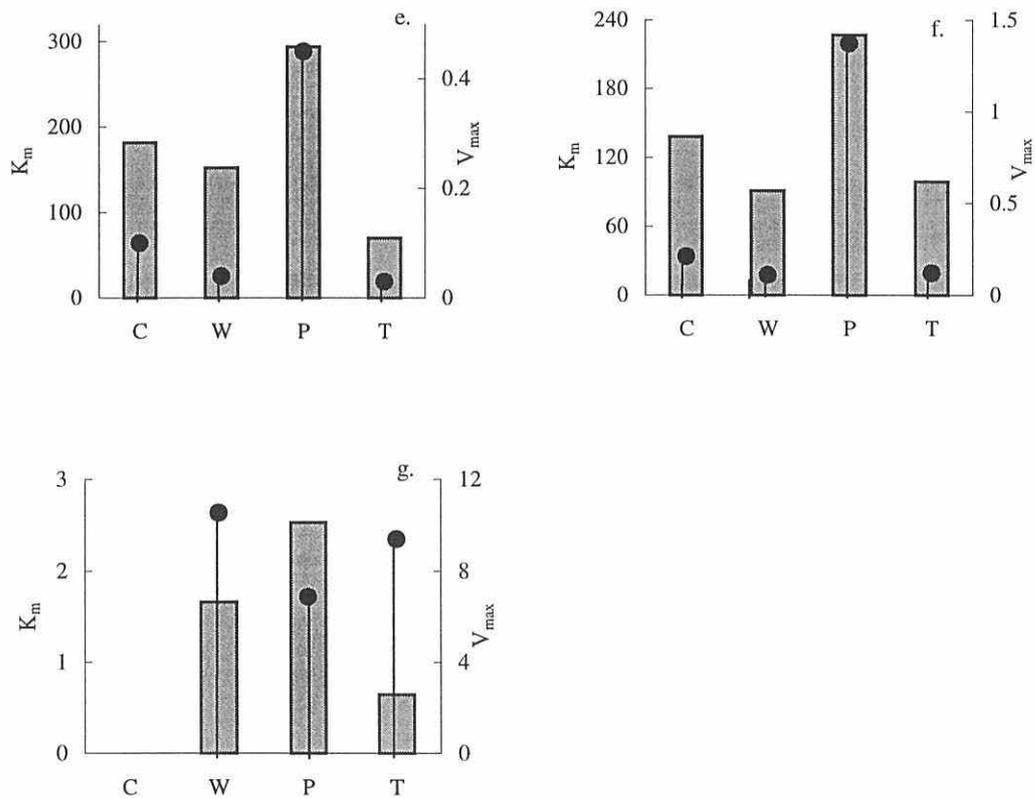


Figure 2.6 contd.

Comparison of K_m (■) and V_{max} (●) values between the four wetland field sites (Centre for Alternative Technology (C), Waun-y-Cwrt (W), Pelelenna (P) and Tollesbury (T)), for sulphatase (e), xylosidase (f) and phenol oxidase (g). For hydrolases K_m units are $\mu\text{mol l}^{-1}$ and V_{max} units are $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$. For phenol oxidase, K_m units are mmol l^{-1} and V_{max} units are $\text{mmol diqc g soil}^{-1} \text{ min}^{-1}$.



Eivazi & Tabatabai (1977) reported values of V_{\max} between 200-625 $\mu\text{g p-nitrophenol h}^{-1} \text{ g}^{-1}$ for acid phosphatase and 124-588 $\mu\text{g p-nitrophenol h}^{-1} \text{ g}^{-1}$ alkaline phosphatase; K_m values for the same enzymes were reported as 1.11-3.40 mM and 0.44-4.94 mM respectively. Phosphatase and sulphatase were reported to follow Michaelis-Menten kinetics by Pettit *et al* 1977. Eivazi & Tabatabai (1988) reported values of K_m between 1.30-2.4 mM for p-nitrophenyl- β -D-glucoside. The differences in K_m and V_{\max} values for the same enzyme in different soils agree with the work of Tabatabai & Bremner (1971), who reported K_m and V_{\max} values for arylsulphatase in a range of different soils, in which the largest were some 400 % and 4500 % greater, respectively, than the smallest. Tabatabai & Bremner (1971) suggested that similar K_m values for enzymes from different sources indicated that the enzymes involved were similar in type or origin, or that K_m was affected by (and made more uniform through) the association of enzymes with soil constituents. However no correlation was found between K_m and pH, organic carbon content, cation exchange capacity, sand or silt content of a soil (Tabatabai & Bremner 1971).

Some of the enzymes showed inhibition at high substrate concentrations, particularly at CAT (Figure 2.1), Waun-y-Cwrt (Figure 2.2) and for phenol oxidase (Figure 2.5). The highest activities were always found in enzymes from the Peledda wetland soil, followed by enzymes from CAT wetland soil.

2.3 Kinetic parameters as indicators of organic matter cycling

The concept of turnover time, T_t , of a substrate was proposed by Gocke (1977) to be the time required by heterotrophs to take up an amount of substrate equal to the naturally occurring concentration. The kinetic parameters of an enzyme are related to the availability of its natural substrate(s) (Chróst & Rai 1993, Button 1994), and the turnover time therefore gives a useful indication of the importance of particular substrates (or of the enzymes which act on them) to the heterotrophic

community, and is related to the efficiency of substrate utilization (Gocke 1977, Hoppe *et al* 1988).

For enzymes whose kinetics obey the Michaelis-Menten equation

$$V = \frac{V_{\max} [s]}{K_m + [s]}$$

the parameter T_t is defined as K_m / V_{\max} . For the substrates investigated in this chapter, T_t was determined using the intersection of the regression line with the y-axis on plots of $t/f (= [s]/V)$ versus $[s]$ (Gocke 1977), using data which did not show substrate inhibition (Table 2.5). These plots are identical to Hanes plots.

Table 2.5

Turnover time for investigated substrates at the four wetland sites (hours)

MUF substrate	CAT	Waun-y-Cwrt	Pelenna	Tollesbury
β -D-cellobiopyranoside	3.0	10	1.33	3.33
β -D-glucoside	0.42	3.33	0.42	1.33
N-acetyl- β -D-glucosaminide	2.5	15.83	0.83	9.17
Phosphate	-	1.0	0.5	1.08
Sulphate	31.67	58.33	10.83	41.7
β -D-xyloside	11.67	16.67	2.67	13.3

Shorter turnover times indicate faster cycling of organic matter, but may also be attributable to more labile substrates, while higher T_t may be associated with slower cycling and/or more recalcitrant material (Romani i Cornet 1997).

Turnover times calculated from the four wetland sites were longest at Waun-y-Cwrt (except phosphatase substrates) and shortest at Pelenna. This suggests that at the Waun-y-Cwrt wetland, organic matter takes considerably longer to be broken down than at any of the other three wetlands; similarly, the short turnover times at the Pelenna wetland suggest that organic matter is recycled much more quickly there. These observations, if substantiated experimentally, may have implications affecting the treatment processes within the wetlands, in particular processes

which immobilize metals as these frequently depend on adsorption to organic matter.

Phosphatase substrates had the shortest turnover time (mean of all sites), and sulphatase substrates the longest; these differences point towards the high lability of phosphate substrates, and the much more stable structure of sulphate compounds such as amino acids, iron-sulphur proteins and lipoic acid (Paul & Clark 1989). Lou & Warman (1992) reported that ester sulphate groups bound to external surfaces of soil humic polymers may be easily accessible to sulphatase, whereas those on inner surfaces of organics are shielded from hydrolysis by size exclusion, and so are more stable. The turnover times were on the whole a factor of ten shorter than those calculated by Romani & Cornet (1997) for extracellular enzymes in Mediterranean streams. This may be because the substrates available in the wetland soils are considerably more labile than those available in the streams.

The increasing T_t of MUF-glucoside, MUF-cellobiopyranoside and MUF-xyloside, substrates representative of carbon substrates of increasing molecular weight, was evidence for the relative recalcitrance of each substrate. Interestingly MUF-N-acetylglucosaminide had a mean T_t similar to that of MUF-xyloside, suggesting that the natural substrates these analogues represent are similarly resistant (or susceptible) to enzymic attack, and provide carbon and nitrogen to the microbial biomass at similar rates. This may be expected however, as otherwise one or other substrate would accumulate faster in the natural environment.

Substrates with faster turnover times (conferred by higher enzyme affinity and lower maximal velocity) will naturally be degraded more quickly than those with slower turnover times. For maximal microbial activity therefore, substrates which represent significant pools of nutrients would ideally have fast turnover times which allowed nutrients to be supplied at rates which maintained an ideal carbon :

nitrogen : phosphorus : sulphur ratio. Clearly this is not always the case, and microbial growth becomes limited by nutrient supply. The ecological significance of enzyme kinetics, and in particular of turnover time, therefore lies in the information regarding rates of nutrient availability which this approach can provide. If the naturally occurring concentration of a substrate can be determined in addition to its turnover time, then the flux rate of the substrate in a particular ecosystem can be calculated (Gocke 1977). Rates for different substrates will vary, as decomposition is not a uniform process, revealing the importance of extracellular enzyme activity in partitioning organic matter into the heterotrophic food chain (Hoppe *et al* 1988).

Conclusions

Some of the soil enzymes investigated showed substrate inhibition, and so concentrations for subsequent assays in this thesis were chosen to obtain maximum activity. The Pelenna wetland soil consistently had the highest enzyme activities, and soil from Waun-y-Cwrt the lowest; these differences may result from the different physical and chemical conditions in each wetland, which may favour high or low (respectively) enzyme production and/or retention. Turnover times calculated from the four wetland sites were longest in soil from the Waun-y-Cwrt wetland (except phosphatase) and shortest in soil from the Pelenna wetland. Thus it would seem that soil from the Pelenna wetland has high enzyme activity which results in fast turnover of organic matter, and that the opposite situation pertains at the Waun-y-Cwrt wetland (low activity, slow turnover). Phosphatase substrates had the shortest turnover time (mean of all sites), and sulphatase substrates the longest. The increasing turnover times of β -glucosidase, cellobiohydrolase and xylosidase substrates, which represent carbon molecules of increasing molecular weight, was supported by current views concerning the relative recalcitrance of each substrate, and was evidence for the validity of the kinetic approach to enzyme study.

Chapter III

Comparison of biogeochemical cycling in constructed wetlands

Comparison of biogeochemical cycling in constructed wetlands

3.1 Introduction

The minutiae of nutrient cycling in constructed wetlands has largely been ignored in the past, as such wetlands have been considered “black box” systems in which actual processes were unimportant as long as the end result was clean water. The routes by which organic pollution may be mineralized to nutrients and gases are the same in constructed wetlands as in dry or waterlogged soil or in sediment; proteins must still be deaminated then broken down to release carbon dioxide, water and nitrogen, lipids still undergo hydrolysis to fatty acids (Hammer & Knight 1994). However in constructed (and other) wetlands, the balance between different transformations is different to that in soil, and the effects of waterlogging on soil enzyme activity are very poorly understood (McLatchey & Reddy 1998).

The microbial degradation of macromolecular organic matter is begun by extracellular enzymatic hydrolysis (Marxsen & Witzel 1991), and so the study of soil enzymes is useful to describe and make predictions about an ecosystem's function, quality and subsystem interactions (Dick & Tabatabai 1993).

Intracellular transformation and utilization of organic matter follows on from extracellular degradation, and results in the emission (and sometimes also consumption) of carbonaceous and nitrogenous gaseous products; therefore observation of soil gas fluxes provides information about soil microbial activity and metabolism.

Seasonal changes in soil enzyme activity and gas fluxes may be expected in constructed wetlands, as they are seen in natural wetlands. Since changes in enzyme activity and gas flux represent changes in nutrient cycling within a soil, variations in treatment efficiency of constructed wetland might be found over the course of a year. In addition, changes which are due to seasonal changes in

temperature may indicate the potential for using constructed wetlands in different climates.

To better understand how constructed wetlands deal with pollution, and to develop hypotheses about the manipulation of constructed wetlands to increase treatment efficiency, the nutrient transformations which occur in their soil must be investigated and clarified; the first step in this is to investigate the relationships which exist between extracellular enzyme activity and environmental conditions such as temperature, pH, nutrient availability and supply of electron acceptors (McLatchey & Reddy 1998). The balance of processes occurring in wetlands is likely to vary according to the composition of the wastewater being treated, and may also be influenced by the construction and operation of each wetland. It is therefore important to consider a range of wetlands, built for different purposes.

The enzymes investigated in the study were chosen to give indications of activity in the extracellular carbon cycle (cellobiohydrolase, β -glucosidase, xylosidase), phosphorus cycle (phosphatase), sulphur cycle (sulphatase) and nitrogen cycle (N-acetyl-glucosaminase), and of aromatic degradation (phenol oxidase). Similarly, the gases nitrous oxide, methane and carbon dioxide were analysed to indicate the functioning of the nitrification/denitrification stage of the nitrogen cycle, anaerobic metabolism and aerobic metabolism respectively.

3.2 Aims and hypotheses

The aims of this field study of constructed wetlands were as follows

- to quantitatively investigate seasonal changes in soil enzyme activity in constructed wetlands which have been built for the treatment of different wastewaters
- to compare the level of this activity with that occurring in natural wetlands
- to investigate soil enzyme activity in relation to soil physico-chemical factors

- to quantitatively investigate seasonal changes in soil gas fluxes from constructed wetlands
- to compare these gas fluxes with those from natural wetlands
- to investigate soil water chemistry in relation to enzyme activity and gas fluxes.

These aims were built around the following hypotheses, which in themselves represent fundamental questions about the nutrient cycling processes in constructed wetlands:

- 1 Constructed wetlands will show similar levels of nutrient cycling (indicated by enzyme activity and gas fluxes) as natural wetlands, because they are designed to emulate the same ecological processes
- 2 Soil enzyme activity will influence water chemistry and gas fluxes
- 3 Soil enzyme activity and gas fluxes will be related to (and explained by) soil physico-chemical factors.

To investigate these hypotheses, a twelve month study of four constructed wetlands to gather data on the seasonal changes in enzyme activity levels, gas emissions and soil water chemistry was completed; this also allowed identification of factors which regulate and influence enzyme activities in constructed wetlands. All samples were collected on a monthly basis to obtain sufficient data over the course of the year. Enzyme activities and water chemistry parameters were measured in the laboratory after transporting soil samples from the field sites; gas fluxes were measured *in situ* to avoid inaccuracies due to soil disturbance.

3.3 Constructed wetland study sites

In order to be able to compare the enzyme activity, gas fluxes and water chemistry of treatment wetlands constructed for different purposes, yet still be able to obtain regular samples from the wetlands, site selection was governed largely by the

availability of constructed wetlands in Wales. Selecting constructed wetlands on the basis of their treatment function meant that a variety of construction types was also selected, including sand and bark substrates, a range of ages, varying sizes and vegetation types, and different hydrologic regimes. Three of the wetlands (Centre for Alternative Technology, Waun-y-Cwrt and Pelenna sites) were small beds used for the treatment of contaminated water of various origins, and the fourth (Tollesbury) was an area of newly created salt marsh used as a “managed retreat” coastal flood defence. This selection of sites encompassed organic (Centre for Alternative Technology, Waun-y-Cwrt) and inorganic (Waun-y-Cwrt, Pelenna) wastewaters, organic (Pelenna) and inorganic (Centre for Alternative Technology, Waun-y-Cwrt) substrate types, vertical (Pelenna) and horizontal (Centre for Alternative Technology, Waun-y-Cwrt) flow beds, and new (Tollesbury, Pelenna) as well as well-established (Centre for Alternative Technology) wetlands.

a. Centre for Alternative Technology

The Centre for Alternative Technology (CAT) near Machynlleth, mid Wales, has a relatively small (approximately 50 m²) constructed wetland. This consists of four *Phragmites australis* vertical flow cells in an upper bed and a single large horizontal flow bed below planted with a mixture of wetland plants; outflowing water descends an aeration cascade, before entering ditches planted with willow saplings. The beds are filled with pebbles at the bottom, graduating to coarse sand at the top which has accumulated a considerable amount of organic matter. Outflowing water discharges to a tributary of the Afon Dulas. The system was constructed in 1991, and receives domestic sewage from the Centre's residential buildings. Solid material is first removed from the raw sewage and composted, before the liquid proceeds to the treatment bed where the four upper cells are used in pairs, alternating on a weekly basis. Samples were taken from fixed locations in three of the cells.

b. Waun-y-Cwrt landfill site, Nantmel

Llysdinam Field Centre, near Llandridnod Wells, Wales (part of the University of Wales, Cardiff), designed and constructed a small (40 m²) wetland at the Waun-y-Cwrt landfill site, Nantmel. Planted with *Phragmites australis* in winter 1993/94, the wetland receives leachate from the adjacent landfill site which was closed and capped in 1990 after 30 years' use. Both parts of the *Phragmites* bed are filled with coarse sand. Leachate drains from the Waun-y-Cwrt landfill at 4,000 l day⁻¹, and is pumped to a holding lagoon before entering the wetland; previously the leachate was transported to the local sewage treatment works almost every day (Kowalik *et al* 1996). There are two units in the wetland, the leachate going first to one and then the other, and subsequently being pumped to one of two willow beds. Occasionally one willow bed receives the raw leachate, operating in parallel with the reed bed.

c. Whitworth No. 1 wetland, Pelelenna catchment

Whitworth No. 1 treatment wetland is the first part of a five-stage construction project initiated and led by West Glamorgan County Council (with the Environment Agency, the Welsh Development Agency and the EC LIFE programme), to improve the water quality and appearance of rivers receiving abandoned mine discharges. This problem is considerable in South Wales, where workings at most coal mines have been abandoned since the 1960s. The Pelelenna valley discharges are typical of South Wales.

The Whitworth No. 1 wetland at the Tonmawr mine was constructed in 1995 to treat a discharge of 23 x 10⁴ litres per day, with an iron load of 6.3 kg day⁻¹. It consists of four wetland cells (total area 900 m²), each with a different combination of substrate and macrophyte; the cell included in this study has a bark substrate and is planted with *Juncus effusus*. Treated water discharges to the Nant Gwenffrwd.

d. Tollesbury constructed salt marsh

The Eastern seaboard of England has historically been prone to tidal flooding during storm events combined with Spring high tides; consequently in the last fifty years it has been extensively defended against inundation by so-called 'hard' sea defences such as sea walls, which also sometimes have the benefit of providing reclaimed ground on the landward side. At Tollesbury marshes in Essex (southern East Anglia), part of this reclaimed land, used for agriculture, was flooded in August 1995 by breaching the sea wall in an attempt to reinstate 'soft' sea defence, as salt marsh. The 21 hectares of previously agricultural land now constitutes a constructed wetland, and its recolonization by species such as *Salicornia*, which dominate neighbouring areas of mature, established salt marsh (Boorman, unpubl.), was just beginning at the time of this study. The study site has a spring tidal range of 4.3 m, and has a silty clay sediment (Boorman *et al* 1994).

The physical and chemical characteristics of the four constructed wetlands are compared in Table 3.1.

Table 3.1
Characteristics of the four wetland sites

Name	Wastewater type	Vegetation	Annual average pH	Organic matter*	Bulk density (g cm ⁻³)
Centre for Alternative Technology	Domestic sewage	<i>Phragmites australis</i>	5.33 (n = 23)	5.386	1.014
Waun-y-Cwrt	Landfill leachate	<i>Phragmites australis</i>	6.6 (n = 10)	0.849	1.077
Whitworth no. 1, Pelenna	Acid mine drainage	<i>Juncus effusus</i>	6.08 (n = 11)	47.812	0.345
Tollesbury salt marsh	Tidal inundation	Colonizing <i>Spartina</i>	7.43 (n = 9)	6.223	1.097

* Determined after ignition at 550 °C for 24 hours, n = 5, given as % by weight

3.4 Methods

a. Field collection of soil, water and gas samples

A wetland sampling method already established by the Bangor University biogeochemistry lab was adopted and followed, with some minor alterations necessary to cope with the soil types and water table at some sites.

At each site five replicate samples, each of three parts, viz. 10 cm³ wetland surface soil, 10 ml subsurface water collected with a soil solution suction sampler (Freeman *et al* 1993), and 10 ml of evolved gas collected using the closed chamber technique of Freeman *et al* (1994), were collected each month. At all sites water samples were unobtainable by suction for at least some part of the year, in which case they were obtained by the centrifugation method of Reynolds (1984). Air temperature at approximately 1 m above ground and soil temperature at 10 cm depth adjacent to each of the five soil samples, background gas

concentrations and a note of prevailing weather conditions were also collected. pH was measured in the laboratory by making a soil solution with distilled water.

b. Laboratory analysis of samples

Fluorimetric determination of hydrolase activity

The activities of six hydrolytic enzymes (β -D-cellobiopyranosidase, β -D-glucosidase, N-acetyl- β -D-glucosaminidase, phosphatase, sulphatase and β -D-xylosidase) were determined in each of the five soil samples from each site each month, using fluorogenic methylumbelliferyl (MUF)-substrates and the method of Freeman *et al* (1995b). MUF substrates were prepared by first dissolving in Cellosolve (ethylene glycol monomethyl ether) organic solvent, using an ultrasonic bath, then topping up with deionized water.

For each of the six enzyme assays, 1 cm³ of soil from each sample from each site was prepared, and equilibrated to field temperature (average for the four sites each month) in an incubator. Seven millilitres of MUF substrate solution (400 $\mu\text{mol l}^{-1}$, except MUF-phosphate 200 $\mu\text{mol l}^{-1}$; also at field temperature) were added to each soil portion and thoroughly mixed with the soil by hand. The resulting slurries were incubated at field temperature for 60 minutes (45 minutes for phosphatase), and mixed by hand every 15 minutes. 1.5 ml of slurry from each sample was pipetted into a separate micro-centrifuge vial, and the enzyme/substrate reaction terminated at exactly 60 minutes by centrifuging at 10,000 g for 5 minutes. 0.5 ml of supernatant from each vial was mixed with 2.5 ml of deionized water in a cuvette, and the fluorescence measured at 330 nm excitation, 450 nm emission (slit width 2.5 nm) using a Perkin Elmer LS50 fluorimeter.

The dry mass of one cubic centimetre of soil from each sample from each site was measured each month after placing samples in a muffle furnace for 24 hrs, to calculate the rate of enzyme activity as $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$.

Spectrophotometric determination of phenol oxidase activity

The method of phenol oxidase activity determination was adapted from that of Pind *et al* (1994), in which the model substrate L-dihydroxy phenylalanine (L-dopa) is used as a substrate for phenol oxidase. In the presence of phenol oxidase enzymes, L-dopa is rapidly converted to the red-coloured product 2-carboxy-2,3-dihydroindole-5,6-quinone (diqc) (Mason 1948), the absorbance of which can be measured at 460 nm. An initial series of incubations were made to determine the optimum interval for product measurement; diqc production increased linearly until 5 minutes' incubation, and so incubation times of 1 and 3 minutes were used.

For each replicate required (three replicates per sample, two temporally separated measurements per replicate; total six replicates per sample), 1 cm³ of peat was mixed with 5 ml of distilled water and the slurries equilibrated to field temperature (determined as above each month) in the incubator. From each slurry, six 0.75 ml aliquots were pipetted into micro-centrifuge vials; 0.75 ml of 5 mM L-dopa solution (at field temperature) was added to three vials for three minutes' incubation, and after two minutes 0.75 ml of 5 mM L-dopa added to the remaining vials for one minute's incubation. The reaction was terminated at exactly three minutes by centrifuging at 10,000 g for 5 minutes.

Immediately, 1 ml of supernatant from each vial was pipetted into a spectrophotometer cuvette, and the absorbance measured at 460 nm with the spectrophotometer. The same cuvette was used for all samples. The amount of product released was calculated using Beer's Law:

$$A = \Sigma cl$$

A = difference in absorbance after 1 and 3 minutes' incubation

Σ = molar absorptivity constant for diqc (3.7×10^4).

c = concentration (mol l⁻¹)

l = pathlength (1 cm)

Carbon and ion content of soil water

Dissolved organic and dissolved inorganic carbon were quantified using a Shimadzu TOC-500 Total Organic Carbon Analyser. Sulphate-S, nitrate-N, ammonium-N and phosphate-P in soil solutions from the wetland samples were analysed using Skalar Auto Analyser continuous segmented flow colorimetry.

Sulphate-S was measured using the methylthymol-blue method, in which sulphate ions react to form barium sulphate and the released methylthymol-blue (the concentration of which is proportional to the concentration of sulphate) is measured at 460 nm. Nitrate-N was measured using the ammonium chloride method; nitrite is reduced to nitrate which reacts with a colour-reagent to form a complex which is measured at 540 nm, being proportional to the nitrate concentration. Ammonium-N was measured using the sodium salicylate/sodium dichloroisocyanurate method, measured at 660 nm. Phosphate-P was measured using the ammonium-molybdate method, in which ortho-phosphate reacts to form phosphomolybdic acid; this is then reduced to a blue complex which is measured at 880 nm and is proportional to the concentration of the phosphate. Each method was calibrated using commercial ion standards to produce a calibration curve, and intermittent random standards during each sample run. Data validity was checked with an independent quality control standard on each run.

Phenolic content of soil water samples

Phenolic content of the soil waters was determined using Folin-Ciocalteu phenol reagent, sodium carbonate (200 mg l^{-1}) and water sample in the ratios recommended by Box (1983) of 10 : 1.5 : 0.5 respectively. The absorbance of the blue product formed was measured at 750 nm after 90 minutes. A standard curve was produced using phenol standards in the range 0 to 10 mg l^{-1} to allow quantification of measured absorbances.

Composition of evolved gases

Gas samples were analysed using an Ai Cambridge model 92 gas chromatograph with nitrogen as the carrier gas. Nitrous oxide was analysed using an electron capture detector, at a flow rate of 70 ml min⁻¹, and carbon dioxide and methane using a flame ionization detector at a flow rate of 13 ml min⁻¹. Background gas concentrations were subtracted from the samples to estimate the flux from each wetland.

c. Statistical analysis of results

Data describing soil enzyme activity, soil gas fluxes, soil water chemistry (sulphate-S, nitrate-N, ammonium-N, phosphate-P, dissolved organic carbon and phenolics), pH and temperature for each site which conformed to the Normal distribution were used in a correlation matrix for that site. Between-site comparisons between variables were made with one-way ANOVA tests, using data which conformed to the Normal distribution and had homogenous variance.

3.5 Results

a. Centre for Alternative Technology

Table 3.2 gives the range of values for activity of each enzyme between February 1997 and December 1998. Soil enzyme activity at the Centre for Alternative Technology over the period February 1997-December 1998 is shown in Figure 3.1 a.

Table 3.2

Enzyme activity at the CAT wetland between February 1997 and January 1998. (Units are $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$ for hydrolases, $\text{pmol diqc g}^{-1} \text{ min}^{-1}$ for phenol oxidase)

Enzyme	Min. value (month)	Max. value (month)	% change
Cel	0.1702 (December)	0.5743 (July)	337 %
Glu	0.4324 (May)	1.2557 (July)	290 %
NAG	0.3505 (December)	0.9554 (September)	272 %
Pho	0.4709 (May)	1.7970 (December)	381 %
Sul	0.0406 (May)	0.4639 (June)	1142 %
Xyl	0.0569 (May)	0.1770 (August)	311 %
Ph. ox	1.17 (July)	25.3 (June)	2162 %

Carbon cycling enzymes cellobiohydrolase and β -glucosidase activities correlated significantly with soil temperature ($r = 0.623$, $p = 0.001$ for cellobiohydrolase, $r = 0.49$, $p = 0.018$ for β -glucosidase), over the period February 1997 to December 1998. Despite the different magnitude of activity between enzymes, all hydrolases showed a distinct decrease in activity in May 1997. This decline was repeated in April 1998 for all hydrolases except phosphatase (for which it appeared in May 1998), and sulphatase (for which it appeared in March 1998). The decreases in activity in these months ranged from 29 % to 80 %, and are given in Table 3.3

Table 3.3

Changes in enzyme activity in springtime at the CAT wetland (given as percentage of year's average activity for each enzyme).

Enzyme	Lowest activity (1997)	% decrease	Lowest activity (1998)	% decrease
Cellobiohydrolase	May	45	April	63
β -glucosidase	May	53	April	57
NAG	May	40	April	66
Phosphatase	May	64	May	29
Sulphatase	May	80	March	37
Xylosidase	May	52	April	41

There was a considerable degree of correlation between enzymes at the CAT wetland, with 13 different inter-correlations (Fig 3.3 a); these did not include phenol oxidase. The combined activity of the carbon cycling enzymes cellobiohydrolase, β -glucosidase and xylosidase correlated significantly with soil temperature ($r = 0.528$, $p = 0.01$), and with carbon dioxide emission ($r = 0.43$, $p = 0.046$).

Gas emissions from the Centre for Alternative Technology are shown in Figure 3.4 a-c. Nitrous oxide and carbon dioxide emissions were significantly correlated with each other ($r = 0.621$, $p = 0.002$). Q_{10} values calculated from the field data were 1.96 for nitrous oxide, and 52.6 for carbon dioxide.

Figure 3.1

Activity of cellobiohydrolase (◆), β -glucosidase (■), N-acetylglucosaminidase (◇), phosphatase ($\times 1/10$ in (b)) (□), sulphatase (○) and xylosidase (●) in soil from (a) the Centre for Alternative Technology wetland, February 1997-December 1998, (b) the Waun-y-Cwrt wetland, February 1997-January 1998. (Mean, $n = 5$, \pm s.e.).

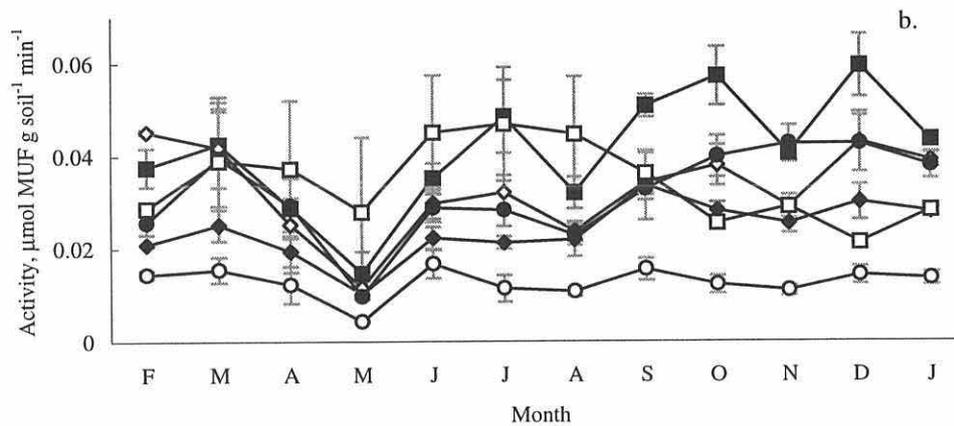
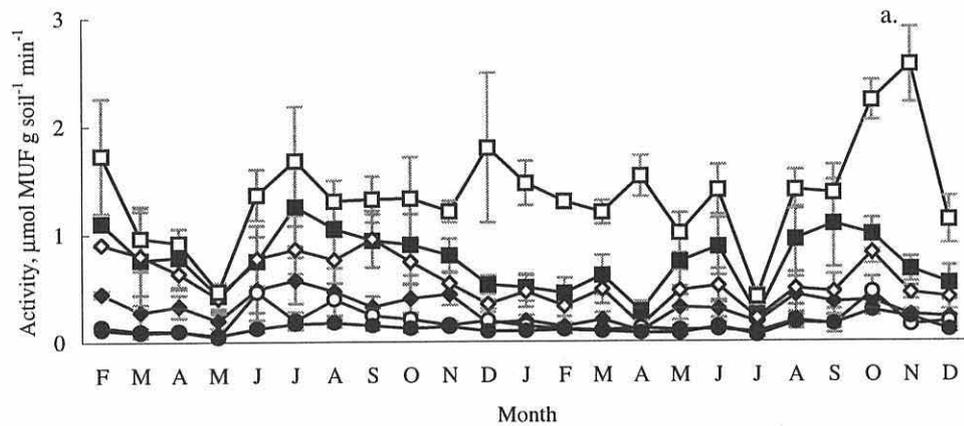


Figure 3.2

Phenol oxidase activity in (a) Centre for Alternative Technology, (b) Waun-y-Cwrt, (c) Pelenna and (d) Tollesbury wetlands over the period February 1997 – December 1998 (a) or January 1998 (b-d). (Mean, $n = 5$, \pm s.e.).

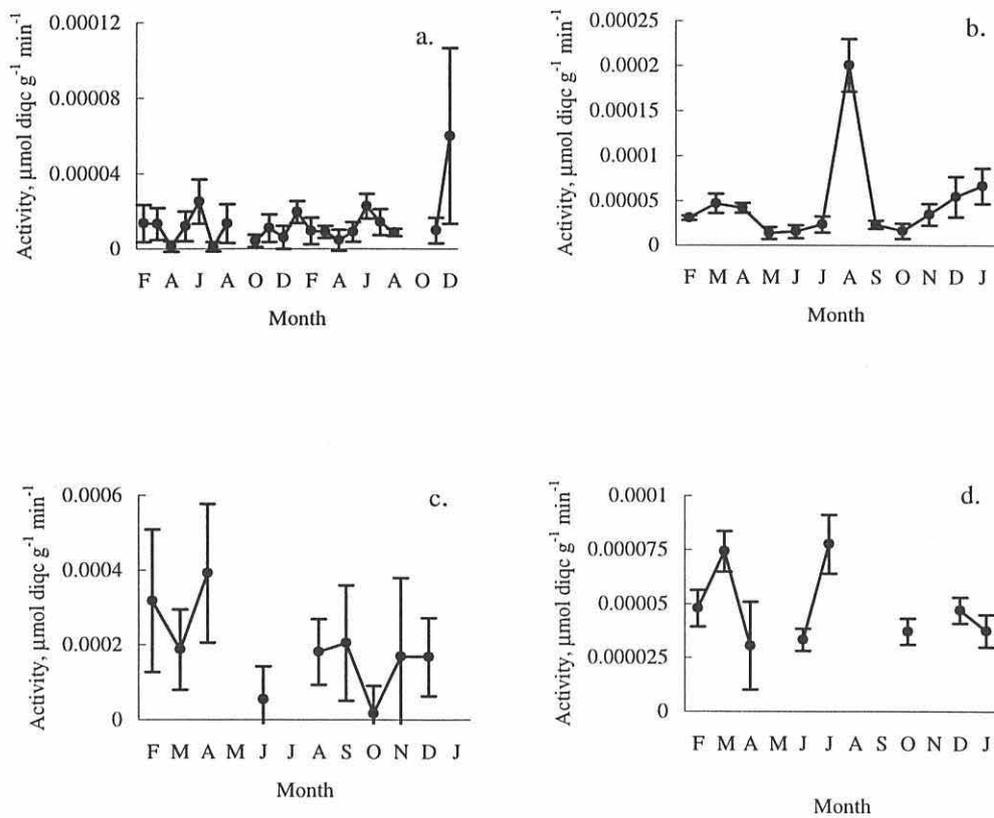


Figure 3.3

Correlations between enzymes at (a) Centre for Alternative Technology, (b) Waun-y-Cwrt, (c) Pelenna and (d) Tollesbury wetlands. (Data which conformed to the Normal distribution: Pearson correlations; non-Normal data: Spearman correlations).

— = correlation significant at $p < 0.05$

- - - = correlation significant at $p < 0.1$

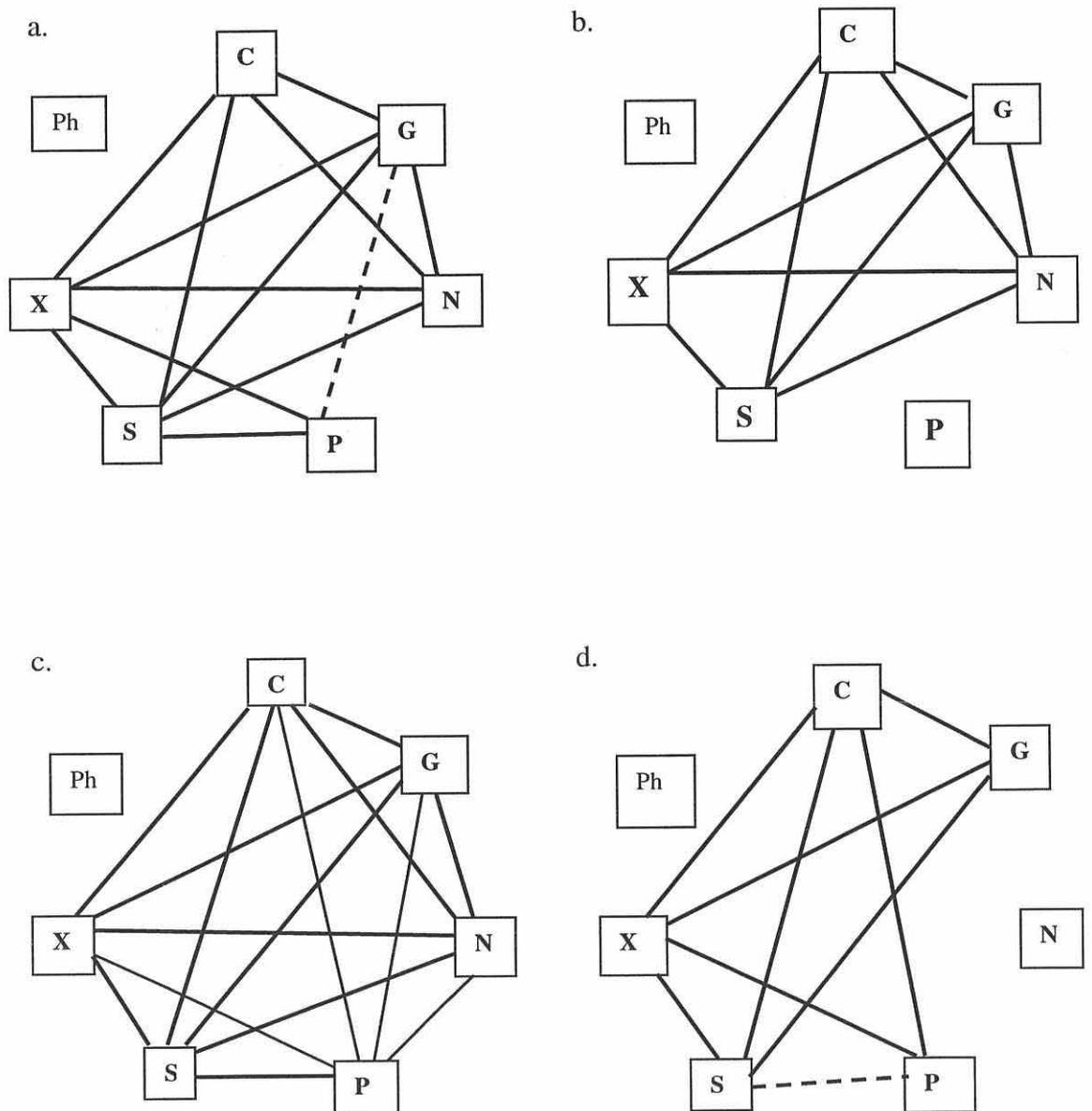
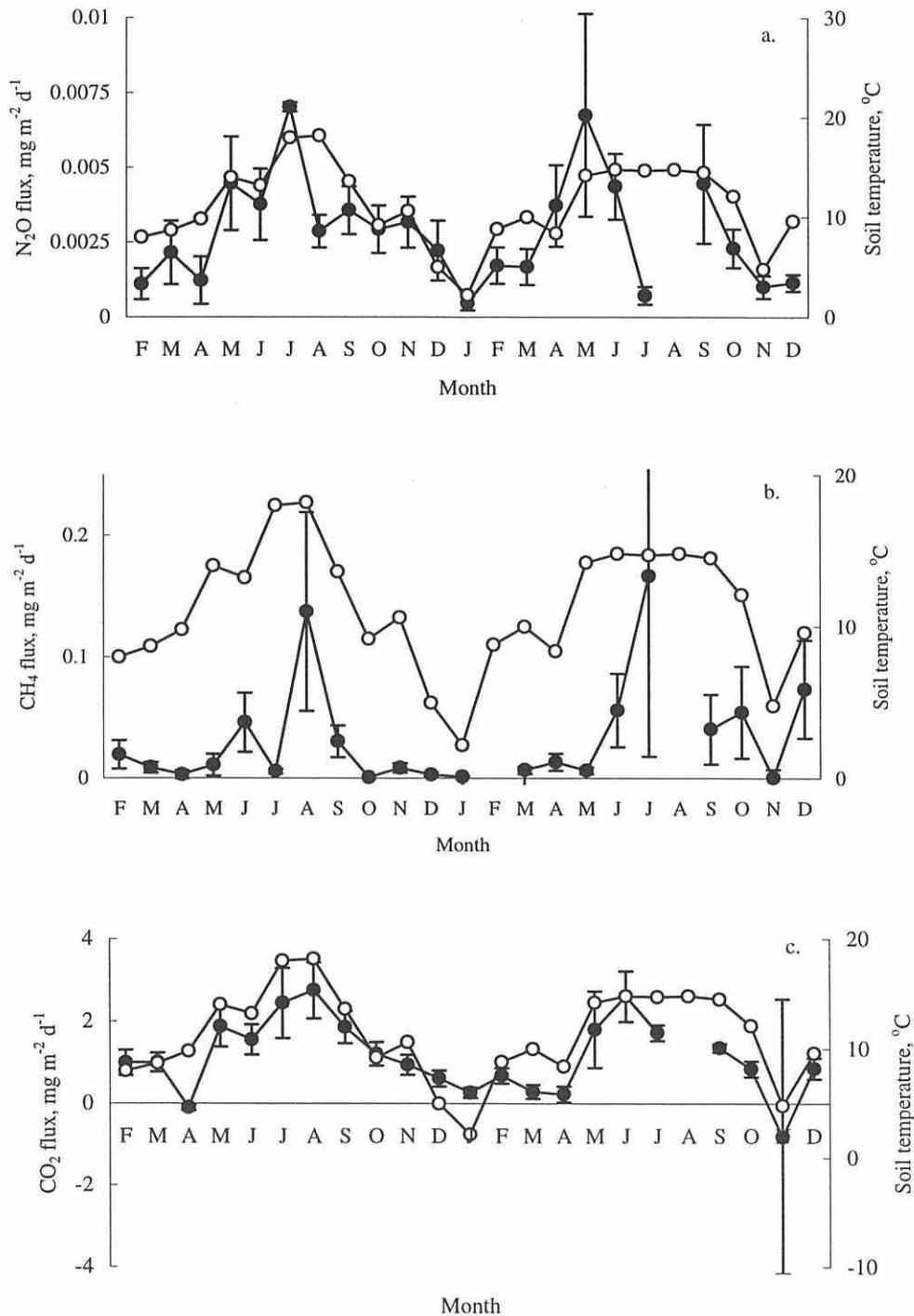


Figure 3.4

Emissions (●) of (a) nitrous oxide (b) methane and (c) carbon dioxide, and soil temperature (○) at the Centre for Alternative Technology wetland between February 1997 and December 1998. (Mean, $n = 5$, \pm s.e. for gases).



Tables 3.4 a and b show significant correlations between temperature, gas emissions, enzyme activities and water chemistry factors at the CAT wetland between February 1997 and December 1998.

Table 3.4 a

Significant correlations between soil enzymes and other variables at the CAT wetland, February 1997-December 1998 (r = Pearson correlation coefficient, p = probability).

		Cel	β -glu	NAG	Pho	Sul	Xyl	Ph.ox.
NO₃-N	r	0.418				0.428		
	p	0.053				0.047		
Phenol	r	0.382	0.411				0.362	
	p	0.072	0.051				0.089	
NH₄-N	r			0.420				
	p			0.046				
DOC	r				-0.448			0.390
	p				0.032			0.089
PO₄-P	r							0.557
	p							0.011
Temp	r	0.534	0.447					
	p	0.009	0.033					

Table 3.4 b

Significant correlations between soil gas fluxes and other variables at the CAT wetland, February 1997-December 1998 (r = Pearson correlation coefficient, p = probability).

		N₂O	CH₄	CO₂
NH₄-N	r	0.388		0.422
	p	0.075		0.051
Cel	r	0.412		0.474
	p	0.057		0.026
β-glu	r	0.393		0.403
	p	0.070		0.063
Pho	r			-0.365
	p			0.095
Phenolics	r		0.472	
	p		0.031	
DOC	r			0.480
	p			0.024
Temperature	r	0.651		0.844
	p	0.001		< 0.001

Nitrogenous substrates (NO₃-N + NH₄-N) correlated significantly with nitrous oxide emission (r = 0.385, p = 0.077) (Figure 3.5 b). Also, soil phosphate-P was significantly correlated with soil sulphate-S (r = -0.327, p = 0.096), and temperature was significantly correlated with nitrate-N (r = 0.361, p = 0.099), phenolics (r = 0.516, p = 0.012) and DOC (r = 0.452, p = 0.030). Carbon dioxide was significantly correlated with nitrous oxide flux (r = 0.621, p = 0.002) and with methane flux (r = 0.472, p = 0.031).

Table 3.5 shows the range of Dissolved Organic Carbon, phenolics and other water chemistry determinands in soil water from the CAT wetland between February 1997 and December 1998. These are illustrated in Figure 3.5 a, b and c.

Table 3.5

Range of water chemistry determinands in soil water from the CAT wetland, February 1997 - December 1998. (Units are mg l⁻¹).

Determinand	Min. value (month)	Max. value (month)
DOC	17.46 (November 1998)	65.54 (December 1998)
Phenolics	0.184 (December 1998)	4.53 (September 1998)
Ammonia-N	0.40 (February 1997)	30.65 (September 1997)
Nitrate-N	9.37 (September 1998)	95.72 (June 1997)
Phosphate-P	<0.01 (June-October 1997)	6.92 (September 1998)
Sulphate-S	28.02 (February 1997)	611.64 (January 1998)

Figure 3.5

Concentrations of (a) ammonia-N (■), nitrate-N (◆), phosphate-P (□) and sulphate-S (○) in soil water samples, and (b) relationship between nitrous oxide emission (○) and nitrogenous substrates (ammonia + nitrate-N) (■) at Centre for Alternative Technology wetland between February 1997 and December 1998. (Mean, n = 5).

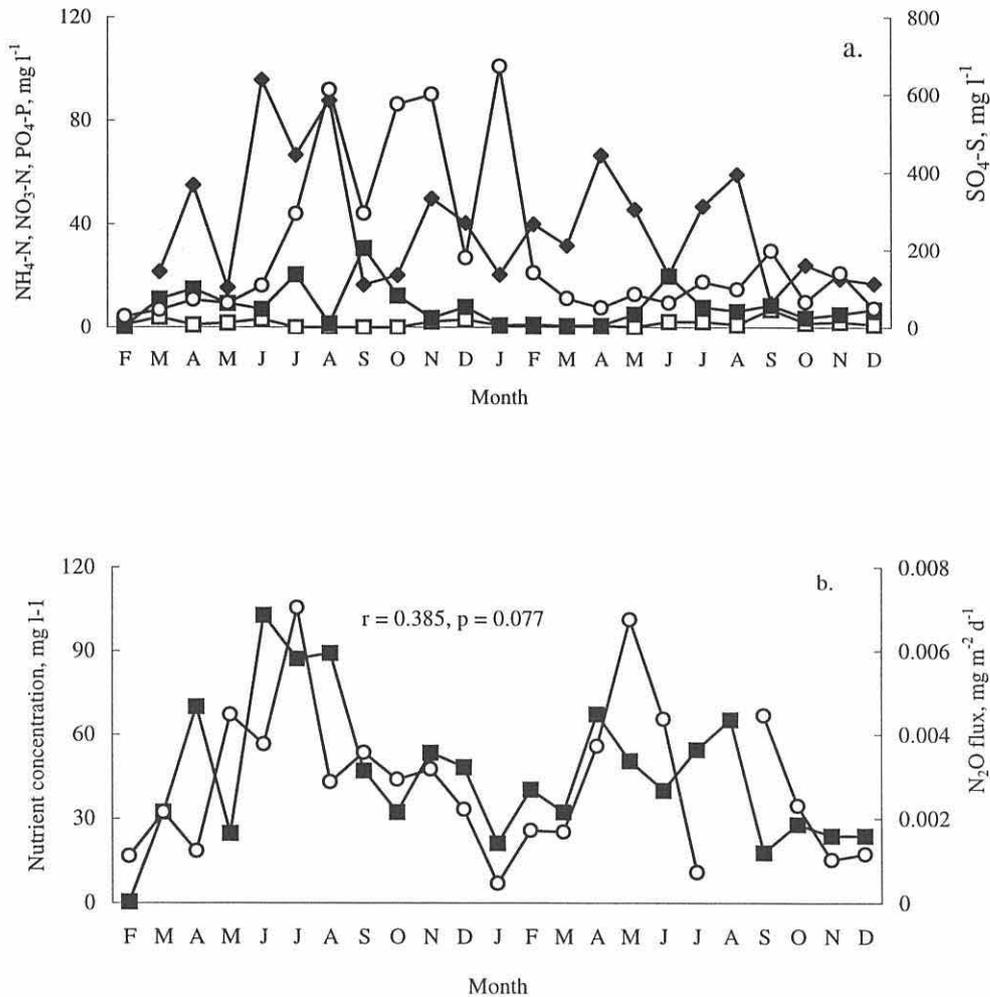
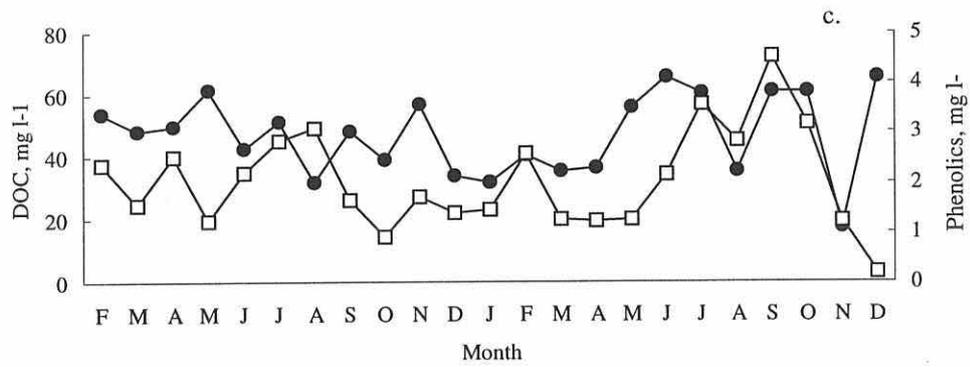


Figure 3.5 contd.

(c) Concentrations of dissolved organic carbon (●) and phenolic substances (□) in soil water samples from the Centre for Alternative Technology wetland between February 1997 and December 1998. (Mean, n = 5).



b. Waun-y-Cwrt landfill site, Nantmel

Soil enzyme activities at Waun-y-Cwrt over the period February 1997-January 1998 are shown in Figure 3.1 b. Table 3.6 gives the range of values for activity of each enzyme between February 1997-January 1998. For all hydrolases, activity was minimal in May 1997.

Table 3.6

Enzyme activity at the Waun-y-Cwrt wetland between February 1997-January 1998. (Units are $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$ for hydrolases, $\text{pmol diqc g}^{-1} \text{ min}^{-1}$ for phenol oxidase)

Enzyme	Min. value	Max. value (month)	% change
Cel	0.0102 (May)	0.0336 (September)	329 %
Glu	0.0147 (May)	0.0594 (December)	404 %
NAG	0.0116 (May)	0.0453 (February)	390 %
Pho	0.2123 (December)	0.4693 (July)	221 %
Sul	0.0043 (May)	0.0167 (June)	388 %
Xyl	0.0097 (May)	0.0427 (November)	440 %
Ph. ox.	13.9 (May)	201.0 (August)	1446 %

All hydrolases at the Waun-y-Cwrt wetland showed a distinct decrease in activity in May 1997. The decreases in activity in these months ranged from 19 % to 69 %, and are given in Table 3.7

Table 3.7

Changes in enzyme activity in springtime at the Waun-y-Cwrt wetland (given as percentage of year's average activity for each enzyme).

Enzyme	Lowest activity (1997)	% decrease
Cellobiohydrolase	May	57
β -glucosidase	May	63
NAG	May	65
Phosphatase	May	19
Sulphatase	May	67
Xylosidase	May	69

There was good inter-correlation between enzymes at the Waun-y-Cwrt wetland, with 10 significant inter-correlations (Figure 3.3 b), which included all enzymes except phenol oxidase and phosphatase. Gas emissions from the Waun-y-Cwrt site are shown in Figures 3.4 d-f. Tables 3.8 a and b show significant correlations between temperature, gas emissions, enzyme activities and water chemistry factors at the Waun-y-Cwrt site between February 1997 and January 1998.

Table 3.8 a

Significant correlations between soil enzyme activity and other variables at the Waun-y-Cwrt wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

		Cel	β -glu	NAG	Pho	Sul	Xyl	Ph.ox.
N ₂ O	r				0.649			
	p				0.022			
Phenol	r				0.563			
	p				0.072			
SO ₄	r	-0.660	-0.711	-0.561			-0.676	
	p	0.020	0.009	0.058			0.016	

Table 3.8 b

Significant correlations between soil gas fluxes and other variables at the Waun-y-Cwrt wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

		N_2O	CH_4	CO_2
Phenolics	r	0.782		
	p	0.004		
SO₄	r	0.583		
	p	0.047		
DOC	r		0.768	
	p		0.004	
NH₄	r		0.503	
	p		0.096	
PO₄	r		0.924	
	p		< 0.001	
Temperature	r	0.534		0.627
	p	0.061		0.029

In addition, phenolics correlated significantly with phenol oxidase activity (r = 0.693, p = 0.018) and soil water sulphate-S concentration (r = 0.679, p = 0.021). Dissolved organic carbon correlated significantly with ammonia concentration (r = 0.657, p = 0.002) and with phosphate-P concentration (r = 0.828, p = 0.002).

Table 3.9 shows the range of Dissolved Organic Carbon, phenolics and other water quality determinands in soil water from the Waun-y-Cwrt wetland between February 1997 and January 1998. These are illustrated in Figures 3.6 a, b and c. Leachate chemistry data were provided by the Waste Management Section of Powys County Council for dates throughout 1997 (Table 3.10).

Table 3.9

Range of water chemistry determinands in soil water from the Waun-y-Cwrt wetland, February 1997 – January 1998. (Units are mg l^{-1}).

Determinand	Min. value (month)	Max. value (month)
DOC	29.08 (August)	110.416 (March)
Phenolics	1.375 (November)	9.44 (August)
Ammonia-N	0.40 (February)	63.8 (April)
Nitrate-N	0.285 (December)	1064.56 (August)
Phosphate-P	<0.01(June-October)	1.55 (March)
Sulphate-S	38.55 (September)	407.64 (August)

Table 3.10

Leachate chemistry data for Waun-y-Cwrt landfill during 1997. Units for all determinands except pH are mg l^{-1} .

Date	pH	SS	BOD	Iron	NH ₃	SO ₄	N(total)	Phenols	PO ₄
			5						
24/4/97	8.1			5600					
7/5/97	6.8	22	4	7.8	58.4	14	2.8	<0.1	
12/5/97	6.0	51	<2	9.4	31.9	<5	2.1	<0.1	
14/10/97	6.76	13.6	6	8.7	17.7				0.07
20/10/97	6.89	25.2	14	6.9	0.36				0.05
27/10/97	7.22	44	14	17.4	0.043				0.06
13/11/97	6.22	23.2	5	22	0.3				<0.02
24/11/97	7.0	6.4	12	6.9	22				<0.02
22/12/97	6.4	54.4	8	26.7	34.9				0.07

Figure 3.4 contd.

Emissions (●) of (d) nitrous oxide (e) methane and (f) carbon dioxide, and soil temperature (○) at the Waun-y-Cwrt wetland, February 1997 – January 1998. (Mean, $n = 5$, \pm s.e.).

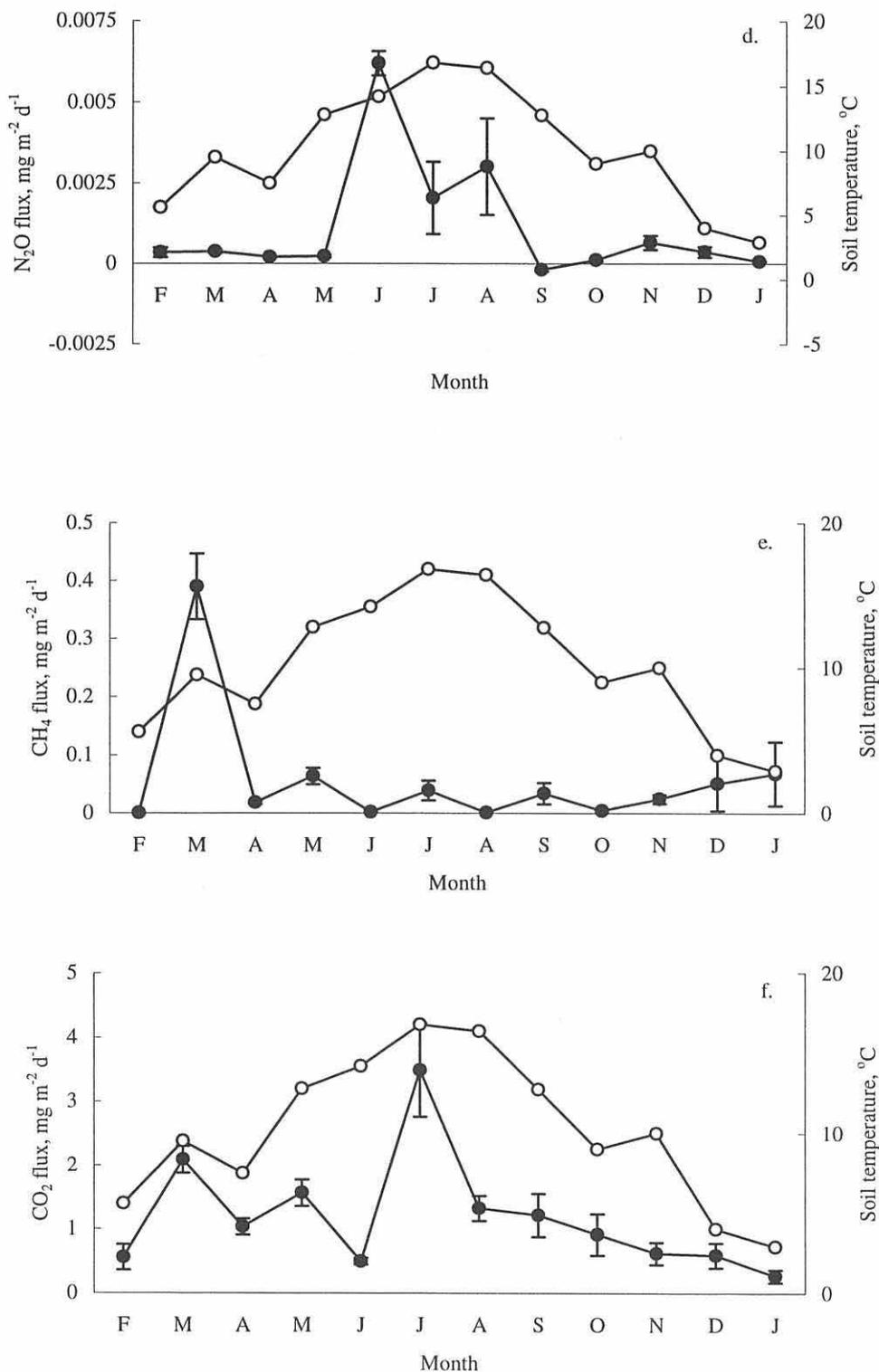


Figure 3.6

Concentrations of (a) ammonia-N (■), nitrate-N (◆), phosphate-P (□) and sulphate-S (○) in soil water samples, and (b) relationship between dissolved organic carbon concentration in soil water (■) and methane emission (○) at Waun-y-Cwrt wetland between February 1997 and January 1998. (Mean, n = 5).

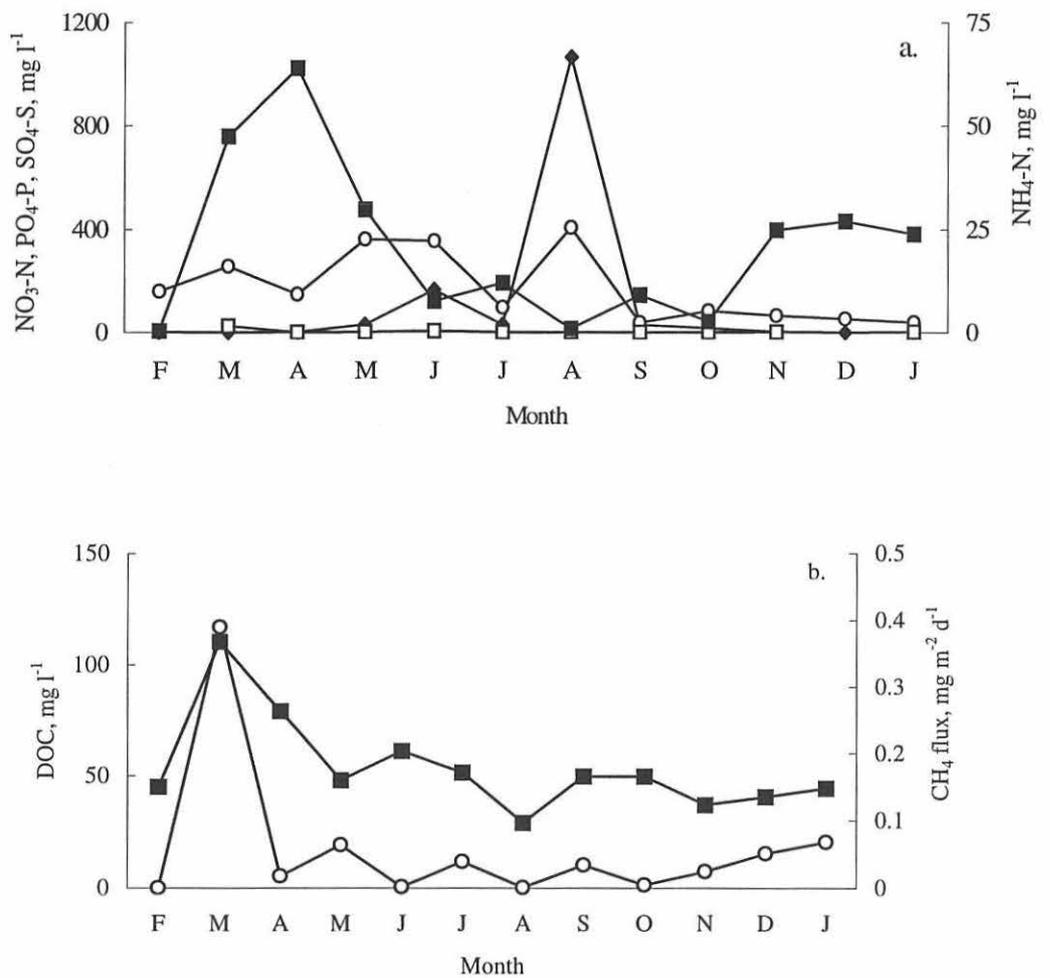
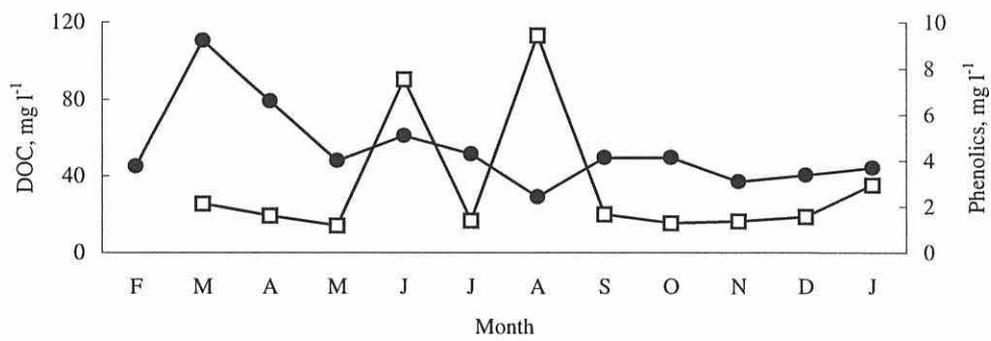


Figure 3.6 contd.

(c) Concentrations of dissolved organic carbon (●) and phenolic substances (□) in soil water samples from the Waun-y-Cwrt wetland between February 1997 and January 1998. (Mean, n = 5).



c. Pelenna

Soil enzyme activity at Pelenna over the period February 1997-January 1998 is shown in Figure 3.1 c. Table 3.11 gives the minimum and maximum values for activity of each enzyme between February 1997-January 1998. For all hydrolases, activity was minimal in May 1997 and maximal in August 1997.

Table 3.11

Enzyme activity at the Pelenna wetland between February 1997 and January 1998. (Units are $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$ for hydrolases, $\text{pmol diqc g}^{-1} \text{ min}^{-1}$ for phenol oxidase)

Enzyme	Min. value	Max. value	% change
Cel	0.0547	0.4993 (August)	912 %
Glu	0.1652	1.266 (August)	766 %
NAG	0.0920	0.9508 (August)	1033 %
Pho	0.2335	2.6754 (February)	1145 %
Sul	0.0123	0.0964 (February)	783 %
Xyl	0.0521	0.5122 (February)	983 %
Ph. ox.	17.0 (October)	392.0 (April)	2305 %

All six hydrolases showed a distinct decrease in activity in May 1997, and similar patterns in activity throughout the year despite their different magnitudes of activity. All hydrolases at the Pelenna wetland showed a distinct decrease in activity in May 1997. The decreases in activity in these months ranged from 79 % to 83 %, and are given in Table 3.12

Table 3.12

Changes in enzyme activity in springtime at the Pelenna wetland (given as percentage of year's average activity for each enzyme).

Enzyme	Lowest activity (1997)	% decrease
Cellobiohydrolase	May	82
β -glucosidase	May	79
NAG	May	82
Phosphatase	May	83
Sulphatase	May	79
Xylosidase	May	82

There were good correlations between different enzymes, with 15 significant inter-correlations; these included all enzymes except phenol oxidase (Figure 3.3 c).

Gas emissions from the Pelenna site are shown in Figures 3.4 g- i. Tables 3.13 a and b show significant correlations between temperature, gas emissions, enzyme activity and water chemistry factors at the Pelenna wetland.

Figure 3.1 contd.

Activity of cellobiohydrolase (◆), β -glucosidase (■), N-acetylglucosaminidase (◇), phosphatase (□), sulphatase (○) and xylosidase (●) in soil from (c) the Pelenna wetland, February 1997-January 1998, (d) the Tollesbury wetland, February 1997-January 1998. (Mean, $n = 5$, \pm s.e.).

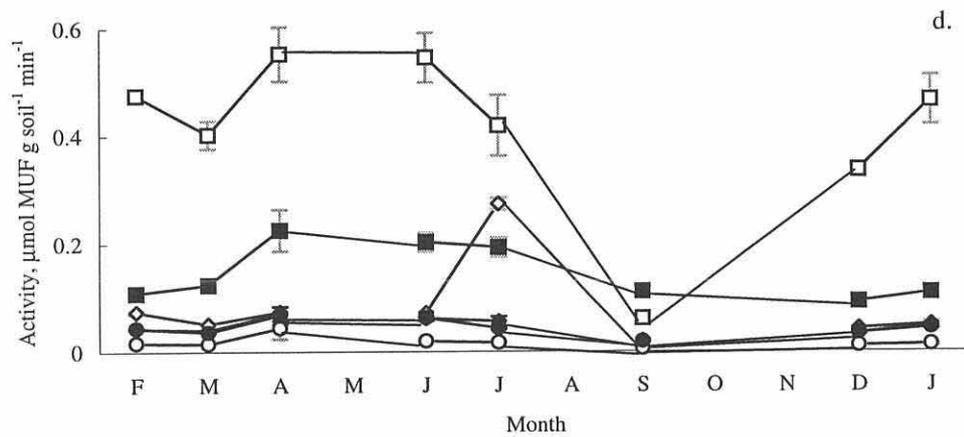
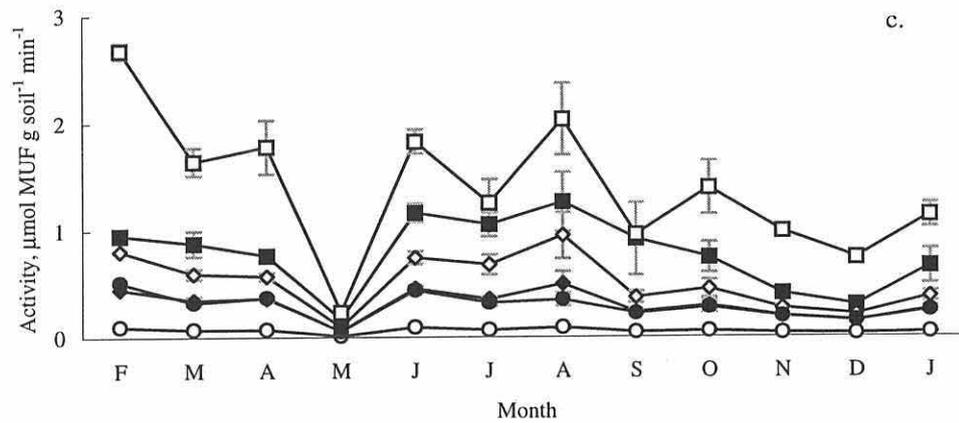


Table 3.13 a

Significant correlations between soil enzyme activity and other variables at the Pelenna wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

		Cel	β-glu	NAG	Pho	Sul	Xyl	Ph.ox.
PO₄	r	0.676		0.751	0.796	0.731	0.747	
	p	0.096		0.052	0.032	0.062	0.054	
DOC	r	0.624	0.562	0.674		0.579		
	p	0.030	0.057	0.016		0.049		
CH₄	r		-0.536	-0.536				
	p		0.073	0.072				
CO₂	r		0.619	0.532				
	p		0.032	0.075				
SO₄	r		-0.516	-0.544				
	p		0.086	0.068				
Temp	r				0.706			
	p				0.010			

Table 3.13 b

Significant correlations between soil gas fluxes and other variables at the Pelenna wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

		N₂O	CH₄	CO₂
NO₃	r		-0.793	0.695
	p		0.004	0.018
SO₄	r			-0.749
	p			0.005
DOC	r			0.654
	p			0.021
Temperature	r			0.542
	p			0.069

In addition, Dissolved Organic Carbon correlated significantly with pH ($r = 0.601$, $p = 0.05$) and sulphate-S concentration ($r = -0.725$, $p = 0.08$), and carbon dioxide flux correlated significantly with methane flux ($r = -0.65$, $p = 0.022$).

Table 3.14 shows the range of Dissolved Organic Carbon, phenolics and other water quality determinands in soil water from the Pelenna wetland between February 1997 and January 1998. These are illustrated in Figure 3.7 a, b, c and d. Sulphate and iron concentrations in the inflow and outflow to the wetland are illustrated in Figures 3.7 e and f respectively (data courtesy of Environment Agency, South Wales).

Table 3.14

Range of water chemistry determinands in soil water from the Pelenna wetland, February 1997 – January 1998. (Units are mg l^{-1}).

Determinand	Min. value (month)	Max. value (month)
DOC	10.19 (September 1997)	74.115 (August 1997)
Phenolics	0.51 (November 1997)	2.98 (August 1997)
Ammonia-N	0.281 (October 1997)	3.16 (September 1997)
Nitrate-N	0.0702 (October 1997)	9.88 (July 1997)
Phosphate-P	<0.01 (July-October 1997)	1.44 (February 1997)
Sulphate-S	71.94 (August 1997)	937.84 (January 1998)

Figure 3.4 contd.

Emissions (●) of (g) nitrous oxide, (h) methane and (i) carbon dioxide and soil temperature (○) at the Pelenna wetland, February 1997-January 1998. (Mean, $n = 5$, \pm s.e.).

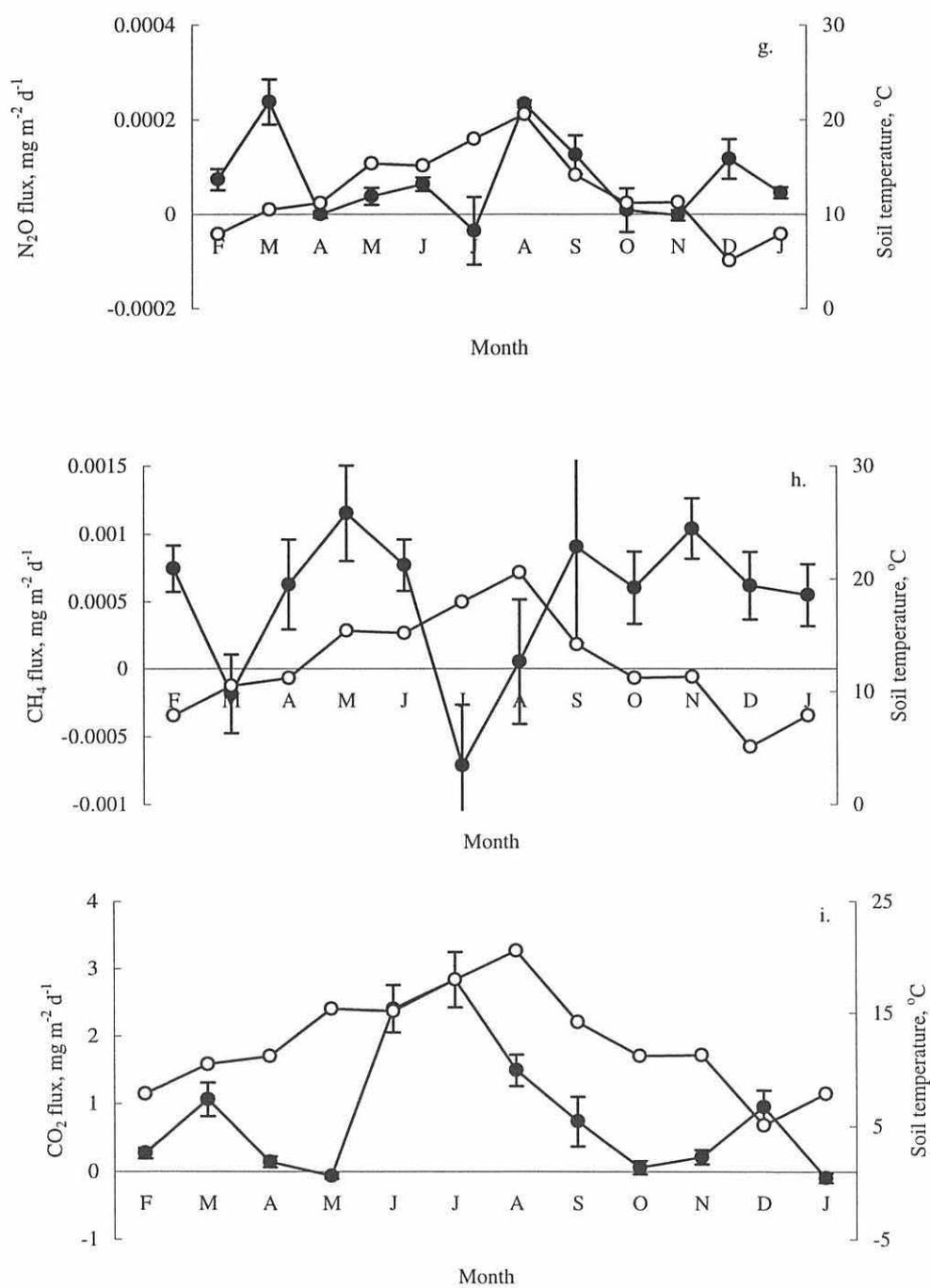


Figure 3.7

Concentrations of (a) ammonia-N (■), nitrate-N (◆), phosphate-P (□) and sulphate-S (○) and (b) dissolved organic carbon (●) and phenolic substances (□) in soil water samples from the Peledda wetland between February 1997 and January 1998. (Mean, n = 5).

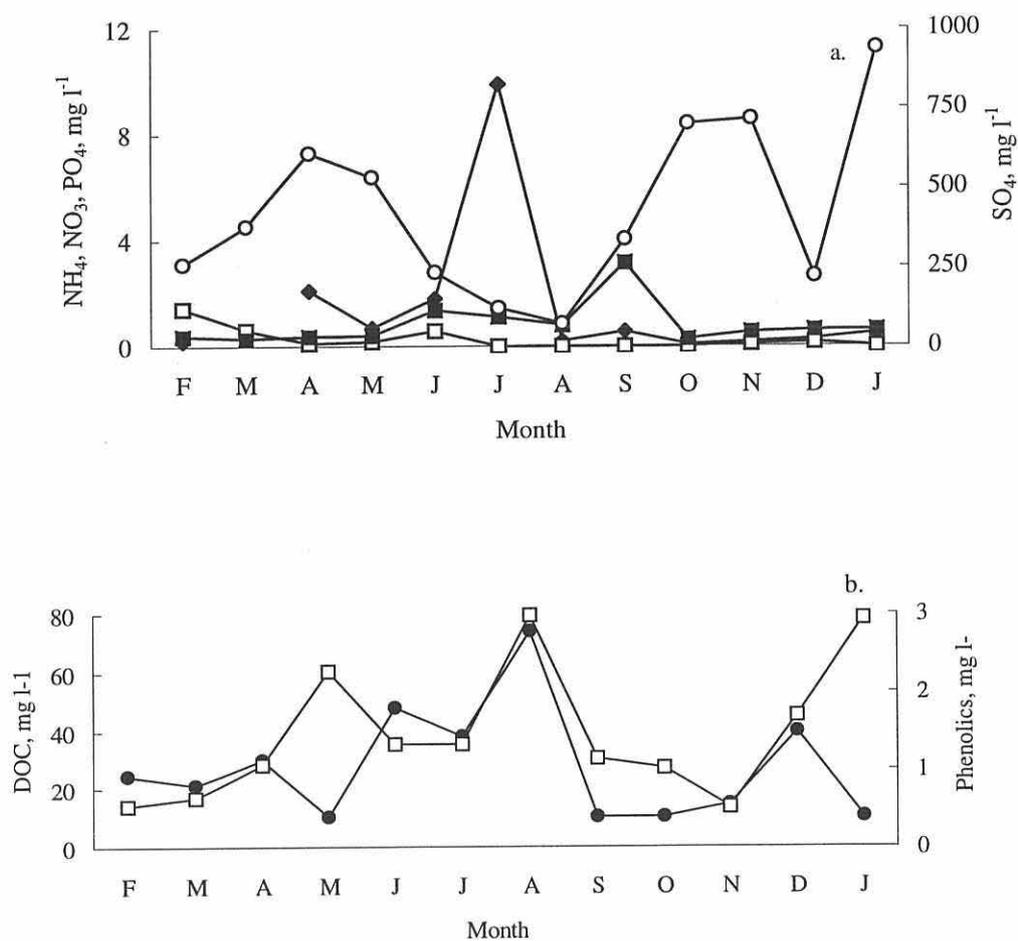


Figure 3.7 contd.

(c) Relationship between methane (x 25) (□) and carbon dioxide (■) emission, and nitrate-N concentration in soil water (○) and (d) relationship between nitrous oxide emission (○) and nitrogenous substrates (ammonia-N + nitrate-N) in soil water (■) at Pelenna wetland between February 1997 and January 1998. (Mean, n = 5).

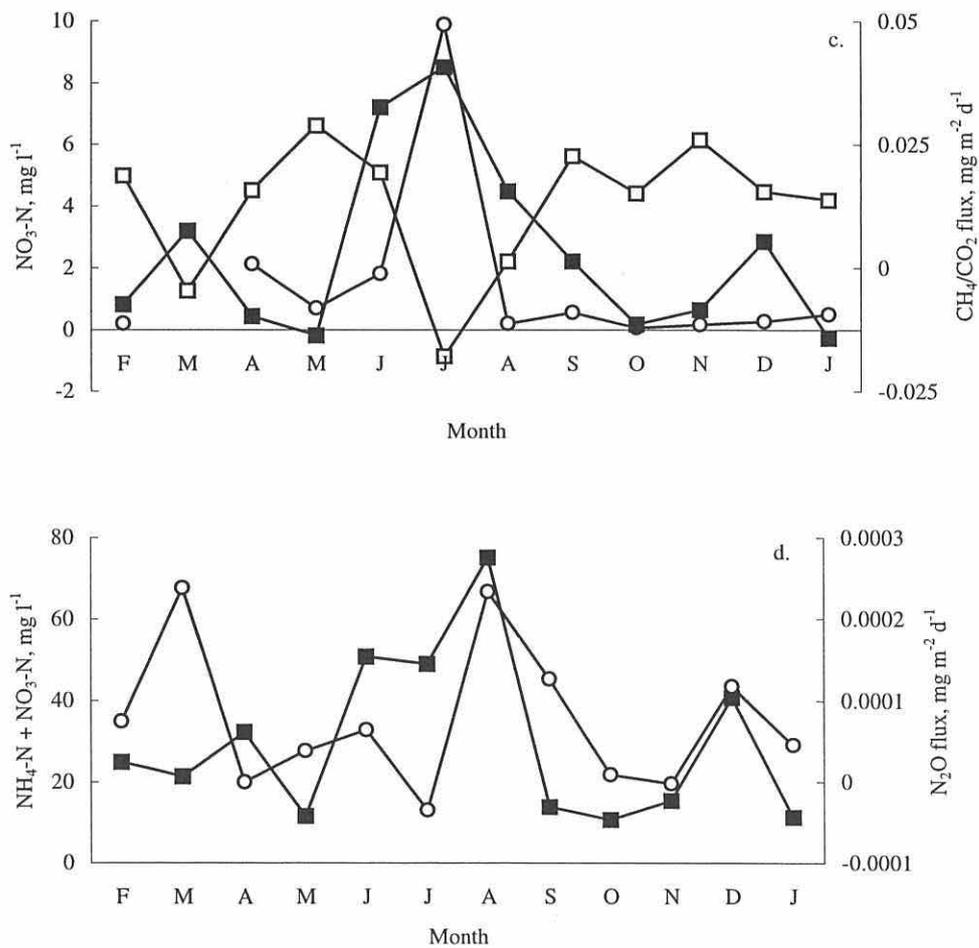
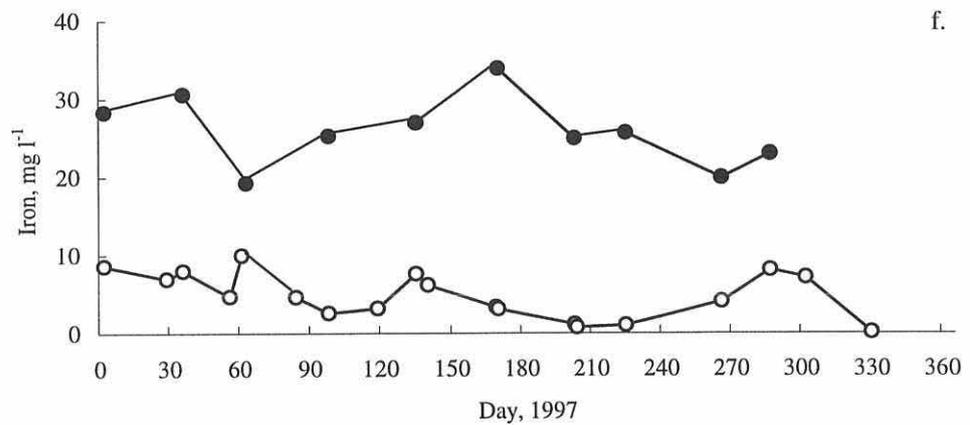
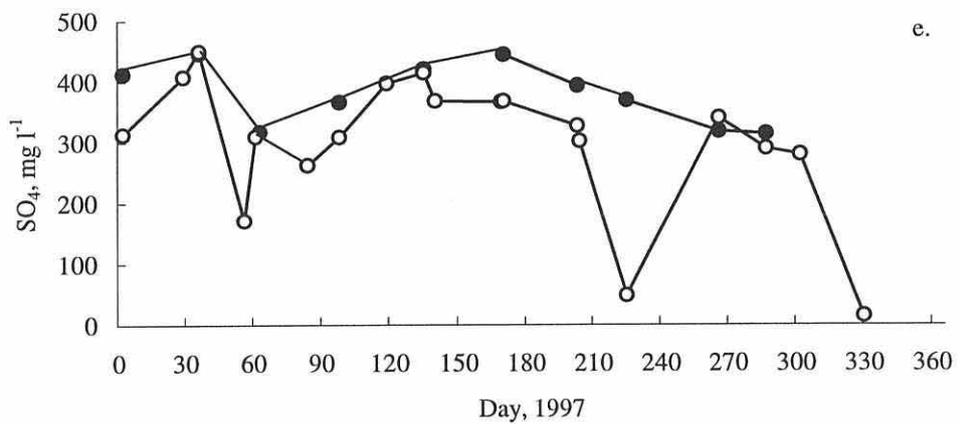


Figure 3.7 contd.

Concentrations of (e) sulphate in inflow (●) and outflow (○) and (f) iron in inflow (●) and outflow (○) to Pelenna wetland, February 1997-January 1998. (Mean, $n = 5$).



d. Tollesbury

Soil enzyme activity at the Tollesbury salt marsh over the period February 1997-January 1998 is shown in Figure 3.1 d. Table 3.15 gives the minimum and maximum values for activity of each enzyme between February 1997 and January 1998.

Table 3.15

Enzyme activity at Tollesbury wetland between February 1997 and January 1998. (Units are $\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$ for hydrolases, $\text{pmol diqc g}^{-1} \text{ min}^{-1}$ for phenol oxidase)

Enzyme	Min. value	Max. value	% change
Cel	0.0199 (September)	0.0716 (April)	359 %
Glu	0.0927 (December)	0.2257 (April)	243 %
NAG	0.0191 (September)	0.2739 (July)	1434 %
Pho	0.0621 (September)	0.5527 (April)	890 %
Sul	0.0101 (December)	0.0192 (June)	190 %
Xyl	0.0183 (September)	0.0686 (April)	374 %
Ph. ox.	30.5 (April)	77.5 (July)	254 %

There was some correlation between different enzymes at the Tollesbury wetland (9 significant intercorrelations), but these did not include phenol oxidase or N-acetylglucosaminidase (Figure 3.3 d).

Gas emissions from the Tollesbury site are shown in Figures 3.4 j-l. Tables 3.16 a and b show significant correlations between temperature, gas emissions, enzyme activities and water chemistry factors at the Tollesbury site between February 1997 and January 1998. Table 3.17 gives the range of Dissolved Organic Carbon, phenolics and other water chemistry determinands in soil water from the Tollesbury wetland between February 1997 and January 1998. Nutrient ion

concentrations are given in Table 3.18, and DOC and phenolics are illustrated in Figure 3.8.

Table 3.16 a

Significant correlations between soil enzymes and other variables at the Tollesbury wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

	Cel	β -glu	NAG	Pho	Sul	Xyl	Ph.ox.
CH₄	r	-0.760			-0.888		
	p	0.079			0.018		
DOC	r	0.701					
	p	0.079					
Phenol	r				-0.777		
	p				0.069		
SO₄	r					-0.990	
	p					0.089	
Temp	r	0.693					
	p	0.084					

Table 3.16 b

Significant correlations between soil gas fluxes and other variables at the Tollesbury wetland between February 1997 and January 1998 (r = Pearson correlation coefficient, p = probability).

	N ₂ O	CH ₄	CO ₂
Phen. oxidase	r	0.830	0.864
	p	0.041	0.027
DOC	r		-0.773
	p		0.071

In addition, temperature correlated significantly with Dissolved Organic Carbon (r = 0.766, p = 0.044), pH (r = -0.878, p = 0.002) and ammonia concentration (r = 0.944, p = 0.016). Carbon dioxide flux correlated significantly with nitrous oxide

flux ($r = 0.690$, $p = 0.058$), and phenolic concentration was inversely correlated with phosphate-P concentration ($r = -0.929$, $p = 0.022$).

Table 3.17

Range of water chemistry determinands in soil water from the Tollesbury wetland, February 1997 – January 1998. (Units are mg l^{-1}).

Determinand	Min. value (month)	Max. value (month)
DOC	25.75 (January 1998)	85.24 (April 1997)
Phenolics	4.96 (April 1997)	33.57 (September 1997)
Ammonia-N	0.81 (January 1998)	4.56 (September 1997)
Nitrate-N	5.17 (January 1998)	15.0 (April 1997)
Phosphate-P	0.21 (March 1997)	1.87 (February 1997)
Sulphate-S	1978.07 (April 1997)	8515.63 (December 1997)

Table 3.18

Nutrient ion concentrations in soil water from Tollesbury wetland, February 1997-January 1998 (mean, n = 5, mg l⁻¹).

	NH ₄ -N	NO ₃ -N	PO ₄ -P	SO ₄ -S
February			1.87	
March			0.21	
April	2.79	15.0	1.38	1978.07
May				
June	3.17	8.45	0.304	2277.33
July				
August				
September	4.56	14.24		
October				
November				
December	1.47	11.27	0.66	8515.63
January				

Figure 3.4 contd.

Emissions (●) of (j) nitrous oxide (k) methane and (l) carbon dioxide, and soil temperature (○) at the Tollesbury wetland, February 1997-January 1998. (Mean, n = 5, ± s.e.).

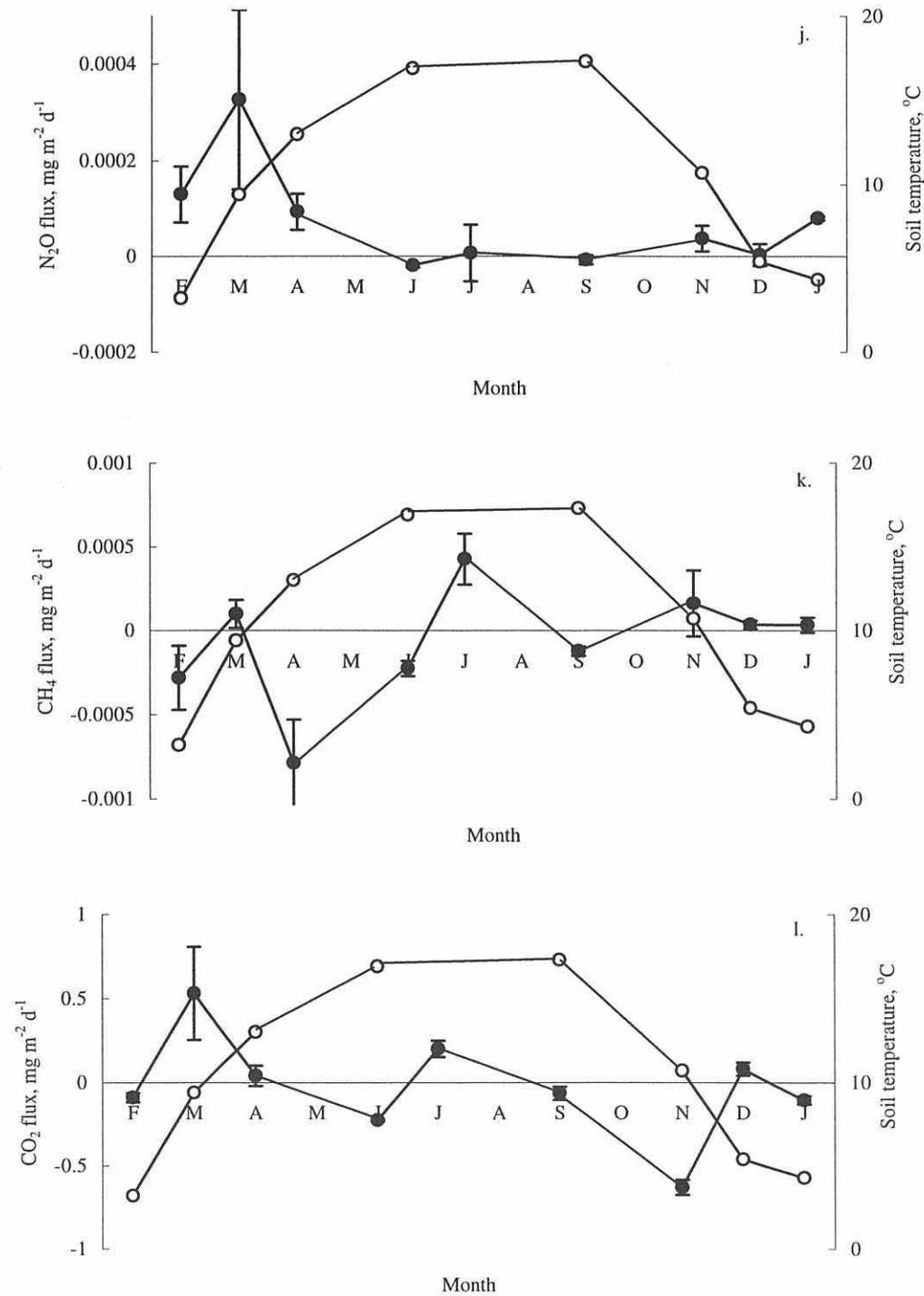
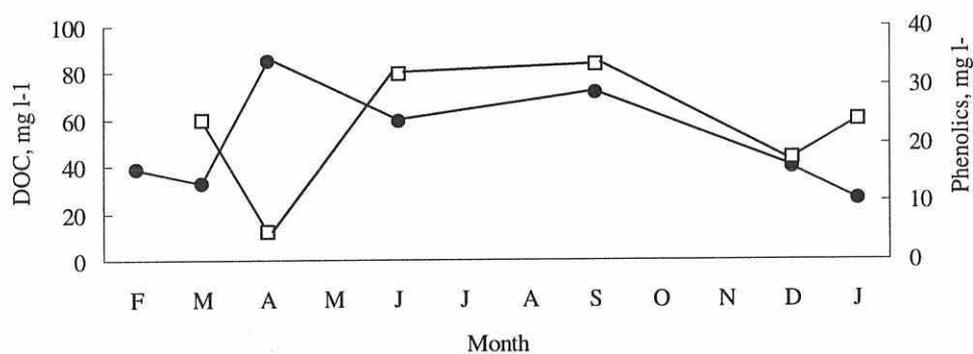


Figure 3.8

Concentrations of dissolved organic carbon (●) and phenolic substances (□) in soil water samples from the Tollesbury wetland between February 1997 and January 1998. (Mean, n = 5).



e. Between-site comparisons

Between-site comparisons of mean annual (1997) enzyme activity using one-way ANOVA tests revealed significant differences between sites for cellobiohydrolase ($F_{3,40} = 25.93$, $p < 0.001$), β -glucosidase ($F_{3,40} = 27.62$, $p < 0.001$), N-acetylglucosaminidase (Tollesbury data was omitted due to non-Normality) ($F_{2,33} = 36.59$, $p < 0.001$), phosphatase ($F_{3,40} = 16.48$, $p < 0.001$), sulphatase (Pelenna data was omitted due to non-Normality) ($F_{2,29} = 18.15$, $p < 0.001$) and xylosidase (Waun-y-Cwrt data was omitted due to non-Normality) ($F_{2,29} = 37.97$, $p < 0.001$). For all hydrolases, the CAT site had the highest activity, and for all except xylosidase, the Tollesbury site had the lowest. There were significant differences between sites in phenol oxidase activity ($F_{2,25} = 18.81$, $p < 0.001$; Waun-y-Cwrt data was omitted due to non-Normality).

One-way ANOVA on gas emissions throughout 1997, using data which were normally distributed, showed significant differences between sites. CAT, Pelenna and Tollesbury showed significantly different annual nitrous oxide emissions ($F_{2,29} = 25.8$, $p < 0.001$), the difference mainly being attributable to the CAT site which had nearly 40 times the emission of Pelenna or Tollesbury. Methane emissions were four times (significantly) higher at the Pelenna site than at Tollesbury ($F_{1,17} = 8.96$, $p = 0.008$) (CAT and Waun-y-Cwrt site data was not normally distributed). Carbon dioxide emissions were significantly different between CAT, Pelenna and Tollesbury sites ($F_{2,29} = 6.66$, $p = 0.04$), being 1.5 times higher at CAT than at Pelenna, and 15 times higher at Pelenna than at Tollesbury. Q_{10} values for hydrolase activities at each site are shown in Table 3.19.

Table 3.19

Q₁₀ values for hydrolase activities at the four constructed wetlands

Enzyme	CAT	Waun-y-Cwrt	Pelenna	Tollesbury
Cellobiohydrolase	1.74	-	1.91	1.54
β-glucosidase	1.65	-	2.45	2.06
NAG	1.33	-	2.79	1.47
Phosphatase	-	1.89	1.4	1.25
Sulphatase	1.17	1.07	1.86	1.82
Xylosidase	1.06	-	1.41	1.57
Phenol oxidase	-	-	-	-

- indicates data unsuitable for Q₁₀ calculation

Measurements of phenol oxidase activity occasionally gave negative results, notably in the autumn (August, September 1997, September, October 1998) at CAT and in the summer (June, July at Waun-y-Cwrt, May, July at Pelenna) at other sites. The negative results could not be explained by other factors measured at these sites, but may be due to anaerobic soil conditions limiting activity of the enzyme. McLatchey & Reddy (1998) detected phenol oxidase activity only under aerobic soil conditions, as the enzyme requires molecular oxygen to be active.

3.6 Discussion

a. Gas emissions from constructed wetlands

Natural wetlands are significant sources of the radiatively active gases methane and nitrous oxide (Clymo 1983), and also of carbon dioxide, via their transformation and mineralization of organic matter. In constructed wetlands the same transformation and mineralization processes occur, and methane, nitrous oxide and carbon dioxide are produced. The significant differences in gas emissions and enzyme activities between the four wetlands monitored in this study indicate the different conditions in each. Gas evolution from a soil can be used to indicate the microbial activity occurring within that soil (Bell 1969), and the results presented here reveal different levels and partitioning of microbial activity in the wetlands.

Nitrous oxide

In soils the production of nitrous oxide is generally attributed to nitrification (Bergstrom *et al* 1994), although it may also be produced as an intermediate in denitrification (e.g. Terry & Tate 1980). Nitrous oxide emission is influenced by hydrology (denitrification requires anaerobic conditions, nitrification requires aerobic), nitrate supply (nitrate accepts electrons during denitrification) and carbon supply (denitrifiers are mainly heterotrophic and require organic carbon) (Groffman 1991). In contrast to previous observations that temperature does not significantly influence soil nitrous oxide emissions (Skiba *et al* 1994), at CAT and Waun-y-Cwrt these two factors were significantly correlated. Whether this is a direct relationship, or whether a third factor is involved, mediated by temperature and so controlling nitrous oxide production (such as microbial nitrate consumption), is unclear. The status of these wetlands as treatment soils, with a much higher nitrogenous input than natural soils, must be influencing their nitrogen dynamics. Denitrification can influence plant productivity in natural wetlands because plants in these are usually nitrogen-limited (Bowden 1987). Better knowledge of what controls denitrification could enable wetland

management to optimize molecular nitrogen production (and minimize production of nitrogen oxides), thus maximizing water quality whilst minimizing atmospheric pollution (Jordan *et al* 1998). Denitrification in wetlands can prevent nitrate pollution, and thus eutrophication, of surface water bodies (Groffman & Hanson 1997).

Bergstrom *et al* (1994) observed high rates of nitrous oxide production in soil with relatively high levels of ammonia and carbon substrate levels, and Freeman *et al* (1997) also state that production is favoured by high mineral nitrogen and organic carbon availability. At the CAT wetland, nitrous oxide emission from the wetland correlated significantly with the total available mineral nitrogen ($\text{NH}_4\text{-N} + \text{NO}_3\text{-N}$) measured in the soil water ($r = 0.385$, $p = 0.077$), and when dissolved organic carbon was also included the correlation improved to $r = 0.516$, $p = 0.014$, supporting these findings. At Waun-y-Cwrt there was no significant relationship between nitrous oxide emission and nitrogenous substrates in the soil water, and at Tollesbury there was insufficient data for analysis. At the Pelenna wetland a significant correlation between nitrous oxide production and $\text{NH}_4\text{-N} + \text{NO}_3\text{-N} + \text{DOC}$ existed for the second half of the year (August – January 1998, $r = 0.862$, $p = 0.027$). These differences between sites with regard to control of nitrous oxide production may be influenced by the relative emissions of nitrous oxide from each site; at CAT flux is always positive, ranging between $< 0.0005 \text{ mg m}^{-2} \text{ d}^{-1}$ and $0.007 \text{ mg m}^{-2} \text{ d}^{-1}$, but at Waun-y-Cwrt, Pelenna and Tollesbury it is occasionally negative and the maximum emission ranges from $0.0065 \text{ mg m}^{-2} \text{ d}^{-1}$ (Waun-y-Cwrt) to $< 0.00001 \text{ mg m}^{-2} \text{ d}^{-1}$ (Pelenna and Tollesbury). At lower levels of emission relationships may be harder to pick out as instrument insensitivity during analysis adds relatively more noise to the data. In addition, ammonia levels at the Waun-y-Cwrt site are extremely high for much of the year, and it seems likely that inorganic nitrogen supply exceeds carbon substrate supply then, making ammonia a poor predictor of nitrous oxide emission because much of it may be volatilized rather than mineralized, or remain untransformed.

Reddy *et al* (1982) found that denitrification in anaerobic soils was proportional to the concentrations of the substrates nitrate and available carbon, and that it was also influenced by the rate at which available carbon was mineralized and therefore made available to microorganisms. They found a significant correlation between nitrate consumption and carbon dioxide production, which was confirmed at the Pelenna wetland (soil water nitrate vs. carbon dioxide emission, $r = 0.695$, $p = 0.018$). Continuously saturated sites like Waun-y-Cwrt and Pelenna may have much less dynamic nitrogen cycling and less denitrification than intermittently wet/dry sites (Groffman & Hanson 1997). The nutrient status of the wetland substrate may also be influential, as nutrient-poor substrates tend to produce low-nutrient vegetation which in turn produces low carbon quality/low nitrogen content litter; this can only support low levels of microbial activity and denitrification (Groffman & Hanson 1997).

Temperature, nitrogenous substrates and dissolved organic carbon were all found to influence nitrous oxide flux, but the relationships varied between different sites. This was not unexpected given the different purposes, water inputs and construction/operation of the wetlands. Nitrate was related to carbon dioxide flux at one wetland.

Carbon dioxide

According to Moore & Knowles (1989), temperature appears to be the main controlling factor of carbon dioxide emissions from soils, largely because (at least in aerobic soils) microbial biomass responds to an increase in temperature with an increase in respiration (Gersberg *et al* 1983). The rate of carbon dioxide efflux from soil can be used as a measure of total soil respiration (Bridgham & Richardson 1992), even though it encompasses respiration by microorganisms, macrofauna and subsurface plant biomass. Yavitt *et al* (1987) found carbon dioxide production changed seasonally in surface peat from a *Sphagnum* bog in West Virginia, although microbial activity is reported to be much less seasonal

than plant activity (Sommerfeld *et al* 1993). Seasonal variation in temperature correlated significantly with variation in carbon dioxide emission at the CAT, Waun-y-Cwrt and Pelenna wetlands, supporting these findings. At the Tollesbury wetland there was no significant relationship, demonstrating a possibly more complex control of carbon dioxide emission, and therefore of microbial metabolism. If carbon dioxide is taken as an indicator (rather than a quantitative measure) of microbial metabolism, the sites can be ranked (on the basis of their annual average emission) in order of microbial activity as Waun-y-Cwrt > Centre for Alternative Technology > Pelenna > Tollesbury. Soil microbial activity is related to climatic conditions and soil type, although the most important factor is the amount and type of the various microorganisms (Goodman *et al* 1992). Variations in microbial biomass within and between wetlands are caused by factors such as organic matter quality and quantity, hydrology, plant type and dynamics, and disturbance (Groffman *et al* 1996); as all of these factors varied between the four wetlands, variations in their respective microbial activities would be expected. Groffman *et al* (1996) go on to report that determining systematic differences between different wetland types would be useful for functional evaluation of wetlands in nutrient cycling, water quality maintenance and wetland construction and restoration contexts.

At CAT, Waun-y-Cwrt and Pelenna wetlands, plants were included within the gas collection chambers, and would therefore have contributed (via their own respiration and photosynthesis) to the carbon dioxide flux; the size of this contribution is highly likely to vary seasonally, possibly with temperature. At these three wetlands carbon dioxide emissions were comparable in magnitude, ranging from 0-3.5 mg m⁻² d⁻¹, with occasional negative fluxes. However at Tollesbury carbon dioxide emission was frequently negative and, when positive, no higher than 0.6 mg m⁻² d⁻¹; the negative fluxes are difficult to explain in terms of micro-algal photosynthesis as they are maximal in winter rather than summer. The diurnal tidal inundation of this site may heavily influence carbon dioxide

emission, since Moore & Knowles (1989) found an inverse relationship between water table and carbon dioxide emission in peat soil. However Moore & Knowles (1989) also reported that methane emissions showed the reverse pattern, but this was not shown at Tollesbury.

At Pelenna, soil water nitrate-N concentration was a far better predictor of carbon dioxide emission than temperature. As the inflowing mine drainage is nutrient poor, it is possible that the microbial biomass is nitrogen limited, and so responds to raised nitrate concentrations with bursts of metabolic activity which in turn raise soil carbon dioxide emissions.

Moore & Knowles (1989) used the molar ratios of carbon dioxide and methane emitted from soil to compare the relative importance of these two gases. They found that even under anaerobic conditions, carbon dioxide was the dominant gas emitted from peat columns. Data from this study did not support these findings of Moore & Knowles.

Temperature had the most marked influence on carbon dioxide fluxes from these wetlands, and variations in carbon dioxide flux between sites indicates variations in the microbial biomass; these may be related to the functions of the different wetlands.

Methane

Most biogenic methane is believed to be derived from acetate cleavage or hydrogen reduction of carbon dioxide (Oremland *et al* 1982). If these compounds are removed by sulphate-reducing bacteria (which are energetically more efficient than methanogens (Oremland *et al* 1982), then methanogenesis will be substrate limited (Yavitt *et al* 1987) and acetate converted to carbon dioxide. Variables which have previously been reported to control methane emission from wetlands include hydrology, soil temperature, quality and quantity of substrates, and water

chemistry (availability of nitrate and sulphate) (Crill *et al* 1991), soil composition, plant productivity and oxygen concentration (Bianchi *et al* 1996), and soil pH and redox status (Crozier *et al* 1995). In this study there was evidence that nitrate and oxygen availability, and dissolved organic carbon concentration, influenced methane flux. Methane production will not respond to an increase in temperature if some other environmental factor is limiting production (Yavitt *et al* 1987).

In a seasonal study, Yavitt *et al* (1987) found that rates of methane emission from a *Sphagnum* bog in West Virginia increased from February to November, and Dise *et al* (1992) reported that methane emission is often correlated with temperature due to activation of methanogens at higher temperatures. Press *et al* (1995) also reported higher rates of methane emission from a peat bog between July and September, but found no correlation between emission and soil temperature. Emissions data from the CAT, Waun-y-Cwrt, Pelenna and Tollesbury sites agreed with this latter work, as there was no evidence of seasonal patterns of emission. However at the CAT wetland, methane emissions peaked briefly in August 1997 and July 1998. Yavitt *et al* (1987) concluded that in winter, methane production must be limited by other factors besides low temperatures, possibly a low population of methanogens or reduced activity of the hydrolytic organisms which convert polymeric organic matter to fermentable substrates. Anaerobiosis is a major condition for the activity of methanogenic bacteria because they are obligate anaerobes (Tiedje *et al* 1984), whereas under aerobic conditions soils consume methane (Moore & Knowles 1989). All four of the wetlands studied had fluctuating water levels which would lead to intermittent aerobic and anaerobic conditions.

Methane production in soils is suppressed by nitrate, even at very low levels (Bell 1969), and because nitrate must be removed before methanogenesis can begin, denitrifying organisms have a primary influence on methanogen activity (Bollag & Czlonkowski 1973). At the CAT and Waun-y-Cwrt wetlands the data describing

methane emission and soil water nitrate-N concentration did not support these findings; however at the Pelenna wetland a significant inverse correlation between these two factors did support the work. At the Pelenna site, nitrate availability appears to influence both carbon dioxide emission (directly) and methane emission (inversely), which seems to point towards switching between aerobic and anaerobic metabolism dominance in this wetland. It is highly likely that this in turn is controlled by the degree of flooding of the wetland, but no quantitative data about this were obtained. According to Moore & Knowles (1989), a reduced water table can reduce methane fluxes but increase carbon dioxide fluxes, presumably by forcing a switch from anaerobic to aerobic metabolism by soil bacteria.

Methane production in soils is suppressed by redox potentials which are too high (Bollag & Czlonkowski 1973). The structure and concentration of available organic substances also influences methane formation; for example Bollag & Czlonkowski (1973) found that low levels of glucose would increase methane production, but higher levels (10 mg compared to 2.5 mg) suppressed it and simultaneously increased carbon dioxide production. It seems likely that the higher glucose supplementation stimulated non-methanogenic bacteria which outcompeted methanogens and/or aerated the soil. At Waun-y-Cwrt dissolved organic carbon correlated significantly with methane emission ($r = 0.768$, $p = 0.004$), agreeing with comments by Bollag & Czlonkowski (1973) and Crill *et al* (1991) that organic substances influence methanogenesis. Since the chemical composition, and therefore the microbial utilization, of DOC varies greatly according to its source (Bianchi *et al* 1996), it should perhaps not be surprising that not all of the wetlands showed correlations between methane production and measured DOC concentration.

Bollag & Czlonkowski (1973) comment that methane formation could be controlled if it caused an ecological problem by adding nitrogenous compounds, to prevent the activity of methanogens. Anaerobic metabolism tends to produce more

dissolved organic carbon products such as organic acids, alcohols, aldehydes and ketones (Ponamperuma 1972), whereas aerobic is more efficient and tends to result in carbon dioxide (gaseous inorganic carbon). Methanogenesis requires stricter environmental conditions than carbon dioxide evolution, so there is greater variability in fluxes (Moore & Knowles 1989); methane is also less soluble in water than carbon dioxide, so is emitted primarily as bubbles. Williams & Crawford (1984) report that methanogenesis is restricted in acid conditions, and in culture methanogens metabolize best between pH 6.7 and 8.0. All the study sites in this survey had soil pH below this, however even in acid conditions there may be neutral micro-sites where methanogenesis occurs (Williams & Crawford 1984).

Plants promote the release of methane from wetlands (Holzapfelschorn *et al* 1986) because methane formed in the anaerobic soil layer would normally be oxidized as it emerged through the aerobic surface soil layer, but this is avoided if the gas enters plants and is released to the atmosphere via the aerenchyma tissue (Roura-Carol & Freeman 1999). Plants may also stimulate methane production by providing carbon sources (via root exudation) in the anaerobic zone which support methanogenic microorganisms (Roura-Carol & Freeman 1999); however as plant roots are also believed to supply oxygen to such anaerobic zones, any exudates would have to diffuse further than the aerobic rhizosphere to provide such stimulation. Methane oxidation has been reported in the rhizosphere (King 1994), but may be limited by competition between root respiration, aerobic microbial metabolism and ferrous iron oxidation for oxygen (Chanton *et al* 1992). Plant metabolism can adversely affect methane emission by allowing methanotrophic bacteria to oxidize methane (Roslev & King 1994), but on the other hand Gerard & Chanton (1993) report that plant-enhanced methane transport is more significant than plant-enhanced methane oxidation. Bianchi *et al* (1996) reported negative methane fluxes following droughts and flooding events, and cited work which found methane oxidation occurred when soils were not flooded.

Dise (1992) reported a sharp decline in methane emission from a northern peatland in late August when the plants senesced, whereas Wilson *et al* (1989) found the opposite, a late summer peak in emission which they also attributed to plant senescence and concomitant release of organic materials. The decline in methane emission at the CAT wetland in late summer contrasts with an increase at the Pelenna site, the sites seeming to respectively support the two studies as plants began to die back after the summer. To accurately predict soil methane emission rates, the size of the labile carbon pool and the effect of soil oxidized zones must be quantified (Crozier *et al* 1995), and to pinpoint the factors dictating methane emission from these wetlands a detailed study of soil aerobic/anaerobic status and methanogenic substrates would be required. Seasonal changes in methane emission may be closely linked to the dynamics of vascular plant communities in the wetlands (Wilson *et al* 1989).

Freeman *et al* (1997) found that addition of sulphatase to peat decreased methane production, probably by releasing sulphate, which stimulated sulphate-reducing bacteria which outcompeted methanogenic organisms. Neither sulphatase nor sulphate correlated with methane emission at the CAT, Waun-y-Cwrt or Pelenna wetlands, suggesting that this mechanism of methane suppression was not active in the wetland soils.

Comparison of the gas emissions from all four constructed wetlands sampled in this study with soil gas emissions from studies of natural wetlands shows that the constructed wetlands have relatively low levels of emission (Table 3.20).

Table 3.20

Other studies of soil gas emissions

Author	Kang <i>et al</i> , 1998b	Freeman <i>et al</i> 1993	Whiting & Chanton, 1993	Williams & Crawford, 1984	This study
Soil type	Welsh fen	Welsh peatland cores	<i>Typha</i> peat bog	Peatland	Sand/peat
N ₂ O	-0.05-0.35 mg m ⁻² d ⁻¹	0-0.25 mg m ⁻² d ⁻¹			-7.5 ng – 0.007 mg m ⁻² d ⁻¹
CH ₄		100-650 mg m ⁻² d ⁻¹	100-360 mg m ⁻² d ⁻¹	200 nmol g ⁻¹ hr ⁻¹	-1.28-0.39 mg m ⁻² d ⁻¹
CO ₂		400-800 mg m ⁻² d ⁻¹	6.5-10 g m ⁻² d ⁻¹		-0.86-3.49 mg m ⁻² d ⁻¹

Nitrous oxide emissions from the studied constructed wetlands are circa 1 % of those in the natural wetlands studied by Kang *et al* (1998b), Freeman *et al* (1993), Whiting & Chanton (1993), and Williams & Crawford (1984), suggesting that nitrogen cycling is much lower in artificial soils than in natural ones. Similarly methane emissions from constructed wetlands were only about 0.1 % of those found in the above studies, and carbon dioxide emissions from constructed wetlands were approximately 1 %, of those measured in the above studies of natural wetlands. This evidence does not support hypothesis 1, which stated that constructed wetlands would have similar fluxes of nitrous oxide, methane and carbon dioxide to natural wetlands. There was some support for hypothesis 2 (which stated that soil enzyme activity would influence soil gas fluxes) as there were 14 significant correlations between enzyme activities and gas fluxes at various sites.

The low levels of methane and carbon dioxide production indicate much lower

levels of microbial respiration in these constructed wetlands than in natural wetlands, which may correspond with the smaller quantity of organic material stored in the substrate of constructed wetlands. They may also reflect the generally low organic content of the artificial substrate (e.g. sand or gravel rather than *Sphagnum* peat or natural soil).

b. Enzyme activity in constructed wetlands

The relationship between soil enzyme activity and temperature has rarely been investigated (McClaugherty & Linkins 1990), despite the evident importance of temperature as a controlling factor in many decomposition processes (Linkins *et al* 1984), including those which are extracellular. Examining the temperature responses of enzymes in constructed wetlands could indicate the potential for decomposition at different latitudes. The results of this field study confirmed a relationship between soil temperature and extracellular carbon cycling enzyme (cellobiohydrolase and β -glucosidase) activity at CAT, between temperature and β -glucosidase and N-acetylglucosaminidase activity at Waun-y-Cwrt, between temperature and phosphatase activity at Pelenna and between temperature and β -glucosidase activity at Tollesbury. The lack of correlation between temperature and activity of other enzymes (60 % of the enzyme data) may be a consequence of the different substrates used to construct each wetland, as the type of binding which secures extracellular enzymes within wetlands not only depends on the substrate and its organic matter content, but also influences their temperature responses (McClaugherty & Linkins 1990). However the degree of inter-correlation between enzyme activities at all sites suggests that one (or two closely-related) factors are controlling the bulk of enzyme activity; temperature is extremely likely to be one of these because of its fundamental influence on chemical and biological processes (Gersberg *et al* 1983).

Phenol oxidase activity did not correlate significantly with any other enzymes at

any of the four sites (Figure 3.3). This suggests that its activity is influenced by entirely different factors to those which control hydrolase activity, and that it does not play a significant part in maintaining the supply of nutrients from organic matter. At the Waun-y-Cwrt wetland phosphatase was similarly uncorrelated with other hydrolases, as was N-acetylglucosaminidase at the Tollesbury wetland. Whilst phosphatase activity was much higher at Waun-y-Cwrt than were other enzymes, the concentration of inorganic phosphate in the wetland substrate was much lower than the concentration of other nutrients. This may tentatively be taken as support for the theory that low levels of inorganic phosphate lead to high levels of phosphatase activity (Nannipieri *et al* 1978, Spier & Ross 1978).

The levels of activity of the enzymes studied varied in range by two orders of magnitude between the four sites, but did not exceed the range previously reported for natural systems by Kang (1999), Chappell & Goulder (1994a, 1994b), Ueno *et al* (1991) and Chróst & Rai (1993) (Table 3.21). In terms of annual average activity ($\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$), the sites were ranked in the order CAT > Pelenna > Tollesbury > Waun-y-Cwrt. However the density of the wetland soils was variable (Table 3.1) and affects the comparison of activities between sites because it was measured as rate of hydrolysis per weight of soil.

Table 3.21

Other fluorogenic studies of extracellular enzyme activity

	This study	Kang 1999	Chappell & Goulder 1994a	Chappell & Goulder 1994b	Ueno <i>et al</i> 1991	Chrost & Rai 1993
Sample type	Sand/peat	Natural wetland soils	Epilithic	Epiphytic	Forest soil	Fresh water
β -glu	0.0012-1.256	0.005-0.0225	1-3.5	254		9-28
NAG	0.00053-0.909	0.003-0.044			0.02-11.4	
Pho	0.0066-2.56	0.02-0.18	0-40	978		
Sul	0.00059-0.457	0.001-0.016	0-0.4	2.99		
Xyl	0.0019-0.322	0.0025-0.017	0.25-1.5	48.3		
Units	$\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$	$\mu\text{mol MUF g soil}^{-1} \text{ min}^{-1}$	$\text{nmol MUF cm}^{-2} \text{ h}^{-1}$	$\text{nmol MUF g dry plant}^{-1} \text{ h}^{-1}$	$\text{nmol MUF cm}^{-2} \text{ h}^{-1}$	$\text{nmol MUF l}^{-1} \text{ hr}^{-1}$

Chappell & Goulder (1994a) measured the activity of freshwater epilithic extracellular enzymes in three streams, and found that the relative activities in all streams fell in the order phosphatase > β -D-glucosidase > β -D-galactosidase > β -D-xylosidase > sulphatase. This was also the order of relative activity of phosphatase, β -D-glucosidase, β -D-xylosidase and sulphatase at the Waun-y-Cwrt, Pelenna and Tollesbury sites in this field study; at the CAT site sulphatase and β -D-xylosidase relative activities were transposed, but still lower than those of both phosphatase and β -D-glucosidase. These results probably indicate the relative importance to the microbial community of the respective products of these enzymes, irrespective of the type of wetland involved. The greater activity towards

cellulosic substrates (β -D-glucosidase) compared to hemicellulosic substrates (β -D-xylosidase) at all four sites suggests the greater importance of cellulose over hemicellulose for the microbial population.

Fluorogenic substrates such as MUF substrates do not penetrate cytoplasmic membranes, but are hydrolysed in the periplasmic space (Martinez & Azam 1993), and therefore measure extracellular enzyme activity (that associated with the periplasmic space, bound to cell walls, and of enzymes released from cells) (Chappell & Goulder 1994a). The enzymes measured in a study such as this are functionally defined by the substrates used, and so each measured activity may be a composite of isoenzymes varying between sites and seasons (Chappell & Goulder 1994a). In addition, the soil enzymes are unlikely to be entirely substrate specific so that for example β -D-glucosidase may act on β -D-galactoside and β -D-xyloside substrates (Chappell & Goulder 1994a).

The products of extracellular enzymatic hydrolysis may be low-molecular-weight organic compounds such as simple sugars and amino acids, or inorganic nutrients such as phosphate and sulphate; all of these are available for microbial uptake (Chappell & Goulder 1994b). As no distinction in analysis was made between high and low molecular weight carbon during this field study, it is impossible to discover seasonal changes in the composition of the soil water DOC. Such a distinction could be extremely useful, for example in helping to explain the marked drop in enzyme activities seen in spring at CAT, Waun-y-Cwrt and Pelenna. Kang (1999) also noted a decline in enzyme activity in natural (vegetated) wetlands, and attributed it to competition between plant root systems and microorganisms for nutrients; Kang suggested that during fast growth, root exudation dropped and rhizosphere bacteria (and thus enzyme activity associated with them) also declined. Wilson *et al* (1989) however cite the opposite case, with active root growth in spring increasing the translocation of photosynthate to, and

thus exudation by, roots. This observation is investigated in Chapter VI.

Carbon cycling enzymes showed a strong positive relationship with temperature at three sites, but the majority of enzyme activity was not significantly related to soil temperature. However, the activity of all enzymes (except phenol oxidase) at each site tended to vary according to the same pattern; phenol oxidase activity appeared to vary independently of other enzymes, as did one or two hydrolases at some sites. The soil enzyme activity of these constructed wetlands was comparable to values reported for soil from natural wetlands, and the relative activities fell in the same order as those in some other ecosystems. This evidence supported hypothesis 1, which stated that constructed wetlands would show similar levels of nutrient cycling to natural wetlands. A marked springtime drop in enzyme activity was unexplained by variables measured as part of the field study.

c. Hydrochemistry and soil enzyme activity in constructed wetlands

The concentration of different ion species in wetland soil could potentially influence extracellular enzyme activity by influencing the growth, survival and/or population structure of the microbial population. In this study, better correlations between enzyme activities and hydrochemistry were found where more data were available. There were no hydrochemical correlations which applied across all four sites.

Despite evidence in previously published work, few correlations were found between the activity of a specific enzyme and its target substrate(s). For example the widely accepted theory that phosphatase activity depends inversely upon the availability of inorganic phosphate (Nannipieri *et al* 1978, Spier & Ross 1978) was not substantiated by data from any site in this study, although there was a strong positive correlation at the Pelenna wetland, and at the Waun-y-Cwrt wetland very high phosphatase levels were matched by very low phosphate concentrations all year. Similar supply and demand theories about sulphatase

activity were not supported by the results either. With the possible exception of nitrate-N for N-acetylglucosaminidase extracellular enzyme activities are apparently not related to the nutrient cycles which they represent.

A better predictor of enzyme activity was found to be DOC, which was significantly correlated with phosphatase activity at CAT (inversely), with cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase and sulphatase activity at Peleenna, and with β -glucosidase activity at Tollesbury. Whether higher levels of DOC lead to higher enzyme activity (e.g. through stimulation of microbial metabolism), or higher enzyme activity causes release of more soluble (low molecular weight) compounds is impossible to determine from this data. The concentration of phenolic materials in the soil water also showed significant correlations with some enzyme activities; at the CAT wetland with cellobiohydrolase, β -glucosidase and xylosidase, at Waun-y-Cwrt with phosphatase and phenol oxidase, and at Tollesbury with sulphatase. All these enzymes may be involved in the primary or secondary breakdown of phenolic materials.

The four constructed wetlands studied did not show consistent correlations between hydrochemistry and enzyme activity; thus there is no support for hypotheses 2 or 3, which state that soil enzyme activity will influence water chemistry, and also bear relation to soil physico-chemical factors. However, dissolved organic carbon was a good predictor of activity for several enzymes at different sites, although the cause and effect could not be separated.

Conclusions

- Constructed wetlands appear to have much lower emissions of the radiatively active gases nitrous oxide, methane and carbon dioxide than natural wetlands.

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- There was some evidence that nitrous oxide production correlated with temperature, and with the level of nitrogenous substrates and dissolved organic carbon available in the wetland.
 - Methane fluxes were not seasonally affected, but there was some evidence that nitrate may be suppressing methane production at one wetland; there was also evidence that organic substrates influenced methanogenesis.
 - Carbon dioxide fluxes were closely related to soil temperature, indicating the increase in microbial respiration during warmer months. There was evidence from one site that nitrate availability influenced carbon dioxide flux.
 - Extracellular enzyme activity in these constructed wetlands was generally lower than in natural wetlands, suggesting slower nutrient cycling. However the relative order of enzyme activities suggested that the importance of the enzymic products to the microbial community was similar to that in natural systems.
 - Faster extracellular cycling at higher temperatures suggested that constructed wetlands may be decompose organic matter more efficiently in warmer climates.
 - Dissolved organic carbon and phenolic materials were good indicators of enzyme activity.

Chapter IV

The role of biogeochemistry in water
quality improvement

The role of plants in water quality
improvement

The role of wetland biogeochemistry in water quality improvement

4.1 Introduction

Water quality improvement in constructed wetlands occurs largely within the soil or substrate of the wetland, and for organic waste depends on nutrient cycling processes identical to those which occur in most soils. For sewage treatment there is the additional requirement of pathogen removal. By monitoring the water chemistry of the wetland inflow and outflow at the same time as the soil nutrient cycling, it should be possible to establish links between effective treatment and the activity of specific enzymes or gas fluxes; for example good removal of nitrogenous substrates should link with high nitrogen cycling, as indicated by high nitrous oxide emission.

The Centre for Alternative Technology wetland was chosen as a study site due to its established treatment function (seven years in 1998), its organic waste inflow (domestic sewage), and reasonable proximity to the laboratory for sample analyses. This wetland receives neat sewage liquid, and discharges clean water which complies with water quality standards. The ability to study reduction of bacteria across the system (due to the sewage origin of the wastewater) was important.

Sampling at the Centre for Alternative Technology wetland continued on a monthly basis from January to December 1998. Soil, gas and water samples were collected from the wetland and analysed in the laboratory using the methods described in Chapter III. In addition, samples of inflowing wastewater and outflowing water were collected each month and analysed for nutrient ions, phenolic compounds, dissolved organic carbon, Biological Oxygen Demand and *Coliform* counts. Both Biological Oxygen Demand (BOD₅) and *Coliform* counts are important and widely used parameters for describing the quality of water discharged from sewage treatment works.

The aims of this fieldwork study were as follows

- to investigate seasonal changes in treatment efficiency over the course of a year
- to investigate the relationships between water quality improvement and nutrient cycling (indicated by soil enzyme activity and gas fluxes), soil water chemistry, and other soil parameters such as pH and temperature
- to establish whether the Centre for Alternative Technology wetland could adequately remove the BOD₅ and *Coliform* count of the inflowing sewage

The following hypotheses were to be tested:

- 1 There will be seasonal variations in the treatment efficiency of the wetland
- 2 Treatment efficiency will be related to soil enzyme activity, gas flux, and possibly other soil parameters

4.2 Methods

a. Determination of five-day Biological Oxygen Demand

The principle of the five-day Biological Oxygen Demand (BOD₅) measurement is that during a set period of incubation (usually five days) in conditions of controlled light and temperature, the microbial population in a water sample will oxidize some or all of the organic matter present, using up the available (dissolved) oxygen of the water sample in the process. The BOD₅ of a water sample gives an indication of its potential to damage an environment by sequestering oxygen which would maintain the aerobic status of the environment or otherwise benefit higher organisms. The measurement is obtained by measuring dissolved oxygen in the water sample before and after incubation.

Controlling temperature, light and duration of incubation is important to allow comparison between samples measured on different occasions. If the dissolved oxygen (DO) of a sample is low to begin with and the BOD₅ high, dilution with

oxygenated water adds sufficient oxygen to obtain a depletion over the incubation period. The appropriate dilution must be found by trial and error, or from previous experience. If the microbial population of the sample is very small, a seed culture is added to obtain the oxygen depletion over the incubation period; the contribution of this seed culture is monitored with seed blanks and taken into account when calculating the BOD₅.

To avoid errors caused by organic matter present or accumulating in the bottles used for BOD₅ measurement, these are regularly rinsed with acidified potassium iodide solution (2.5 g l⁻¹ iodine, 12.5 g l⁻¹ potassium iodide in 1% m/V sulphuric acid) and stored unrinsed. Just before use the bottles are rinsed with tap water.

Water samples for BOD₅ determination were collected from the wetland inflow and outflow by completely filling 2 litre capacity wide-neck containers, sealing them with cling film stretched over the water surface and screwing the lids on. During sample collection care was taken that no air bubbles were entrained by the water stream. Samples were transported back to the lab in an insulated box with ice packs, and sample preparation was begun as soon as possible (usually within 1 hour).

Water samples were checked for pH neutrality; if they were outside the range 6.0 - 8.0, alkali or acid was added dropwise to obtain pH 7.

Deionized water for sample dilution was bubbled at 20°C for thirty minutes with compressed air to saturate it with oxygen. 1 ml l⁻¹ each of phosphate buffer solution (8.5 g l⁻¹ KH₂PO₄, 21.75 g l⁻¹ K₂HPO₄, 33.4 g l⁻¹ Na₂HPO₄·4.7H₂O, 1.7 g l⁻¹ NH₄Cl in distilled water), magnesium sulphate solution (22.5 g l⁻¹ MgSO₄·7H₂O in distilled water), calcium chloride solution (27.5 g l⁻¹ anhydrous CaCl₂ in distilled water) and ferric chloride solution (0.25 g l⁻¹ FeCl₃·6H₂O in distilled water) were added just before the water was used.

Samples were diluted ten and one hundred fold (occasionally one hundred and one thousand fold) for oxygen measurement immediately and after five days' incubation. No seed culture needed to be added to either inflow or outflow samples as there was always a sufficient microbial population to oxidize the organic matter in the samples.

Diluted samples were siphoned into 250 ml dark glass bottles which were filled brim full, then closed to exclude air bubbles by introducing the stopper at an angle. Two replicates of each dilution from each sample were prepared for each of the immediate and five-day oxygen determinations (total 16 samples). Two identical bottles were also filled with prepared dilution water to check for oxygen depletion in this over the five-day incubation.

Dissolved oxygen (DO) in the samples was determined with a Jenway Model 9015 oxygen meter, calibrated with sodium sulphite solution (20 g l^{-1}) for zero oxygen, and 100% relative humidity (electrode held 1 cm above shaken distilled water) for 100% oxygen. Dissolved oxygen in half the diluted samples and in one blank dilution water was measured immediately. The remaining diluted samples and blank dilution water were incubated for five days at 20°C in the dark. Dilutions which showed a residual DO of at least 1 mg l^{-1} and a depletion of at least 2 mg l^{-1} were used for calculations.

Calculation

Biological Oxygen Demand when seeding is not required

$$\text{mg l}^{-1} \text{ 5 days}^{-1} \text{ BOD} = \frac{D_1 - D_2}{P}$$

where D_1 = DO of diluted sample 15 minutes after preparation

D_2 = DO of diluted sample after incubation

P = decimal fraction of sample used

b. Enumeration of *Coliform* organisms

Enumeration of *Coliform* micro-organisms in wetland water samples was carried out by incubation on the specific medium McConkey agar. Specificity for *Coliforms* is due to bile salts which inhibit growth of non-*Coliforms*, lactose which nurtures lactose-fermenters, and neutral red which stains *Coliform* colonies reddish; *Salmonella* species are also culturable on McConkey agar, but appear white.

Samples of water from the inflow and outflow to the wetland were collected in clean (not sterile) jars and kept at less than 4°C until use (within three hours of collection). Sterile collecting jars were not considered essential due to the rarity of *Coliform* organisms in general laboratory storage; culture of bench swabs confirmed their absence.

McConkey agar was prepared according to manufacturer's directions (50 g l⁻¹), autoclaved and poured into sterile Petri plates. Once gelled, these were inverted and left for two days to dry. Before use the plates were incubated at 37°C for one hour, open but agar side down, to further prevent condensation during the growth time.

Dilutions of inflow water samples were made at 1000, 10,000 and 100,000 times, and of outflow water samples at 1, 10 and 100 times, using sterile phosphate buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ in 800 ml of deionized water; adjust to pH 7.4 with conc. HCl, dilute to 1000 ml). Three replicate plates were inoculated with 100 µl each of a diluted sample using aseptic techniques (total 18 plates); these plates and two uninoculated controls were incubated upside down at 37°C for 24 hours.

Plates with between 30 and 300 colony forming units (cfu) are generally considered accurate for counting. The average number of cfu ml⁻¹ of sample was calculated. Immediately after counting all inoculated plates were autoclaved and disposed of.

c. Water chemistry

Nutrient ions (SO₄-S, NO₃-N, NH₄-N and PO₄-P) were determined using Skalar Autoanalyser continuous segmented flow colorimetry, as described in Chapter III. Dissolved organic carbon was also measured using Skalar colorimetry, using ultra-violet digestion followed by reaction of the carbon dioxide thus evolved with a pH-sensitive colour reagent, and measurement at 550 nm. Phenolics were measured using the Box assay described in Chapter III. All analyses were applied to inflowing and outflowing water, and to soil water.

d. Statistical analysis of results

The data describing soil enzyme activity, soil gas fluxes, soil water chemistry, temperature and pH were used in Pearson correlation matrices, where data conformed to the Normal distribution. Data which were not Normally distributed were analysed using Spearman correlations. Further correlation matrices were constructed to analyse data describing inflow and outflow water chemistry.

4.3 Results

A correlation matrix was used to analyse the inflow, soil and outflow data. Significant results are presented in Tables 4.1 a-e. Upright type indicates Pearson correlations, italic type indicates Spearman correlations.

Table 4.1 a

Significant correlations between soil water chemistry factors, CAT wetland, January-December 1998 (r = correlation coefficient, p = probability).

	Soil PO ₄ -P	Soil NH ₄ -N	Soil SO ₄ -S	Soil phenolics	Soil DOC
Soil NO ₃ -N	r -0.687				
	p 0.014				
Soil DOC	r 0.576				
	p 0.050				
Temp	r 0.572	-0.607	0.544	0.686	
	p 0.052	0.036	0.068	0.014	

Table 4.1 b

Significant correlations between soil enzyme activities and water quality factors, CAT wetland, January-December 1998 (r = correlation coefficient, p = probability).

	Cel	β-glu	NAG	Pho	Sul	Xyl	Ph.ox.
CH ₄	r -0.554						
	p 0.097						
CO ₂	r -0.587						
	p 0.058						
Soil PO ₄ -P	r 0.563					0.682	
	p 0.056					0.030	
Soil NO ₃ -N	r -0.503						-0.578
	p 0.096						0.080
Soil DOC	r 0.560						0.560
	p 0.092						0.092
Outflow BOD ₅	r 0.669						
	p 0.017						
Temp	r 0.559						
	p 0.059						

Table 4.1 c

Significant correlations between inflow water quality factors, CAT wetland, January-December 1998 (r = correlation coefficient, p = probability).

	Inflow	Inflow	Inflow	Inflow	pH	Temperat
	PO ₄ -P	NO ₃ -N	DOC	NH ₄ -N		ure
Inflow r	0.822	0.820	0.801	0.606	-0.689	
Phenolics p	0.002	0.002	0.003	0.048	0.019	
Inflow r	0.829	0.740		0.765	-0.608	
DOC p	0.002	0.009		0.006	0.047	
Inflow r						0.796
BOD p						0.002

Table 4.1 d

Significant correlations between outflow water chemistry factors, CAT wetland, January-December 1998.

	Outflow	Outflow	Outflow	Outflow	Outflow	pH
	PO ₄ -P	SO ₄ -S	NH ₄ -N	phen	BOD	
Outflow r	0.579	0.790				-0.701
NO ₃ -N p	0.062	0.004				0.016
Outflow r	0.760			0.553		
NH ₄ -N p	0.007			0.078		
Outflow r	0.736		0.653	0.733		
DOC p	0.01		0.029	0.010		
Outflow r	0.692		0.615	0.558		0.658
BOD p	0.018		0.044	0.074		0.020
Outflow r					0.673	0.661
Coliforms p					0.047	0.053
Temp r			0.617	0.641	0.744	
p			0.043	0.034	0.006	

Table 4.1 e

Significant correlations between soil gas fluxes and water quality factors, CAT wetland, January-December 1998 (r = correlation coefficient, p = probability).

		N₂O	CH₄	CO₂
CO₂	r	0.547		
	p	0.081		
Outflow PO₄-P	r	0.684		
	p	0.029		
Outflow NH₄-N	r	0.655		
	p	0.040		
Temperature	r	0.593		0.833
	p	0.054		0.001
Inflow BOD₅	r	0.666		0.703
	p	0.025		0.016
Outflow BOD₅	r	0.776		0.716
	p	0.005		0.013
pH	r	0.582		
	p	0.060		
Outflow NO₃-N	r		0.819	
	p		0.007	
Soil NH₄-N	r			0.724
	p			0.012
Soil DOC	r		0.619	0.848
	p		0.057	0.001

Five-day Biological Oxygen Demand of inflowing water increased steadily in the first six months of the year, and thereafter declined with a slight rise in September. In outflowing water the pattern was very similar, and the Spearman correlation between inflowing and outflowing BOD₅ was significant ($r = 0.682$, $p = 0.014$) (Figure 4.1 a). The reduction in BOD₅ ranged from 0 % (June) to 94 % (November), averaging 61 % per month.

Coliform counts were only successful from April to December (Figure 4.1 c). There was a reduction of over 91% in all months of the year except June; in this month, inflow counts were very low and outflow counts very high. The highest reduction was 99.992% (November 1998); however this still left some 66 colony forming units per ml of water, which vastly exceeds water quality recommendations. Outflowing BOD₅ correlated significantly with outflowing *Coliform* counts (Spearman correlation $r = 0.673$, $p = 0.047$) (Figure 4.1 b).

Figure 4.1

(a) Biological Oxygen Demand in inflow (●) and outflow (○), and (b) outflow Biological Oxygen Demand (○), dissolved organic carbon x 5 (●) and *Coliform* counts (□), at the Centre for Alternative Technology wetland between January and December 1998. (Mean, $n = 2$ for BOD samples, 5 for DOC samples and 3 for *Coliform* counts).

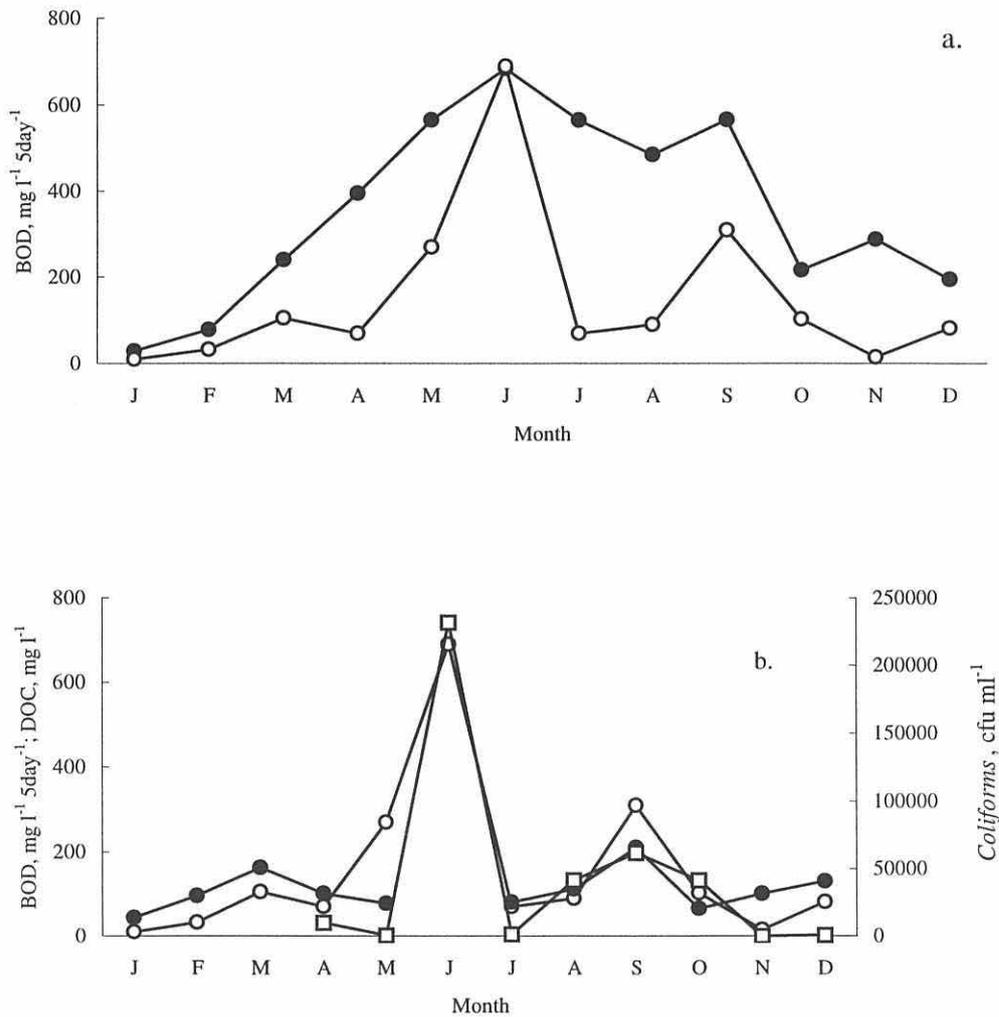
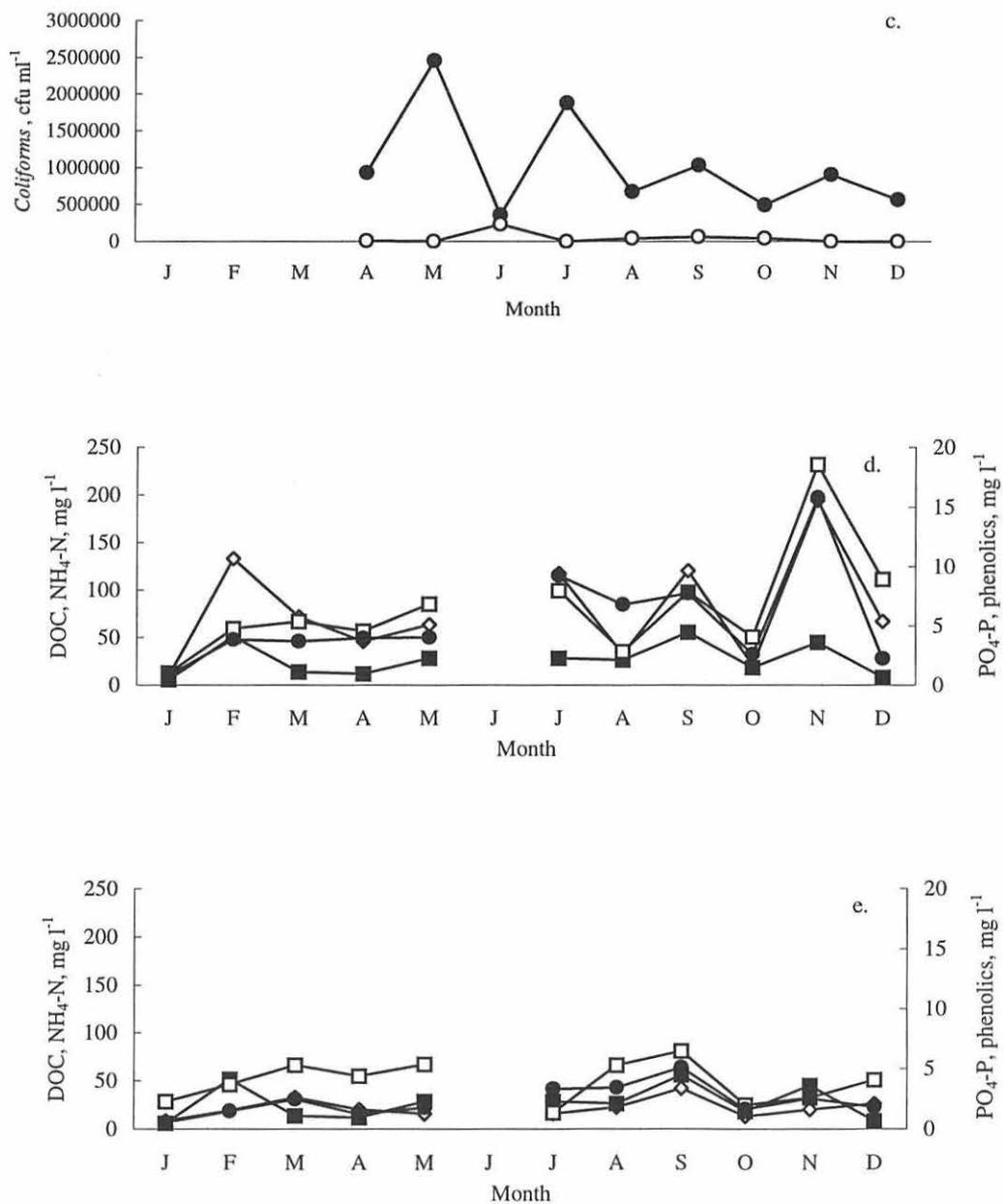


Figure 4.1 contd.

(c) *Coliform* counts in inflow (●) and outflow (○); (d) dissolved organic carbon (◇), ammonium-N (■), phosphate-P (□) and phenolics (●) in inflow; and (e) dissolved organic carbon (◇), ammonium-N (■), phosphate-P (□) and phenolics (●) in outflow, to Centre for Alternative Technology wetland, between January and December 1998. (Mean, $n = 3$ for *Coliform* counts, 5 for water chemistry samples).



Temperature correlated significantly with both inflow ($r = 0.796$, $p = 0.002$) and outflow (Spearman correlation $r = 0.744$, $p = 0.006$) BOD₅.

Inflow and outflow levels of phosphate-P (Figure 4.1 d, e) were similar in the first half of the year (January – May), and different from each other in the second half (July – December), with a large peak in inflowing phosphate-P in November. Outflowing levels were usually, but not always (8 of 11 months) lower than inflowing levels. Sulphate-S levels in both inflowing and outflowing water (Figure 4.1 g) were stable throughout the year with the exception of high inflow sulphate-S in March. Inflowing nitrate-N was extremely low (Figure 4.1 a), as might be expected in sewage, with outflowing nitrate-N nearly always (10 of 11 months) higher than inflow, indicating mineralization of ammonium; this agrees with ammonium-N measurements, which in 9 of the 11 months are higher in inflowing water than in outflowing (Figure 4.1 d, e). Other significant correlations are given in Table 4.2.

To summarise these results, DOC was correlated significantly with phosphate-P, ammonium-N and phenolics in inflow and outflow water (Figure 4.1 d, e). Inflow phenolics were also significantly correlated with phosphate-P and ammonium-N, while in outflowing water ammonium-N and phosphate-P were significantly correlated. In the wetland soil, nitrate-N and phosphate-P were significantly correlated.

Dissolved organic carbon was consistently (and significantly ($t = 3.41$, $p = 0.0028$)) higher in inflowing water compared to outflowing, but fluctuated considerably during the year (Figure 4.1 d, e). Phenolics in inflowing water were consistently (and significantly ($t = 2.43$, $p = 0.025$)) higher than in outflowing water throughout the year.

Table 4.2

Significant correlations between water chemistry factors, CAT wetland, January – December 1998 (r = correlation coefficient, p = probability; upright type indicates Pearson correlations, italic type Spearman correlations).

		Outflow	Soil	Soil DOC	Inflow	Outflow	Outflow
		phenolics	phenolics		BOD₅	<i>Coliforms</i>	BOD₅
Inflow	r	0.546					
phenolics	p	0.082					
Soil	r	0.674					
phenolics	p	0.023					
Inflow	r	0.593	0.573				
NH₄-N	p	0.054	0.065				
Soil	r	0.741			0.722	0.887	<i>0.515</i>
NH₄-N	p	0.009			0.008	0.001	<i>0.087</i>
Outflow	r				0.575		
NH₄-N	p				0.064		
Inflow	r	0.688					
BOD₅	p	0.019					
Soil	r						<i>0.606</i>
DOC	p						<i>0.037</i>
Inflow	r			-0.524			
NO₃-N	p			0.098			

Figure 4.1 contd.

(f) nitrate-N concentration in inflow (●), soil water (□) and outflow (○) and (g) sulphate-S concentration in inflow (●), soil water (□) and outflow (○), of Centre for Alternative Technology wetland between January and December 1998. (Mean, $n = 2$)

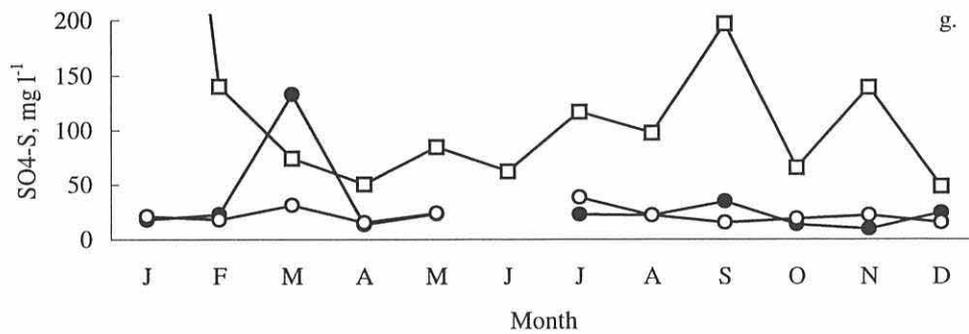
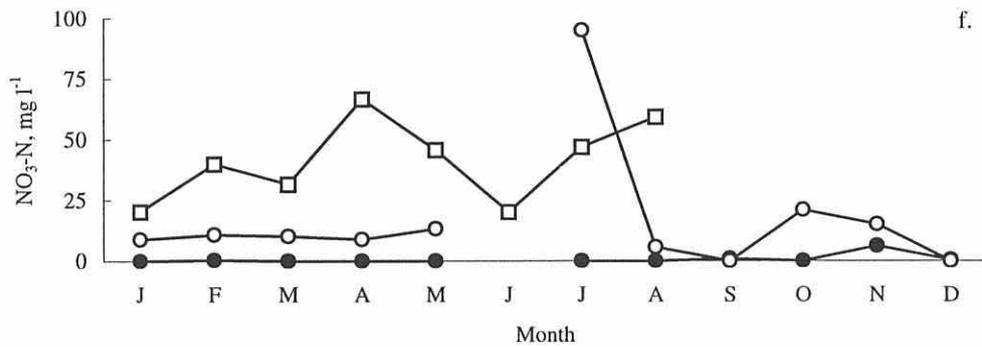
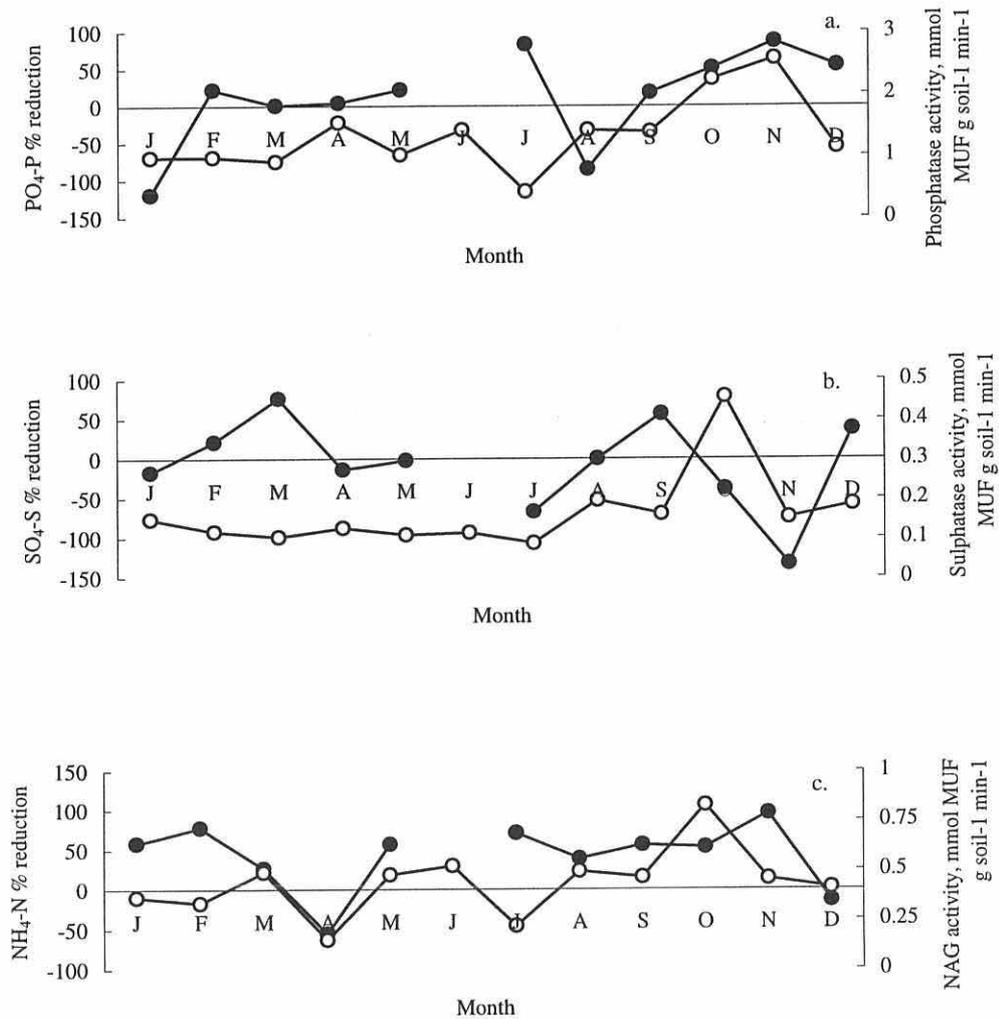


Figure 4.2

(a) Percentage reduction of phosphate-P, compared to phosphatase activity, (b) percentage reduction of sulphate-S, compared to sulphatase activity and (c) percentage reduction of ammonium-N, compared to N-acetylglucosaminidase activity. (Mean, n = 5 for enzyme activities).



4.4 Discussion

Data regarding enzyme activity and soil gas emissions from this wetland during 1998 have already been discussed in Chapter III. The present discussion will focus on water quality improvement and the relationship of soil enzyme activity to changes in nutrient concentration.

A reduction in BOD₅ indicates the removal of solids and stabilization of organic matter (Williams *et al* 1995). In this study, the annual mean removal of Biological Oxygen Demand (BOD₅) during 1998 was 61%, resulting in a mean BOD₅ of 153.8 mg l⁻¹. Gersberg *et al* (1984) reported an annual mean BOD₅ removal of 78% in artificial wetlands constructed to treat primary wastewaters, resulting in a mean BOD₅ of 33 mg l⁻¹ after treatment. The considerable difference between the studies is likely to be due to the larger wetlands (average area 542 m²) used in the study by Gersberg *et al*, compared to the smaller area of the wetland at the Centre for Alternative Technology (160 m²); larger wetlands generally present a greater area for breakdown of organic material, and therefore achieve greater pollutant reductions for a given wastewater. For further comparison, Cooper & Green (1995) reported BOD₅ reductions of 64-96% for various sites in the United Kingdom.

The significant correlation between outflowing BOD₅ and β-glucosidase activity is a possible indication that the labile product (glucose) of β-glucosidase activity is not being taken up by wetland soil microbes prior to its release in the outflow. If this is so, and glucose is contributing to outflow BOD₅ load, a simple solution would be to extend the flow path of the water before it leaves the wetland. The demand placed on this wetland may now be exceeding its design capacity as it was constructed almost ten years ago.

The significant correlation between outflowing BOD₅ and *Coliforms* at the Centre for Alternative Technology wetland during 1998 indicated a link between these

factors. Dissolved organic carbon may be the determining factor for both *Coliform* and BOD₅ levels; although there was not a significant correlation between either DOC and *Coliforms*, or DOC and BOD₅, the three measurements follow each other closely (Figure 4.9 b). When outflow DOC is high, *Coliforms* and BOD₅ become high. There was no such correlation between inflowing BOD₅, DOC and *Coliform* counts.

Williams *et al* (1995) found a significant correlation between faecal *Coliform* counts and BOD₅ in a wetland treatment system, and suggested that adsorption (the predominant removal mechanism of BOD₅ in biofilm systems (Gray 1989)), was probably a more important method of pathogen removal than sedimentation. Qureshi & Qureshi (1990) reported 99-100 % removal of three indicators of faecal pollution (total *Coliforms*, faecal *Coliforms*, coliphages) from secondary and tertiary effluents by wetland treatment. In their study, the mean input of *Coliforms* was 8×10^4 - 130×10^4 colonies ml⁻¹, whereas at the Centre for Alternative Technology it was 35×10^4 - 245×10^4 colonies ml⁻¹. The maximum numbers of *Coliforms* were obtained during the cooler months of December to February (Qureshi & Qureshi 1990) (which contrasts with maximum numbers at the CAT wetland in July-August), which was thought to be due to less general water usage during this period, giving a more concentrated sewage. At the CAT wetland *Coliform* reduction ranged from 35 - 99.99 %, which probably reflects the cooler soil temperature (annual average 11 °C) at this wetland compared to that studied by Qureshi & Qureshi (annual average 20 °C).

For total nitrogen (NO₃ + NO₂ + NH₄), Gersberg *et al* (1984) reported a mean removal of 80 %, with a mean outflow concentration of 4.3 mg l⁻¹. At the CAT wetland, total nitrogen output was on average higher than inflow, with an average increase over the year of 36 % (average outflow 28.4 mg l⁻¹). This poor nitrogen reduction is likely to be due to the predominance of ammonium-N in the inflowing water; when this is the case, low nitrification rates in wetlands limit

subsequent denitrification (van Oostrom & Cooper 1990). For improved nitrogen removal, the inflow would need to be previously nitrified so that organic carbon present in the system could be effectively utilized in denitrification (van Oostrom & Russell 1994). Insufficient anaerobic conditions will also limit denitrification (Gale *et al* 1993).

The concentration of nitrate-N in public water supplies is recommended to be < 10 mg l⁻¹ (Gersberg *et al* 1983). Nitrate removal efficiency has been found to be best in spring and summer, when plant growth and nutrient uptake are greatest, and also reflecting the increased ambient temperature and bacterial activity (Gersberg *et al* 1983). The CAT wetland tended to release more nitrate than it received due to nitrification of ammonium, and to poor denitrification performance. Plant uptake only accounts for a small proportion of the overall nitrogen removal in constructed wetlands, as the major process is denitrification (Gersberg *et al* 1983). Green *et al* (1997) report that nitrogen removal in wetlands is governed by microbial nitrification and denitrification, and that plant uptake and ammonia volatilization are generally less important.

The water chemistry results, particularly those for inflow and outflow chemistry, point towards a strong relationship between dissolved organic carbon and other nutrients (phosphate-P, ammonium-N and nitrate-N in the inflow, phosphate-P and ammonium-N in the outflow), which would be logical. Concentrations of nutrient ions in inflow and outflow waters all tend to follow the same patterns of fluctuation, possibly depending on the concentration of the sewage entering the wetland.

Performance data from constructed wetlands are traditionally analysed as percentage reduction in key nutrient ions. This approach gave mixed results during 1998 at the Centre for Alternative Technology wetland (Figure 4.2 a – c), with no clear annual trend in nutrient removal. For example removal was not

consistently better during the warmer summer months, when increased microbial activity may be expected to result in greater uptake of inorganic ions. Carbon dioxide emissions suggested that increased summer temperatures did lead to increased microbial activity at this wetland (*sensu* Bell 1969, Bridgham & Richardson 1992). Treatment wetlands have been reported to be more efficient in warmer climates (Gersberg *et al* 1983) for this reason. For phosphate-P and sulphate-S there was sometimes a release, rather than an uptake, of ions when outflow was compared to inflow, suggesting that the wetland may on some occasions be a net source, rather than a net sink, of nutrients. Cooper & Green (1995) also reported that no significant phosphate removal had been noted in constructed wetlands in the United Kingdom. No correlations between percentage reduction and the activity of associated enzymes was significant (Figure 4.2 a - c), although at a few points during the year there appeared to be a positive relationship between higher enzyme activity and greater nutrient removal (for example phosphatase and phosphate-P between September and December, and N-acetylglucosaminidase and ammonium-N between March and May).

On the whole there were not obvious relationships between enzyme activity and nutrient removal, although five of the seven enzymes studied showed significant correlations with one or more hydrochemical factors. The activities of the carbon cycling enzymes xylosidase and phenol oxidase correlated significantly with phosphate-P, nitrate and dissolved organic carbon soil water concentrations suggesting that these two enzymes release nutrients which are otherwise bound up in complex molecules. Higher levels of phenol oxidase may have resulted in higher soil water concentrations of phosphate-P, nitrate-N and dissolved organic carbon; these nutrient increases were not reflected in the outflow from the wetland, indicating that they may be used within the wetland by soil microbes, animals or plants.

Correlations between phenol oxidase and phosphate-P, nitrate-N (inverse correlation) and dissolved organic carbon, were not evident during the 1997 field study. Given the correlations between dissolved organic carbon and phosphate-P, ammonium-N and nitrate-N in the inflow, it is possible that phenol oxidase is involved in the decomposition of organic matter which contains these nutrients – higher inflow of this organic matter gives higher phenol oxidase activity and higher release of nutrients. Other correlations between hydrochemical factors in the outflowing wastewater suggest an association of some description between different nutrients. Phosphate-P was correlated with nitrate-N, ammonium-N, DOC and BOD₅, and ammonium-N with DOC and BOD₅.

Phenolics in the inflowing water were significantly correlated with dissolved organic carbon, phosphate-P, nitrate-N and ammonium-N, and in the outflow with dissolved organic carbon, ammonium-N and BOD₅. This suggests that dissolved organic carbon and phenolics may be physically associated in both inflow and outflow, and be passing through the wetland unmodified; this may make a considerable contribution to the Biological Oxygen Demand of the outflow, unless both, or the complex they form, are refractory. Although phenolic materials form part of the dissolved organic carbon, the two factors would not necessarily vary together, unless they were physically associated.

There was a lack of correlation between percentage reduction of nutrient ions and soil enzyme activity, and therefore no evidence to support hypothesis 2.

Correlations between enzyme activity in the soil and the percentage reduction across the wetland system would have indicated the importance of extracellular enzymes to nutrient cycles which are important in water treatment. The absence of such correlations may simply show that extracellular enzymes are generally incidental to nutrient cycling in this wetland, and are not accurate indicators of the turnover of elements in the soil. Due to the residence time of this wetland, the chemical composition of outflowing water probably does not accurately reflect the

processes or chemistry occurring in the wetland soil on the day of sampling. More frequent sampling, for example on a weekly basis, would be required to clarify the relationships which must surely exist between substrates and extracellular enzymes in this wetland.

More conclusive results may have been obtained by monitoring inflow and outflow chemistry on a daily basis, and relating these data to soil enzyme activity data taken on a more regular basis; however even with ideal sampling methods, it can be extremely difficult to understand enzyme activity (Overbeck 1991). Since the inflowing water is sewage, and likely to contain amounts of urea, assaying for soil urease activity would also have been interesting.

McLatchey & Reddy (1998) did find significant relationships between soil enzyme activities and the mineralization rates of carbon, nitrogen and phosphorous in laboratory experiments. Seasonal variations in treatment efficiency were not evident from the percentage removal data obtained from the CAT wetland, although Newman & Clausen (1997), for example, found significant variation in the percentage reduction of suspended solids, phosphorous and nitrogenous compounds by a treatment wetland receiving milkhouse waste. There was therefore no evidence to support hypothesis 1. Efficiency was reduced in winter, reportedly by plant senescence, and ammonium-N release also increased after autumn plant senescence, probably as a result of plant matter mineralization. The influence of extracellular enzymes on the rates of decomposition and solubilization of organic matter in soil are widely discussed (Asmar *et al* 1994, Dilly & Nannipieri 1998), so it is puzzling that this wetland does not show these patterns.

The significant correlation between nitrous oxide emission and Biological Oxygen Demand in both inflow and outflow water may indicate that much of the BOD₅ is removed by nitrification and/or denitrification during the passage of the

wastewater through the wetland. However inflowing Biological Oxygen Demand correlates significantly with outflowing. Lack of correlation between BOD₅ and nitrate-N in the outflow might suggest that the nitrogenous component of the inflowing BOD₅ is not being sufficiently removed, particularly as outflowing BOD₅ and ammonium-N are significantly correlated. Alternatively, ammonium transformation to nitrous oxide may be pre-empting nitrate-N outflow; high levels of outflow ammonium-N did correlate significantly with high soil nitrous oxide emissions, and also with outflow BOD₅, so this wetland may have insufficient nitrogen removal capacity when there are high concentrations of nitrogen in the inflow.

Conclusions

- The concentration of BOD₅ entering and leaving the wetland was closely associated with the numbers of total *Coliforms* detected
- Reduction of BOD₅ and *Coliforms* is satisfactory, although lower than that achieved in some other United Kingdom wetlands.
- There were some correlations between soil enzyme activities and the concentrations of nutrients in the soil water, although percentage removal of nutrients appeared to be unrelated to the soil enzyme activity.
- The CAT wetland showed good nitrification, but poor nitrate-N removal due to a high ammonium-N load in its inflow. The wetland also showed poor phosphate and sulphate uptake, being a source rather than a sink for these nutrients.
- Xylosidase and phenol oxidase correlated significantly with phosphate-P, nitrate-N and dissolved organic carbon within the wetland.

The role of plants in water quality improvement

4.5 Introduction

The twelve month field study during 1997 of the Centre for Alternative Technology, Waun-y-Cwrt, Pelenna and Tollesbury wetland sites (Chapter III) provided the first information about the seasonal changes in the role of soil enzymes in constructed wetlands. Chapter IV a investigated the role of wetland biogeochemistry in water quality improvement; to further investigate the role of wetland substrate factors on water quality improvement, and in particular whether plants had any influence on enzyme activity in the wetland substrate, a third field study was carried out. There have been varying reports of the importance of plants for wetland treatment, and it was therefore of interest to compare the performance of identical planted and unplanted systems. For some types of treatment, plants are believed to be unnecessary or even detrimental; for example in acid mine drainage treatment, iron reduction is undesirable, but may be fuelled by carbon release from plant roots (King & Garey 1999). For other types of treatment, particularly organic wastes, plants are believed to enhance treatment (Brix 1994, 1997). The release of oxygen from the roots of aquatic macrophytes such as *Phragmites* and *Carex* has been well documented (Brix 1994), and is higher in younger roots (Armstrong & Armstrong 1988). Organic decomposition proceeds faster under aerobic conditions (Reddy & Patrick 1975). As the plants grow their root and shoot systems expand, and so the roots are capable of releasing increasing amounts of oxygen to the rhizosphere; this may raise the redox potential of the wetland substrate.

Plants may also influence extracellular enzyme activity by providing carbon substrates (via root exudation (Lynch & Whipps 1990)) which help to sustain a microbial population, which is itself capable of releasing extracellular enzymes. Enzymes have also been reported to form part of root exudates (Burns 1977). If greater water quality improvement can be achieved when extracellular enzyme activity is higher, then using plants to increase the pool of extracellular enzymes

available for decomposition of organic matter would be beneficial. Macrophytes may also take up some inorganic products of extracellular enzyme activity, helping to minimize the nutrient output of a wetland (Boyt *et al* 1977, Brix 1994).

Rather than using a full-scale, on-line treatment wetland for this investigation, mesocosm experimental units were chosen, as these allowed precise control of the input to the wetlands, without physical disturbance or variation in flow or input composition. To obtain consistent wastewater chemistry for application to the wetland mesocosms, an artificial sewage was applied to the wetlands, after they had been inoculated with bacteria by dosing with cattle slurry. Mesocosms with and without plants were monitored in parallel, so that the effects of plants on nutrient cycling and water quality improvement in artificial wetlands could be investigated. Using planted and unplanted mesocosms side by side also provided a means of observing whether the presence of plants affected the springtime decline in enzyme activity, which was seen during 1997 and 1998 (Chapters III and IV a), and also reported by Kang (1999).

The activities of three extracellular enzymes in the substrate of the wetland mesocosms were assayed to indicate activity in three major nutrient cycles (Sinsabaugh 1991); the carbon cycle (β -glucosidase), phosphorous cycle (phosphatase) and sulphur cycle (sulphatase). The relative activities in each of these were gauged by the activity of the wetland substrate towards fluorescent analogue substrates (using the method described in Chapter III). In addition, fluxes of the gases nitrous oxide, methane and carbon dioxide were measured *in situ*, as indicators of the intracellular nitrogen cycle, and anaerobic and aerobic microbial activity, respectively. Soil carbon dioxide flux can be used as an indicator of aerobic microbial respiration (Bridgham & Richardson 1992). Gas flux measurements were made *in situ* to avoid inaccuracy due to gravel disturbance and water loss. The water chemistry of the artificial wastewater, substrate (gravel) water, and outflowing water were measured to monitor the improvement in water quality provided by the wetlands. The pH, temperature and

redox potential of the gravel substrate in each bed were monitored to indicate the physico-chemical condition of the flooded gravel, as all of these factors have been reported to influence soil enzyme activity (Pettit *et al* 1977, Frankenberger & Johanson 1982, McClaugherty & Linkins 1990, Pulford & Tabatabai 1988).

The aims of this study were as follows

- to assess the influence of plants on enzyme activity in the wetland substrate
- to assess the influence of plants on redox potential in the wetland substrate
- to assess water quality improvement in relation to substrate redox potential
- to investigate the differences between planted and unplanted mesocosms with regard to the springtime decline in enzyme activity.

To achieve these aims, the following hypotheses were tested:

- 1 Mesocosms containing plants will show higher levels of extracellular enzyme activity than those without
- 2 Mesocosms containing plants will show higher (more positive) redox potential in the substrate
- 3 Water quality improvement will be better at higher redox potential

4.6 Study site

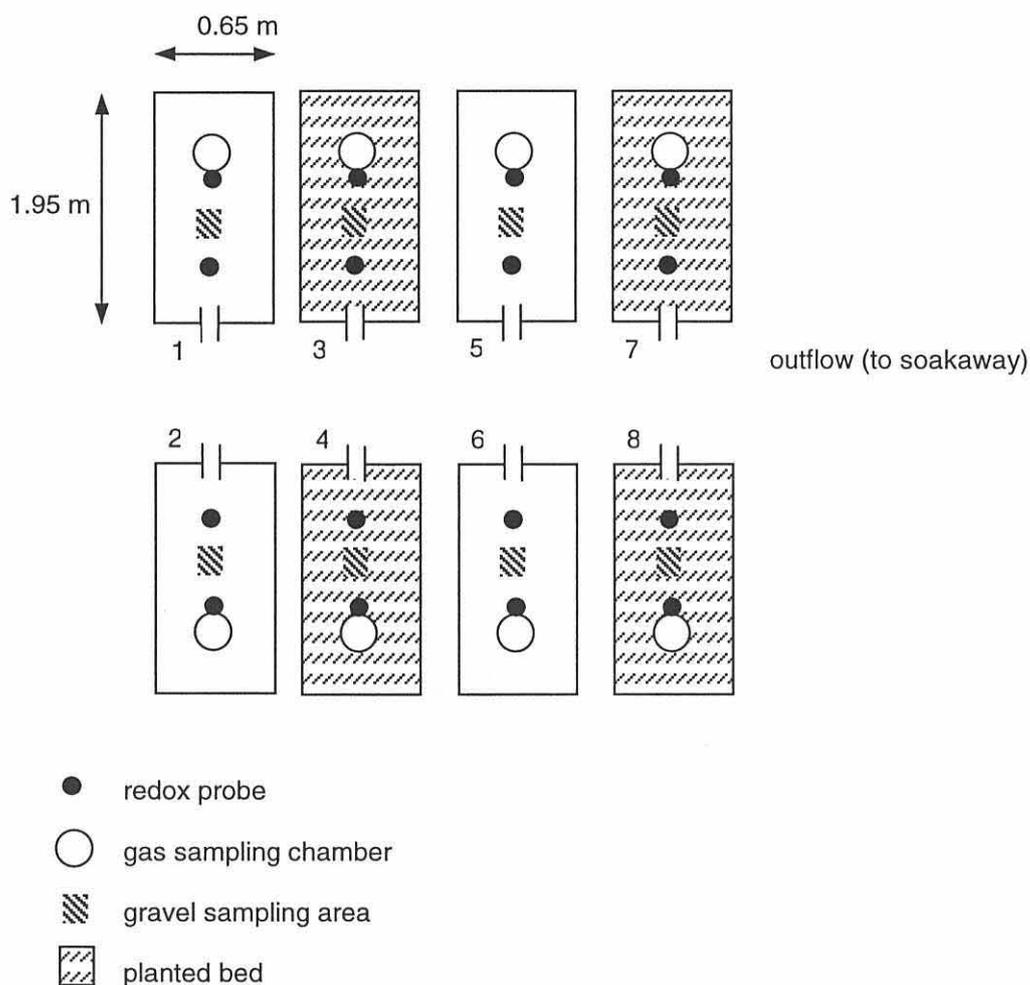
Eight purpose-built constructed wetland mesocosms were used to investigate the influence of redox potential on wetland biogeochemistry. The wetlands were built at the University of Wales' College Farm, Abergwyngregyn, near Bangor, and were identical with respect to construction and substrate. Each measured 1.95 m x 0.65 m, was 0.4 m deep and filled with gravel (10 mm diameter) to a depth of 0.38 m (Figure 4.3).

Four beds were planted with *Phragmites australis* (beds 3, 4, 7, 8) at a density of 4 plants m⁻², and four were left unplanted (beds 1, 2, 5, 6). The wetlands were constructed in November 1998, when some were planted with young reed plants,

and all eight were flooded to a depth of 0.02 m above the gravel surface. The wetlands were then left to overwinter.

Sampling, including the application of artificial sewage, began on 2nd March 1998, and continued for 13 weeks until 1st June 1998. Samples were collected on days 1, 7, 22, 29, 43, 50, 63 and 78. Outflow valves from the beds were closed between site visits.

Figure 4.3
Site plan of constructed wetlands at College Farm, Abergwyngregyn



4.7 Site monitoring

On each site visit, the pH, redox potential (at two points), gas emissions, and water from the inflow, substrate and outflow were sampled. pH was measured with an Orion pH electrode, calibrated using standards of pH 4 and 7. Redox measurements were taken using the method described below. Gas samples were collected using the method of Freeman *et al* (1994), described in Chapter III. A 150 cm³ sample of gravel was collected from each bed for later enzyme analysis. Samples of inflow, substrate and outflowing water were collected using subsurface solution samplers (Freeman *et al* 1993), and filtered (0.2 μm). The air

temperature at 1 m above the ground, and soil temperature at 10 cm depth, and rainfall since the previous visit (collected in a graduated subsurface collector) were recorded.

a. Artificial sewage application

On the initial site visit the eight beds were inoculated with 25 litres of cattle slurry, diluted 1:1 with tap water. At the start of each site visit, 3-5 cm of surface water from each bed was drained to a soakaway, to leave room for artificial effluent addition. After gas, gravel and water samples had been collected, 25 litres of artificial sewage solution, made to a recipe supplied by Unilever plc (Table 4.3, originally adapted from Painter & Viney 1959), were added to each bed, with samples kept for chemical analyses.

Table 4.3

Artificial sewage solution

Ingredient	Concentration
Peptone	70 mg l ⁻¹
Urea	25 mg l ⁻¹
Sucrose	35 mg l ⁻¹
Soluble starch	35 mg l ⁻¹
Ammonium sulphate	140 mg l ⁻¹
Mixed acids (Table 4.4)	105 mg l ⁻¹
Potassium hydrogen phosphate	28 mg l ⁻¹
Ferrous ammonium sulphate	21 mg l ⁻¹
Trace metals solution (Table 4.5)	1 ml

In addition, carboxymethylcellulose was added at approximately 50 mg l⁻¹

Table 4.4

Mixed acids solution

Acid	Concentration
Sodium acetate	136 g l ⁻¹
Sodium propionate	28 g l ⁻¹
Sodium butyrate	12 g l ⁻¹
Sodium benzoate	100 g l ⁻¹
Sodium citrate	44 g l ⁻¹

Table 4.5

Trace metals solution

Metal	Concentration
CuCl ₂ ·2H ₂ O	0.25 g l ⁻¹
Co(NO ₃) ₂ ·6H ₂ O	0.25 g l ⁻¹
Na ₂ B ₄ O ₇ ·10H ₂ O	0.25 g l ⁻¹
ZnCl ₂ ·2H ₂ O	0.25 g l ⁻¹
MnSO ₄ ·H ₂ O	1.0 g l ⁻¹
K ₂ Mo ₄	0.25 g l ⁻¹
NH ₄ VO ₃	0.1 g l ⁻¹

The sewage solution was made up at ten times concentration and diluted *in situ* before application to the beds.

b. Redox probes

Redox measurements were taken with probes which were left in position throughout the sampling period. These were constructed using a method developed at the Royal Holloway Institute of Environmental Research, Royal Holloway and Bedford New College, London, as follows (R. Hunter, pers. comm.).

The main body of each probe was standard insulated copper core electrical cable (2.5 mm core diameter rigid solid conductor, type BU, RS Components part no. 359-043), cut to the length required, plus 20 cm, with pliers. Using wire strippers, 1.5 cm of the sleeving was removed from each end, to expose the copper core. The electrode tip was 0.5 mm diameter (25 gauge) platinum wire (Johnson Mathey chemicals, tel. 01763 253000, product code 944670000), which is corrosion resistant and gives the correct potential. A 1.3 cm piece of platinum wire was sweated on to one end of the copper conductor, using 60/40 tin/lead multicore solder (RS Components part no. 554-917), covering only $\frac{1}{3}$ rd of the length of platinum with solder (Figure 4.4 a and b). Ideally, the sweated joint had no solder between the copper and platinum, as solder reduces the electrical conductivity of the probe.

Figure 4.4 a

Cross section of sweated joint between copper conductor and platinum tip of redox probe

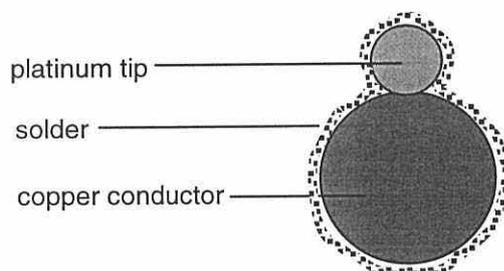
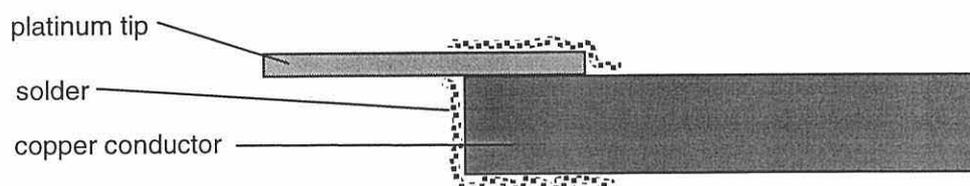


Figure 4.4 b

Vertical cross section of sweated joint at tip of redox probe



The platinum-tipped end of the probe(s) was then placed in a bath of 15% hydrochloric acid for 10-15 minutes to clean away remaining flux, and dried with a warm air source. Two pieces of 3.2 mm internal diameter heat-shrinkable tubing (RS Components part no. 397-809), 2 cm and 1 cm long were cut. The 2 cm piece was applied to entirely cover the copper wire and platinum/copper joint, and to overlap the cable sleeving leaving approximately 6 mm of the platinum wire protruding. The 1 cm piece was applied to overlap the cable sleeving at the other end, leaving a little over half of the copper core exposed. Shrinkwrap tubing ensured that the probe was fully waterproof.

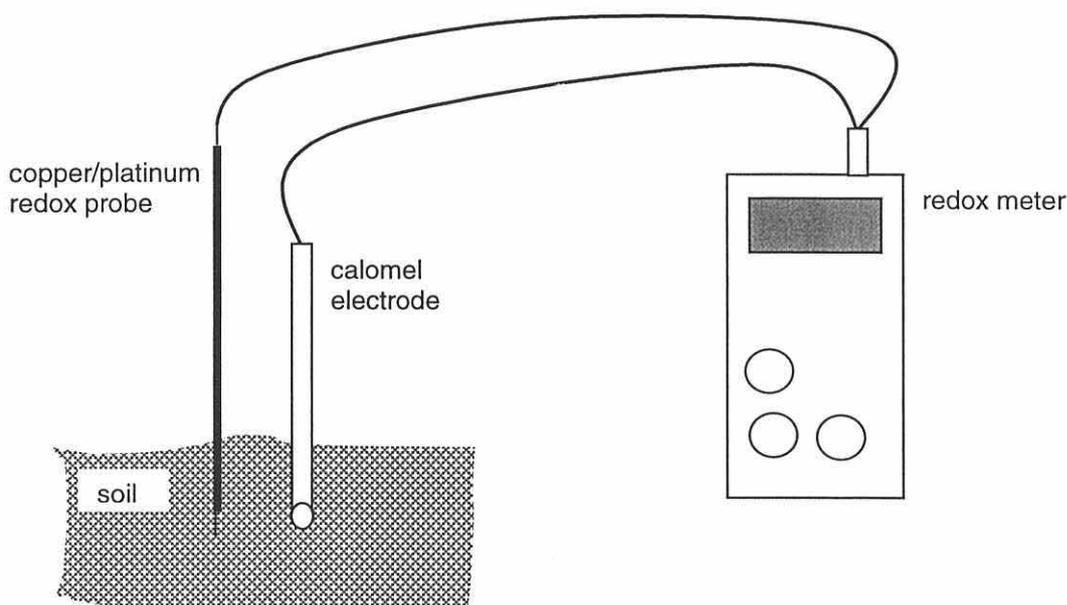
Before use the electrodes were tested using a standard redox potential solution (10.211 g l⁻¹ potassium hydrogen phthalate dissolved in deionized water, then saturated with quinhydrone) of 218 ± 20 mV, a standard calomel electrode and a pH/mV meter. For these measurements, and for field use, a long reach rigid probe (RS Components part no. 423-469) and a two-way adaptor (RS Components part no. 455-999) were required to allow the calomel electrode and redox probe to be connected together. Probes which gave readings in the standard redox potential solution of between 198 and 238 mV (20% error) were acceptable for use.

To position the probes in the field, a hole was made with a metal spike to the depth required for redox measurement, a probe was threaded in, and the hole was tamped down. Bentonite clay can be used half way up the probe to form a seal around it, but in this case was not. Approximately 20 cm of the probe was left out of the soil, and bent over and curled round flat on the soil surface to reduce the chances of disturbance. The probes were left *in situ* for at least 14 days before any field measurements were taken, and thereafter were left for many months.

To take a redox measurement in the field, a small hole was made in the soil, in the middle of the sited probes, and a slurry was mixed in this using distilled water. The exposed copper end of the redox probe was cleaned with emery paper to remove any oxides. The calomel electrode, connected to the pH meter, was then

held in the slurry while the redox probe was connected to the pH meter. After a few seconds the reading was stable (Figure 4.5).

Figure 4.5
Redox measurements using *in situ* probes



4.8 Laboratory analyses

a. Enzyme activity

The activity of β -glucosidase, phosphatase and sulphatase enzymes were assayed using MUF fluorescent substrates and the method described in Chapter III. One gram of gravel was mixed with 7 ml of substrate ($200 \mu\text{M l}^{-1}$ MUF-glucoside, MUF-phosphate or MUF-sulphate) and incubated at field temperature.

b. Water chemistry

DOC

Dissolved organic carbon in all water samples was determined using Skalar autoanalyser colorimetric analysis, as described in Chapter III.

Nutrient ions

Nitrate, phosphate, sulphate and ammonium concentrations were determined using a Dionex DX120 ion chromatograph. Anions were determined using an

AS5A-5 μ column with a self-regenerating suppressor, and cations with a CS12 (10-32) column, also with a self-regenerating suppressor. Eluent flow rate was 1.04 ml min⁻¹, using 1.8 mM sodium carbonate/1.7 mM sodium hydrogen carbonate for anions, and 31 mM sulphuric acid for cations.

Phenolics

Phenolic compounds in all water samples were determined using the Box assay and Folin-Ciocalteu reagent, described in Chapter III.

c. Gases

Nitrous oxide, methane and carbon dioxide concentrations were determined using a Cambridge Ai model 92 gas chromatograph (for specification and analysis conditions see Chapter III). A gas standard of 1 ppm nitrous oxide, 10 ppm methane and 100 ppm carbon dioxide, supplied by Cryoservice (Worcester, UK), was used for calibration.

d. Statistical analysis of results

Data were used in correlation matrices; those which conformed to the Normal distribution were analysed using Pearson correlations, and non-conforming data were analysed using Spearman correlations. Factorial ANOVAs were used to test for differences between planted and unplanted wetlands.

4.9 Results

Enzyme activity (Figure 4.6 a, b and c). β -glucosidase activity was initially twice as high in planted beds as in unplanted, but by day seven and thereafter there was no difference in the activity of β -glucosidase, phosphatase or sulphatase between planted and unplanted beds. Activity remained stable throughout the course of the study. Phosphatase and sulphatase showed a consistent upward trend with time; sulphatase activity was approximately 60 % lower than that of β -glucosidase or phosphatase.

Gases

Nitrous oxide Fluxes of nitrous oxide did not vary significantly between planted and unplanted beds, indicating similar levels of nitrogen cycling (Figure 4.7 a). The only exception was day 21, on which nitrous oxide consumption in planted beds was much greater than in unplanted.

Methane There were no significant differences between planted and unplanted beds, and no significant variations with time (Figure 4.7 b).

Carbon dioxide After an initial drop in emission in unplanted beds, which was not seen in planted beds, there were no significant differences in carbon dioxide fluxes. As the study progressed, positive flux increased (Figure 4.7 c).

Figure 4.6

Activity of β -glucosidase (a), phosphatase (b) and sulphatase (c) in unplanted (○) and planted (●) wetland beds at College Farm, during the field study. Day 0 was 2nd March 1999, day 78 was 1st June 1999. (Mean, $n = 4, \pm$ s.e.).

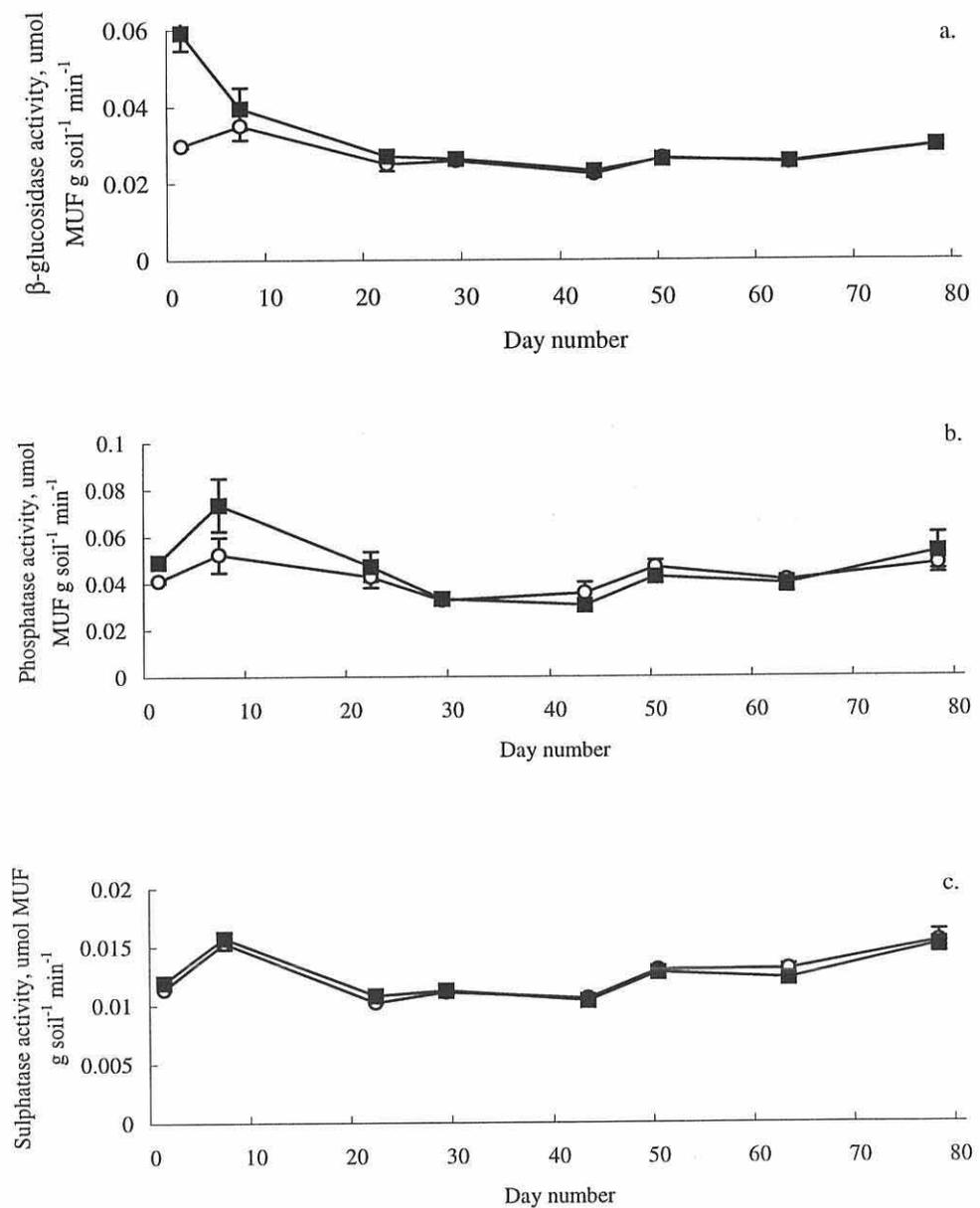
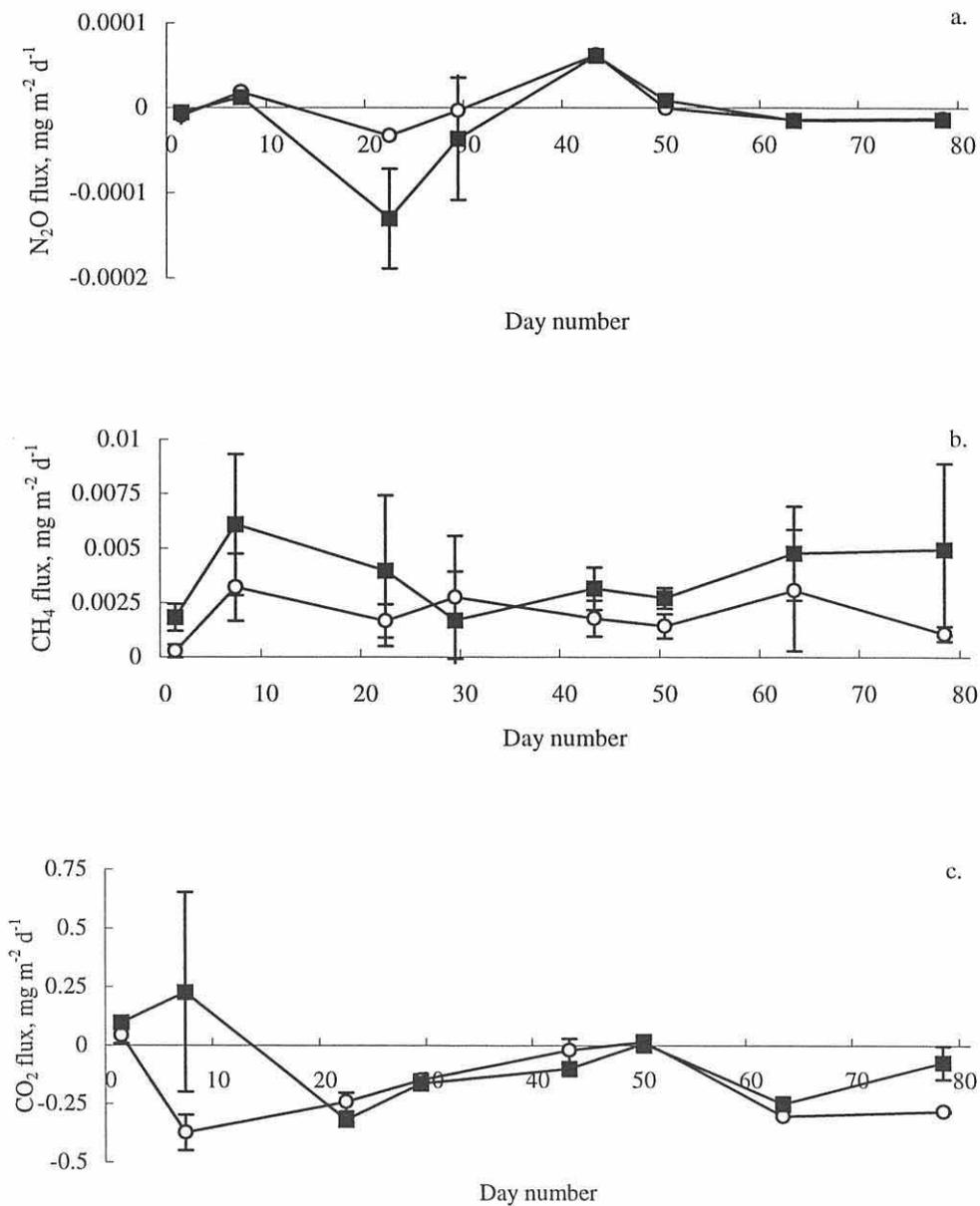


Figure 4.7

Nitrous oxide (a), methane (b) and carbon dioxide (c) emission from unplanted (○) and planted (●) wetland beds at College Farm during the field study. Day 0 was 2nd March 1999, day 78 was 1st June 1999. (Mean, n = 4, ± s.e.).



Dissolved organic carbon Showed a large peak in the bed waters at day 43, and was otherwise fairly stable. Outflowing DOC concentrations were similar from planted and unplanted beds; outflow concentrations were higher than bed water concentrations to begin with, but later dropped to identical levels (Figure 4.8 a).

Phenolics Phenolics in all water samples declined during the study period (Figure 4.8 b). Factorial ANOVA showed significant differences in phenolic concentration over time ($F_{7,96} = 167.6$, $p < 0.001$) and between planted/unplanted and bed/outflow data ($F_{3,96} = 64.19$, $p < 0.001$), but not between time and water origin ($F_{21,96} = 10.29$, $p < 0.001$).

Nutrient ions Ammonium concentration followed similar patterns in planted and unplanted beds, but was higher in outflowing water than in bed water. After an initial decline, there was a peak in ammonium concentration in both bed and outflowing water on day 29, followed by decline again (Figure 4.8 c). Nitrate concentrations were higher in outflowing water than in bed water, where both data sets were available for comparison (Figure 4.8 d). Phosphate concentrations in unplanted bed water rose initially, in contrast to concentration in planted beds and all outflowing water, which showed a decline (Figure 4.8 e). All waters showed a peak in phosphate concentration on day 50, followed by a decline. Sulphate concentration was initially high in unplanted and planted bed and outflowing waters, but dropped rapidly to a low level, and remained low throughout the course of the study (Figure 4.8 f).

Table 4.6

Inflow, bed and outflow average concentrations of ammonium, nitrate, phosphate and sulphate in planted and unplanted mesocosms at College Farm. (Mean, n = 8, standard error in brackets).

	Inflow		Bed		Outflow	
	Planted	Unplanted	Planted	Unplanted	Planted	Unplanted
NH ₄	20.61 (1.69)	20.33 (1.95)	10.72 (2.89)	9.75 (2.62)	17.46 (5.16)	17.04 (5.11)
NO ₃	1.27 (0.59)	1.62 (0.79)	0.25 (0.15)	0.25 (0.08)	0.57 (0.29)	0.64 (0.31)
PO ₄	152.3 (16.89)	146.92 (18.96)	3.18 (1.04)	8.86 (1.98)	3.91 (1.45)	4.45 (1.56)
SO ₄	9.52 (1.62)	9.59 (2.06)	2.13 (1.77)	2.32 (1.59)	1.64 (2.43)	1.49 (1.79)

Table 4.7

Inflow, bed and outflow average concentrations of dissolved organic carbon and phenolic substances in planted and unplanted mesocosms at College Farm. (Mean, n = 8, standard error in brackets).

	Inflow		Bed		Outflow	
	Planted	Unplanted	Planted	Unplanted	Planted	Unplanted
DOC	8.32 (0.88)	8.21 (0.34)	52.16 (7.16)	52.71 (8.84)	63.09 (7.92)	56.36 (6.25)
Phenolic substance	1.04 (0.17)	1.22 (0.203)	11.72 (2.19)	8.78 (1.55)	15.07 (3.06)	16.35 (4.07)

Redox potential During the first half of the study redox potential increased from less than -1000 mV to around 0 mV. This rise was followed by a decline to around -500 mV, and a further rise, to approximately +250 mV. There were no significant differences in redox potential between planted and unplanted beds (Figure 4.10 a).

Figure 4.8

Dissolved organic carbon (a), phenolics (b) and ammonium (c) concentration in unplanted (●) and planted (○) bed waters, and in unplanted (■) and planted (□) outflow from wetlands at College Farm during the field study. Day 0 was 2nd March 1999, day 78 was 1st June 1999. (Mean, n = 4, ± s.e.).

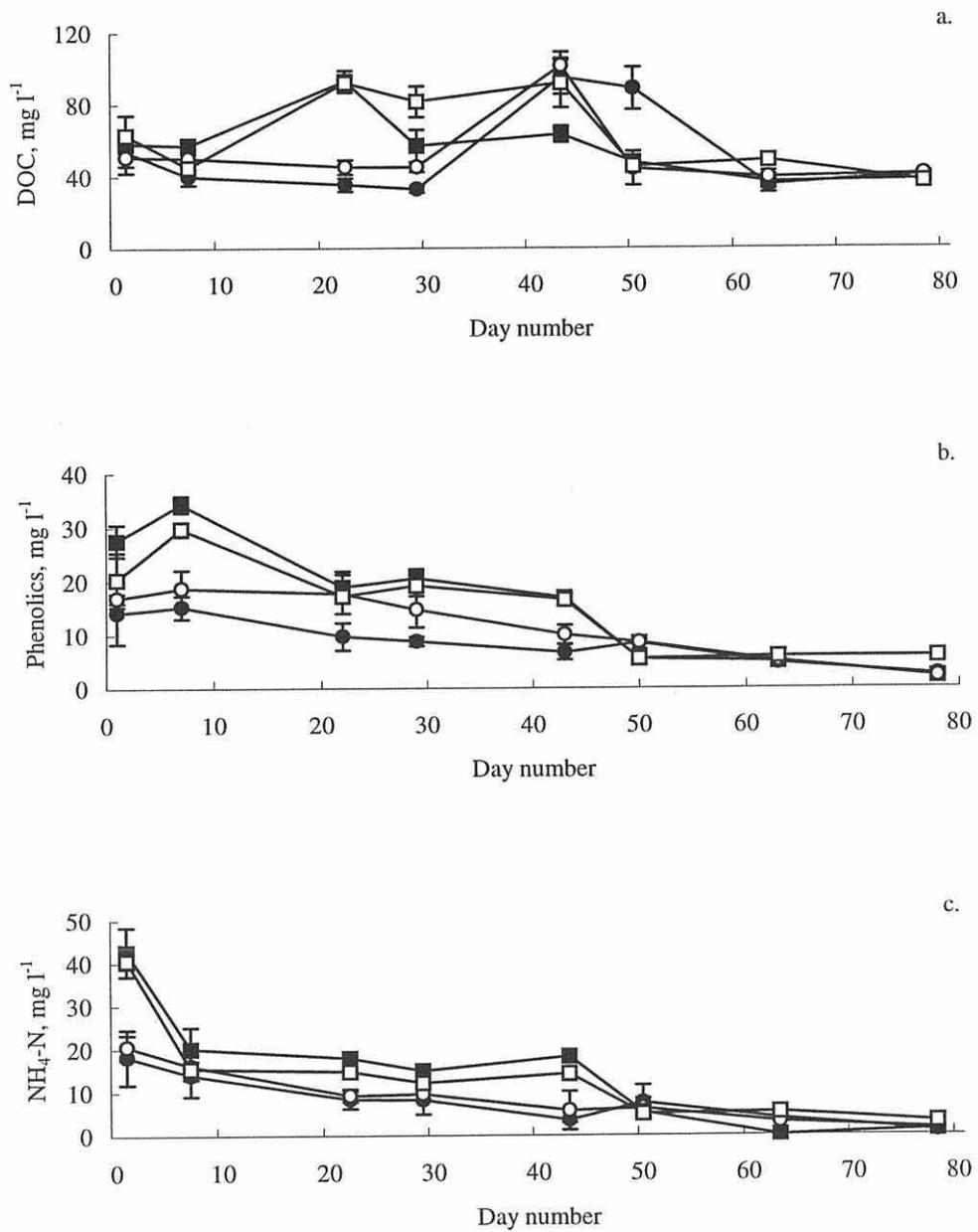
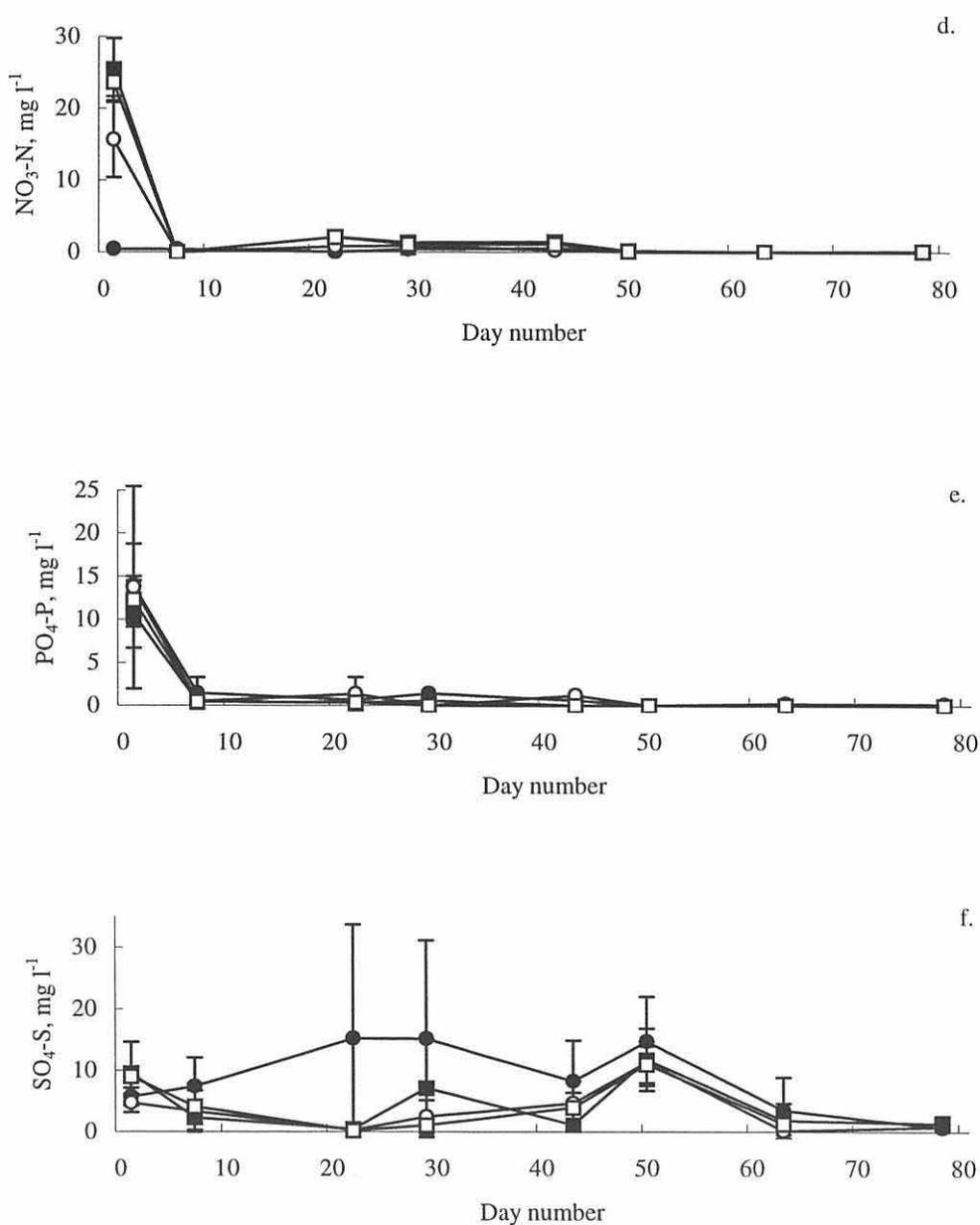


Figure 4.8 contd.

Nitrate (d), phosphate (e) and sulphate (f) concentration in unplanted (●) and planted (○) bed waters, and in unplanted (■) and planted (□) outflow from wetlands at College Farm during the field study. Day 0 was 2nd March 1999, day 78 was 1st June 1999. (Mean, n = 4, ± s.e.).



All data were used in a correlation matrix (Table 4.8). Non-Normal data were compared to other data using Spearman correlations. (Water chemistry data are for the bed only; data from unplanted and planted beds were used together as there were no significant differences between them).

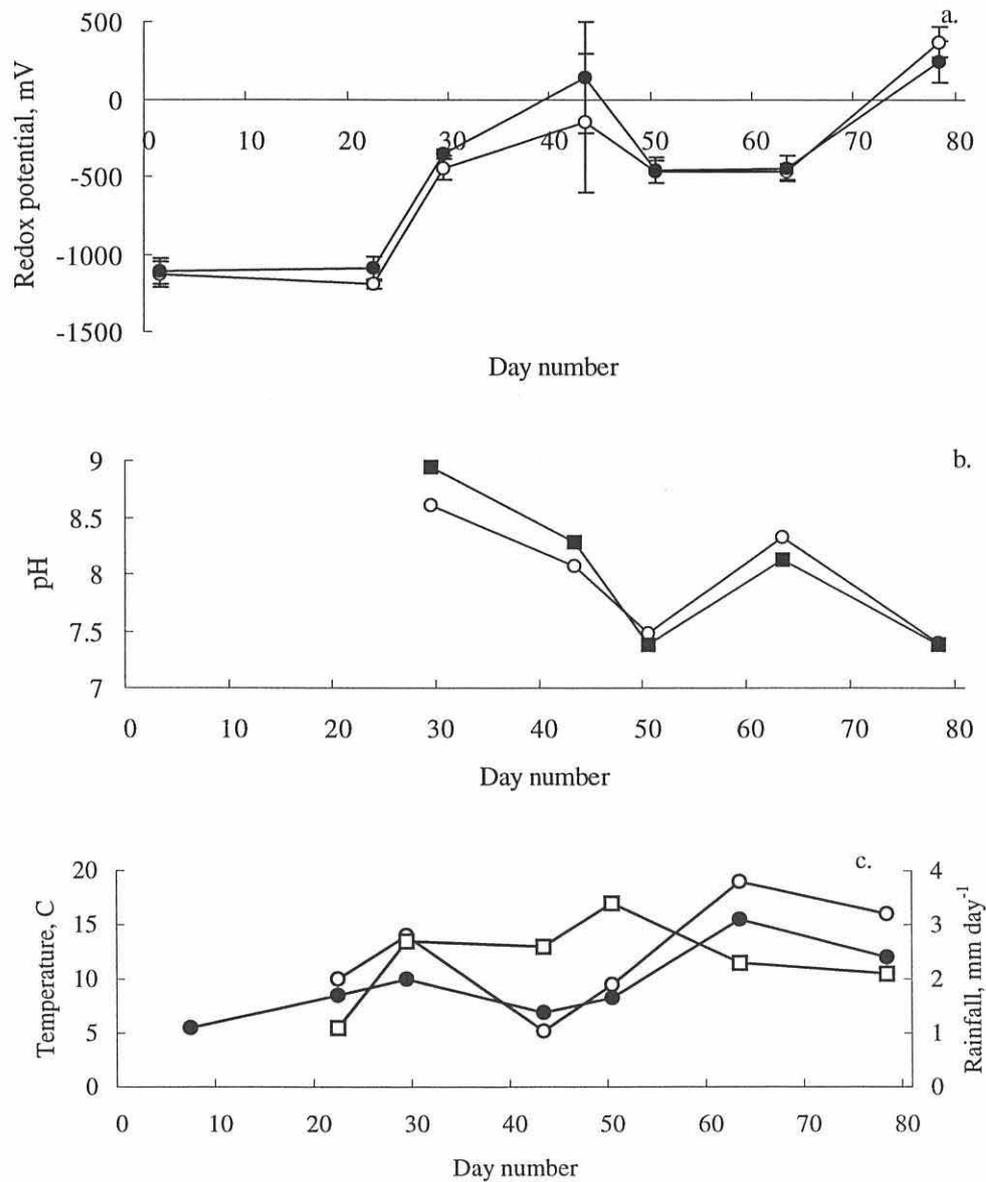
Table 4.8

Correlation matrix for enzyme and water chemistry data from the College Farm wetlands. *Italics* = significant at $p < 0.1$; upright = significant at $p < 0.05$ (r = correlation coefficient, p = probability).

	β -glu	Pho	Sul	N ₂ O	CO ₂	DOC	Phenolics	NO ₃	NH ₄	PO ₄	Redox
Pho	<i>r</i> 0.849										
p	0.000										
Sul	<i>r</i> 0.672	<i>r</i> 0.687									
P	0.004	0.003									
DOC				<i>r</i> 0.464	<i>r</i> 0.601						
p				<i>r</i> 0.070	<i>r</i> 0.014						
NO₃			<i>r</i> -0.49				<i>r</i> 0.518				
p			<i>r</i> 0.049				<i>r</i> 0.040				
NH₄	<i>r</i> 0.459						<i>r</i> 0.895	<i>r</i> 0.484			
p	<i>r</i> 0.073						0.000	0.058			
PO₄							<i>r</i> 0.565	<i>r</i> 0.544	<i>r</i> 0.573		
p							<i>r</i> 0.023	<i>r</i> 0.029	<i>r</i> 0.020		
Redx						<i>r</i> 0.581				<i>r</i> -0.52	
p						<i>r</i> 0.048				<i>r</i> 0.084	
pH	<i>r</i> -0.57	<i>r</i> -0.79					<i>r</i> 0.620		<i>r</i> 0.606		<i>r</i> -0.69
p	<i>r</i> 0.084	<i>r</i> 0.007					<i>r</i> 0.056		<i>r</i> 0.063		<i>r</i> 0.028
Temp				<i>r</i> -0.77		<i>r</i> -0.59	<i>r</i> -0.64		<i>r</i> -0.58		
p				<i>r</i> 0.001		<i>r</i> 0.026	<i>r</i> 0.013		<i>r</i> 0.031		
Rain				<i>r</i> 0.638			<i>r</i> -0.62		<i>r</i> -0.82		
p				<i>r</i> 0.026			<i>r</i> 0.032		<i>r</i> 0.001		

Figure 4.9

Redox potential (a), and pH (b) in unplanted (○) and planted (●) wetland beds, and field temperature (in soil (●) and air (○)) and rainfall (□)(c), at College Farm during the field study. Day 0 was 2nd March 1999, day 78 was 1st June 1999. (Mean, n = 4, ± s.e. for redox potential).



4.10 Discussion

Enzymes

The highly significant correlations between activity of the three measured enzymes (β -glucosidase, phosphatase and sulphatase) indicates that all three are controlled by the same factors. As there were no significant differences in the activity of any enzyme between unplanted and planted beds, such a factor (or factors) is unlikely to be plant-related, despite the observed influence of plants on enzymes such as phosphatase (Khan 1970, Neal 1973). There was therefore no evidence to support Hypothesis 1, that mesocosms containing plants would show higher enzyme activity. Spier (1977) found a very significant correlation between soil sulphatase and phosphatase, which is also seen in this data set.

Surprisingly, and in contrast to the results of the twelve month field study (Chapter III), there was no correlation between enzyme activity and soil temperature. As the same fluorescent substrates were used for assays here as for the temperature behaviour determinations in Chapter V a, results from the laboratory experiment were cautiously applied to predict the temperature-induced increase in activity during the experimental period. Soil temperature at the College Farm experimental site rose from 5.5 °C in March 1999 to 16 °C in May 1999, which falls within the range investigated in the laboratory experiments. In these, Q_{10} for β -glucosidase was found to vary between 1.99 (Pelenna soil) and 3.03 (CAT soil), averaging 2.51 which (assuming that β -glucosidase in the College Farm wetland substrate showed similar behaviour) predicts at least a doubling in activity during the study period. Similarly for phosphatase, Q_{10} varied between 1.39 (Pelenna soil) and 3.9 (CAT soil), averaging 2.59 which also predicts at least a doubling of activity during the study. An increase in soil temperature should produce an increase in enzyme activity even without a corresponding increase in the size of the enzyme pool, and so it is concluded that enzyme activity in the College Farm wetlands may have been inhibited in some way.

The springtime decline in enzyme activity which was seen during the field studies in 1997 and 1998, and also reported by Kang (1999), was not observed in either planted or unplanted mesocosms, despite the sampling period spanning the season when it was expected to occur (March – June). As there were no differences in enzyme activity between planted and unplanted mesocosms, plants may not have a significant influence in the occurrence of the springtime decline in enzyme activity seen in Chapter III. However, possible reasons for the absence of the springtime decline are the low levels of microbial activity (indicated by the low gas fluxes), and the early growth stage which the reeds are at.

Levels of enzyme activity in the gravel substrate of the mesocosms were between 1/100th and 1/10th of the activities recorded between March and June in the constructed wetlands studied in the 1997 field work. This may be a result of the short time over which the mesocosms have been operational, which has not yet allowed an accumulation of extracellular enzymes from the microbial population. The short operation time also results in very little accumulation of organic matter, which is an important requirement for the stabilization and immobilization of extracellular enzymes (Burns 1982).

Hydrogen ion concentration (pH) has already been shown to have considerable influence on a variety of extracellular enzyme activities in soils (Speir & Ross 1978, Frankenberger & Johanson 1982, Nannipieri *et al* 1982). In Chapter V a, laboratory experiments showed that in soils from three other constructed wetlands the activities of β -glucosidase, phosphatase and sulphatase were lower at pH values greater than 8 than at pH 7. The pH of both unplanted and planted beds at College Farm ranged from pH 7.3 to 8.95, and was on average pH 8; it seems likely that this alkalinity contributed to the enzyme activities remaining low as temperature increased.

As with data from both the twelve month field study (Chapter III) and the further field work at the Centre for Alternative Technology (Chapter IV a), there were no significant relationships between the activity of enzymes and their products (e.g. between phosphatase and phosphate). This suggests that the system investigated is not limited by a lack of microbially available substrates, so that extracellular enzyme activity is not the rate limiting step in nutrient removal. Galstyan & Bazoyan (1974) (in Spier & Ross (1978)) reported that the activity of sulphatase does not correlate significantly with the inorganic sulphur content of soil, but (positively) with the organic fraction; whilst the results from this study confirm that sulphatase does not correlate with inorganic sulphur, neither do they support the hypothesis that soil organic matter and sulphatase are related. Tabatabai & Bremner (1970) found that phosphate availability decreased soil arylsulphatase activity, but although there was a negative correlation between these two factors, it was not significant.

Beta-glucosidase activity correlated negatively with substrate pH, suggesting that organic matter decomposition proceeds faster in more neutral conditions than in alkaline ones, which may be a product of increased enzyme denaturation at high pH. Phosphatase activity also correlated negatively with pH, which could potentially lead to increased phosphate availability at neutral, rather than alkaline, pH, but only if other factors governing phosphate adsorption and release were not affected by pH.

Hydrochemistry

Differences in nutrient availability between unplanted and planted beds were more marked for nitrate and phosphate than for ammonium and sulphate. Phosphate in unplanted beds was particularly high, suggesting that uptake of phosphate by plants or rhizosphere bacterial communities may be important.

The percentage removal of ammonia and nitrate were poor at the start of the study, becoming slightly better towards the middle of the period, when input (and

therefore calculations of water quality improvement) ceased. Both ammonia and nitrate showed increases in concentration between inflow and outflow on several sampling occasions, which have several possible causes; poor ammonia removal corresponds with a high pH (pH 9) in the beds, and removal may have been inhibited as it improved when pH dropped to 7.5. Poor nutrient removal may be due to a lack of much organic matter in the mesocosms, which are relatively young; also, the operational regime of continuous flooding may inhibit organic matter decomposition, which generally tends to proceed faster under aerobic conditions than anaerobic (Reddy & Patrick 1975). The presence of plants did not appear to provide greater improvement in water quality.

Temperature in the wetland substrate correlated significantly and inversely with dissolved organic carbon, nitrous oxide and phenolics. At lower soil temperatures microbial metabolism is likely to be lower, reducing the rate of microbial carbon uptake and enzymic turnover and so leaving more organic carbon within the wetland soil and dissolved in the bed water.

Redox potential

As redox potential generally increased throughout the study period, the wetlands have become more oxidized. The plants showed noticeable growth during the eight week Spring-Summer sampling period; however no quantitative data on plant growth or bed oxygen concentration were collected. There was no significant difference in redox potential between planted and unplanted beds, which suggests that other mechanisms contributed to the rising redox potential. Hypothesis 2 (that mesocosms with plants would have higher redox potentials) was not supported. The most obvious cause of the rising redox potential is the addition each week of oxygenated effluent, after release of stagnant water; prior to the sampling period the beds had lain dormant and undisturbed for some four months.

The significant negative correlation between phosphate concentration and redox potential, suggested that phosphate removal was better at more positive redox potentials. Hypothesis 3 (that water quality improvement would be better at higher redox potentials) was therefore supported by phosphate water quality data. Dissolved organic carbon concentrations correlated significantly (positively) with redox potential; this may indicate that the degradation of complex carbon molecules to dissolved carbon is improved at more positive redox potential, and can tentatively be taken to also support Hypothesis 3.

The general lack of distinction between planted and unplanted mesocosms in terms of enzyme activity, pH, water quality improvement, redox potential and gas fluxes (with the exception of methane) could be a result of the plants not being well established at the time of the study. Although they had over-wintered, at the time of this study the reeds had not had a growing season, and so their root systems may not have been extensive enough to effect significant changes on the biogeochemistry of the planted mesocosms.

The negative correlation between redox potential and phenolics, nitrate, ammonia and sulphate in the wetland bed water may be related to the processes which are enabled at different redox potentials. According to Kralova *et al* (1992), ammonia concentration increases as redox potential decreases, because ammonia is more stable under reducing conditions; under aerobic conditions it may be rapidly converted to nitrite and nitrate. This may also explain the high concentrations of nitrate at low (negative) redox potentials in the bed water. As redox potential becomes more positive, more nitrate would be expected through nitrification; but denitrification, which is favoured by more reducing conditions, would be inhibited and less nitrate thus converted to gaseous products.

Redox potential ranged from -1200 mV to $+250$ mV in field measurements taken at College Farm, and from -450 mV to $+100$ mV in lab experiments (Chapter V a). Of the three enzymes measured in the College Farm wetlands (β -glucosidase,

phosphatase and sulphatase), only phosphatase showed any distinct response to change in redox potential in the laboratory experiments. At College Farm, no enzyme activity correlated significantly with redox potential, suggesting that a factor other than redox was considerably inhibiting enzyme activity.

Gases

The more oxidized substrate conditions suggested by the rising redox potential were reflected in the greater production of carbon dioxide (from aerobic respiration) as the study progressed, although there was no significant correlation between these factors. The contribution of plants to carbon dioxide emission, although not sufficient to give a significant difference between planted and unplanted beds, is evident on several sampling occasions (Figure 4.6 c). Redox potential also correlated significantly and negatively with pH, which would be expected according to chemical principles and agrees with the work of Patrick (1964), Bohn (1971) and Kralova *et al* (1992).

As oxidation of the wetland beds increased, there was no corresponding decrease in methane emission; this may indicate that methane production was occurring deeper than the redox probes were positioned, and as planted beds had greater methane production than unplanted, plant transport from deep layers may have been significant (Roura-Carol & Freeman 1999). A two-sample *t*-test showed significantly higher emissions from planted beds ($T = -1.84$, $p = 0.071$). Holzapfelschorn *et al* (1986), Whiting & Chanton (1992) and others, reported greater methane emissions from vegetated soil than unvegetated, due to the role of plants in transporting methane (via their internal tissues) through the upper oxidized soil layers, and thus preventing methane oxidation; the results of this experiment agree with this work.

Fluxes of all three gases were considerably lower from these mesocosm wetlands than from the constructed wetlands studied in Chapter III. Fluxes from the 1997

field study are compared with fluxes from the College Farm mesocosm in Table 4.9 .

Table 4.9

Gas fluxes from the College Farm mesocosms compared to those from the constructed wetlands included in the 1997 field study (Chapter III), during the period March - June. (Units are mass m⁻² d⁻¹).

Wetland substrate	1997 field study	Unplanted	Planted
	Sand/peat	mesocosms	mesocosms
		Gravel	Gravel
N ₂ O	-0.17 ng – 6.0 µg	-33 – 63 ng	-1.3 – 60 ng
CH ₄	-0.2 µg – 0.39 mg	0.2 - 3.0 µg	1.7 – 6.1 µg
CO ₂	-0.09 – 2.4 mg	-0.37 – 0.043 mg	-0.32 – 0.23 mg

Consumption of nitrous oxide in the College Farm mesocosms was greater than in the wetlands studied in 1997, but production was very much lower, at approximately 1/100th of the level, in both unplanted and planted mesocosms. This would suggest that intracellular nitrogen cycling in the mesocosm wetlands was extremely low compared to the constructed wetlands included in the field study, resulting in poor removal of nitrogenous substrates; this conclusion is confirmed by the mean inflow and outflow concentrations of nitrate and ammonia (Table 4.6), although slightly more nitrate was removed than ammonia.

Methane fluxes were always positive, which was in contrast to those from the 1997 study which were commonly negative. The lack of methane consumption may be a result of there being no methanotrophs in the mesocosms, or alternatively any negative fluxes may be disguised by positive. This latter possibility would mean that positive fluxes are larger than they appear to be. However, methane emissions were only circa 1/100th those of the wetlands studied

in 1997, indicating again that anaerobic microbial activity in the mesocosms was probably much lower than in other constructed wetlands.

Consumption of carbon dioxide was slightly higher in the mesocosms than in the constructed wetlands studied in the 1997 survey, but emissions from the planted mesocosms were only about $1/10^{\text{th}}$ of those from the other constructed wetlands, and from unplanted mesocosms about $1/100^{\text{th}}$. Once again, there appears to be lower microbial respiration within the mesocosms than in previously studied constructed wetlands. The higher emissions from planted wetlands are most likely to be due to contributions from photosynthesis.

Conclusions

- Organic matter cycling may proceed faster at neutral pH than at alkaline pH.
- At low redox potentials, nitrate may accumulate in wetlands, potentially posing a water quality problem.
- Mesocosms containing plants did not show higher enzyme activities or redox potentials than those without plants.
- There was evidence that for some nutrients, water quality improvement was better at higher redox potentials.

Chapter V

Manipulation of enzyme activities

Carbon supply and enzyme
activity regulation

Manipulation of enzyme activity

5.1 Introduction

The susceptibility of enzymes to factors which influence their activity is well known, and the extent of the possible activity modification by, for example, hydrogen ion concentration (Frankenberger & Johanson 1982), temperature (McClagherty & Linkins 1990), excess substrate (Chróst 1991), or combinations of factors, has been extensively investigated in diverse fields of research. In soil research, there have also been many reports of enzyme activity manipulation by various factors, perhaps most notably pH (e.g. Pettit *et al* 1977, Nannipieri *et al* 1982).

If a particular level of enzyme activity is beneficial to treatment of a particular wastewater, and that level does not correspond with the naturally occurring activity level, manipulation of wetland soil enzyme activity may enable a more efficient treatment process, and in turn a possibly smaller land requirement. To try and clarify the best approaches to manipulating wetland enzyme activity for increased treatment efficiency, laboratory investigations into the influence of pH, temperature, redox potential, oxygen level, phenolics and carbon supply were made. Some of these factors tend to be interdependent in wetland soils, and adjusting one may induce changes in another.

In each of the manipulation experiments described below, the effect on six hydrolases and one aromatic degradative enzyme were investigated. These enzymes are representative of the carbon cycle (cellobiohydrolase, β -glucosidase, xylosidase), nitrogen cycle (N-acetylglucosaminidase), phosphorous cycle (phosphatase), sulphur cycle (sulphatase) and aromatic degradation (phenol oxidase). Fluorescent substrates as described in Chapter III were used for enzyme assays.

The wetland soils used for the manipulation experiments were taken from the constructed wetlands studied in Chapter III. The Centre for Alternative Technology (CAT) wetland treats domestic sewage, the Waun-y-Cwrt wetland treats landfill leachate, and the Pelenna wetland treats mine drainage water. It was important to use soil from wetlands constructed for a variety of treatment purposes in order to assess the feasibility of manipulating factors such as those investigated in real situations.

5.2 Soil pH

Hydrogen ion concentration (pH) is widely recognized as affecting the activity of enzymes in all manner of organisms and situations, including the activity of soil enzymes (Pettit *et al* 1977, Nannipieri *et al* 1982, Frankenberger & Johanson 1982). Whilst the pH of a wetland soil is likely to be determined largely by that of the wastewater it receives, it may be possible to alter the inflow pH to optimize the activity of particular enzymes according to the nature of the pollutants in the wastewater. Enzymes are ionic molecules, and therefore susceptible to changes in pH. However, the pH stability of soil enzymes is often overlooked, but pH studies are important because exposure to extreme pH values may irreversibly inactivate enzymes which play an essential role in nutrient (carbon, nitrogen, phosphorous and sulphur) transformations and humus formation (Frankenberger & Johanson 1982).

Addition of acid and alkali to soil slurries was used to manipulate pH, which avoids the problem of an enzyme's optimum pH being affected by the pKa of any buffer used (Frankenberger & Johanson 1982).

a. Methods

Twelve soil slurries from each of three compound samples (one from each of CAT, Waun-y-Cwrt and Pelenna wetlands) were prepared by thoroughly mixing 1 cm³ of fresh soil with 1 ml of deionized water.

Initial and subsequent measurements of slurry pH were made with an Orion 720A pH meter. After the initial measurement, the pH of four samples at a time (to avoid pH drift in well-buffered soils) was changed using concentrated hydrochloric acid or sodium hydroxide (0.04 g l^{-1}). pH modified slurries were assayed immediately using the standard MUF method (Chapter III) for cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase, xylosidase and phenol oxidase activity. MUF free acid controls were assayed at a range of pHs for each soil, to check the effect of pH on fluorescence; where necessary, results were corrected using the curves obtained.

b. Results

Each of the six hydrolases, and also phenol oxidase, showed a distinct change in activity in response to changes in pH (Figure 5.1 a-g). These changes varied between soils from different wetland sites. Generally, the response of enzymes in soil from the CAT and Pelenna wetlands was greater than that of enzymes in soil from the Waun-y-Cwrt wetland; at the CAT and Pelenna wetlands the average range of activity was 386 % and 398 % of the minimum, while at the Waun-y-Cwrt wetland it was 198 % of the minimum. Optimum pH values determined by the experiments are shown in Table 5.1. Soil from the Waun-y-Cwrt wetland was very well-buffered and its pH did not respond to addition of hydrochloric acid, so no results were obtained at $\text{pH} < 6$; neither could pH optima be determined for enzymes in this soil.

Table 5.1

Optimum pH of enzymes in constructed wetland soil. Units are pH units

Enzyme	CAT soil	Peleenna soil
Cellobiohydrolase	7	5
β -glucosidase	7	5
N-acetylglucosaminidase	7.5	5.5
Phosphatase	4.5	7.5
Sulphatase	<3	<3
Xylosidase	7	6

Phenol oxidase showed similar results in response to pH changes in all three soils; as pH increased, enzyme activity increased. Peleenna soil showed the greatest phenol oxidase activity for a given pH, followed by CAT, and then Waun-y-Cwrt, soil.

Regression analysis (Minitab v.12) showed significant relationships between pH and enzyme activity for all enzymes at all sites (for r and p values see Figures 5.1 a-g). Standard F tests (Table 5.2) showed that significant differences existed between curves for one enzyme tested at different sites.

Figure 5.1

Response of (a) cellobiohydrolase and (b) β -glucosidase activity to changing pH in soil from Centre for Alternative Technology (\diamond), Waun-y-Cwrt (x 20) (\square) and Pelenna (\circ) wetlands.

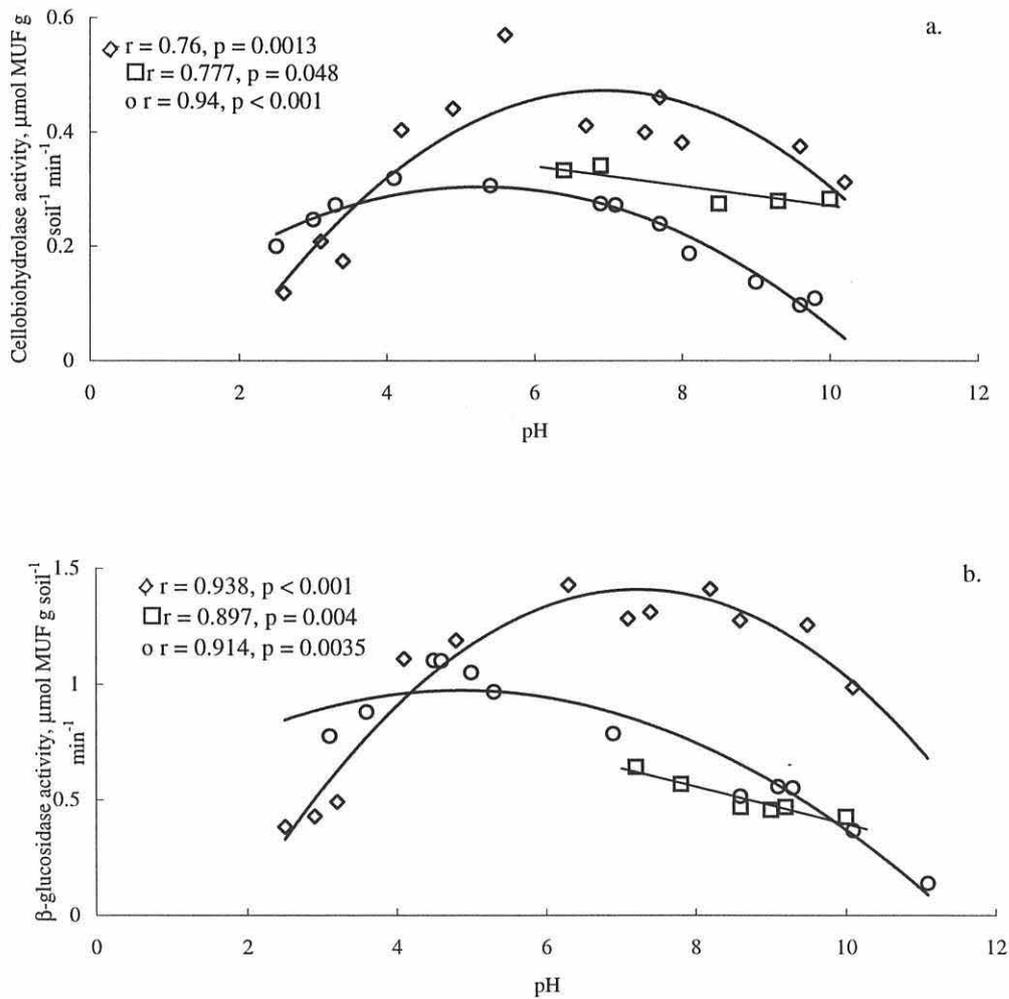


Figure 5.1 contd.

Response of (c) N-acetylglucosaminidase and (d) phosphatase activity to changing pH in soil from Centre for Alternative Technology (\diamond), Waun-y-Cwrt (x 20 in (c) and x 3 in (d)) (\square) and Pelelenna (\circ) wetlands.

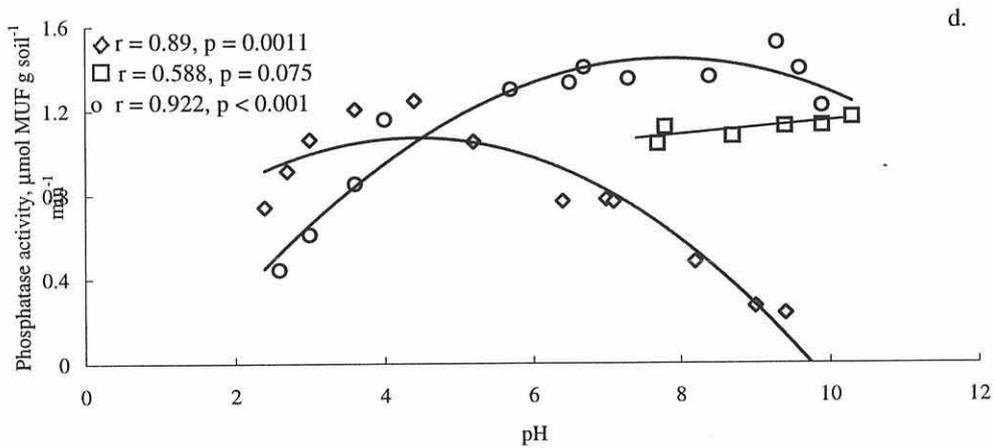
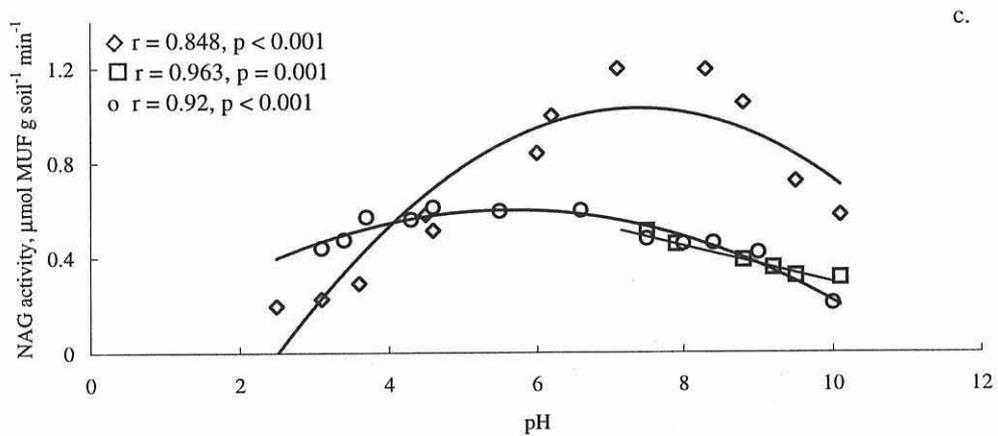


Figure 5.1 contd.

Response of (e) sulphatase and (f) xylosidase activity to changing pH in soil from Centre for Alternative Technology (\diamond), Waun-y-Cwrt (x 50 in (e) and x 10 in (f)) (\square) and Pelenna (x 5) (\circ) wetlands.

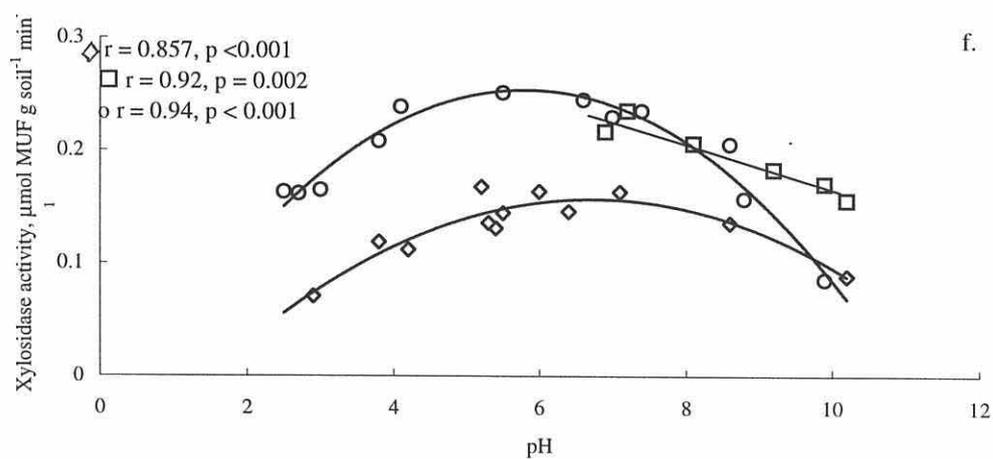
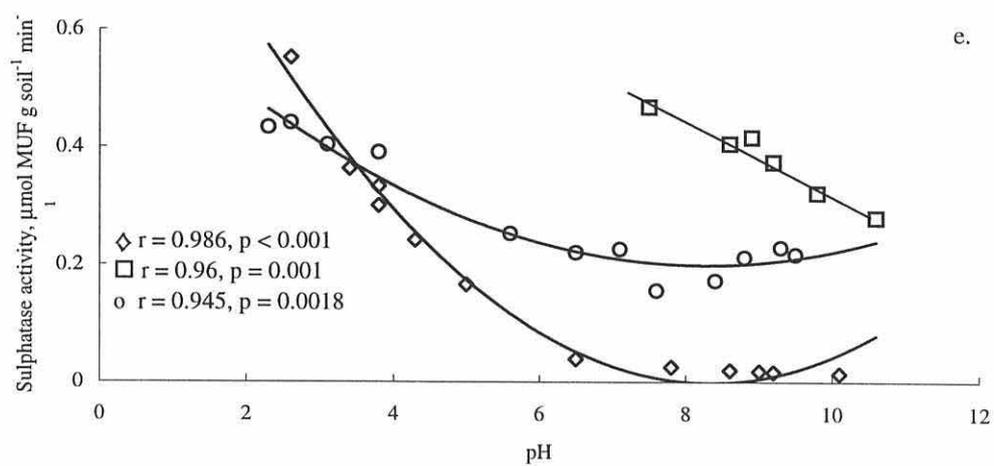


Figure 5.1 contd.

(g) Response of phenol oxidase activity to changing pH in soil from Centre for Alternative Technology (x 2) (\diamond), Waun-y-Cwrt (x 5) (\square) and Pelelenna (\circ) wetlands.

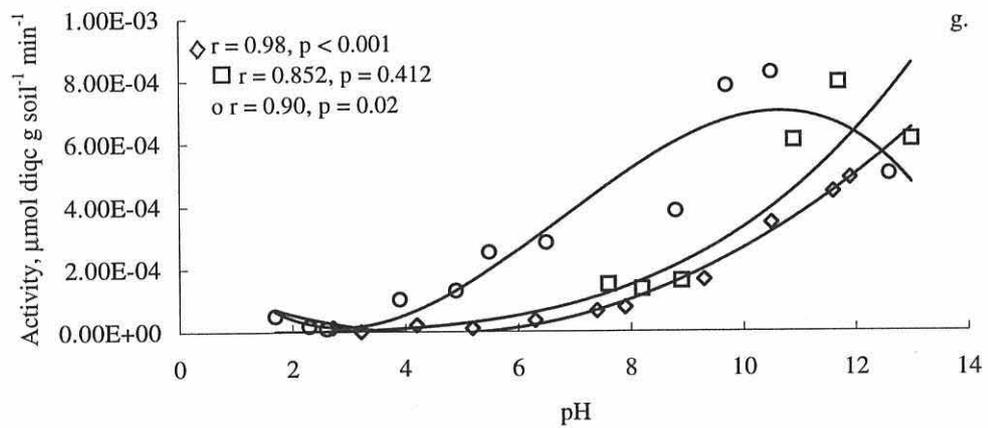


Table 5.2

F value, degrees freedom and p value for each enzyme compared between sites

Enzyme	F value (degrees freedom)	p value
Cellobiohydrolase	35.80 (6,20)	< 0.001
β -glucosidase	93.37 (6,21)	< 0.001
N-acetylglucosaminidase	38.91 (6,21)	< 0.001
Phosphatase	64.00 (6,21)	< 0.001
Sulphatase	175.12 (6,21)	< 0.001
Xylosidase	799.44 (6,21)	< 0.001
Phenol oxidase	12.62 (6,19)	< 0.001

The pH of CAT wetland soil usually lies between 5 and 7, but only three of the six hydrolases showed pH optima between these values (cellobiohydrolase, β glucosidase and xylosidase). The remaining hydrolases had optimal activity at pH 7.5 (N-acetylglucosaminidase), pH 4.5 (phosphatase) and pH <3 (sulphatase). Waun-y-Cwrt wetland soil pH usually lies between 5 and 8. The pH of Peledda wetland soil is usually between 5.5 and 7, as the inflowing mine drainage is not particularly acidic (average pH 6.4 (October 1996)). Only two of the hydrolases (N-acetylglucosaminidase and xylosidase) had pH optima which fell within this range, the others being lower at pH 5 (cellobiohydrolase and β -glucosidase) and pH <3 (sulphatase), or higher at pH 7.5 (phosphatase).

c. Discussion

The pH optimum of an enzyme is usually confined to a narrow range of pH, determined by the pK's of the ionizing groups of the catalytic site (Frankenberger & Johanson 1982); however the experiments described above frequently showed broad, flat peaks of activity in soil enzymes. Enzymes, like all other proteins, are subject to different states of ionization depending on the pH and ionization constants of their various reactive groups.

Exposure to a high hydrogen ion (pH 1-2) or hydroxyl ion (pH 12-14) concentration tends to disrupt the ionic and hydrogen bonds needed to maintain the active conformation of enzyme proteins (Frankenberger & Johanson 1982). Significant changes in the properties of denatured enzyme proteins include decreased solubility at the enzyme's isoelectric point, loss of biological activity, increase in reactivity of chemical groups previously masked by folding in the native protein, increase in levo-rotation, increase in asymmetry and increase in susceptibility to hydrolysis by proteolytic enzymes (Frankenberger & Johanson 1982). The hydrogen ion concentration in the reaction system may also influence the ionization state of the substrate. Thus the affinity constant of the enzyme becomes affected by the pH of incubation (Frankenberger & Tabatabai 1980).

The pH optima of N-acetylglucosaminidase, phosphatase and sulphatase in CAT wetland soil did not coincide. This suggests that some forms of nitrogen, phosphate and sulphate could be cycled more quickly in this wetland if the soil pH was different. However, changing the soil pH would inevitably change the activity of other enzymes, and thus the balance of nutrient availability. Similarly, at the Pelelwa wetland the activities of cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase and sulphatase may be increased by changes in pH. Carbon cycling in this wetland may be sub-optimal as two of the enzymes not operating at their optimal pH are cellobiohydrolase and β -glucosidase; however as mine drainage treatment requires available carbon to act as an electron acceptor, and does not in itself require carbon mineralization for treatment, this may be unimportant.

Phenol oxidase in wetland soil from all three sites increased with increasing pH. The rapid increase in activity at about pH 10 was in contrast to the results of Pind *et al* (1994), who found that phenol oxidase had an optimum pH range of 8-8.5. McLatchey & Reddy (1998) also attributed low phenol oxidase activity to acidic soil conditions. The increase at pH 10 may be related to the pKa of phenol, which

is 10. At pKa, half of the phenol molecules are protonated and half are unprotonated. When the phenol ring is unprotonated, it becomes much more susceptible to degradation and although pH 10 is unlikely to be found in the environment as a whole, the phenol oxidase system seems to be adapted to working at a pH which enhances vulnerability of its substrate.

At the CAT wetland, phosphatase activity appeared to be dominated by acid phosphatase, and at the Pelenna site by alkaline phosphatase. However Nannipieri *et al* (1982) reported that neutral, rather than acid or alkaline, phosphatase is frequently found in soils, with an optimum pH of around 7, and a broad range of activity. This usually represents a mixture of acid and alkaline phosphatase (Speir & Ross 1978). Alef & Nannipieri (1995) cite reports that the pH optimum of cellulase in soil is generally 5-6, so while the optimum of pH 5 found in the Pelenna wetland falls within this range, pH 7 at the CAT wetland does not. Alef & Nannipieri (1995) also cite reports of the pH optimum of xylanase as pH 5-7, which the optimum pH of xylosidase at both the CAT and Pelenna wetlands fell within.

The variation in the pH stability of enzymes among soils may be due to the adsorption properties of the soils, and to the diversity of sources which contribute to enzyme activity (vegetation, micro-organisms and soil fauna) (Frankenberger & Johanson 1982). The clay and humus are generally negatively charged and readily adsorb the positively charged reactive groups of proteins below the protein's isoelectric point (pI). The presence of cations in the soil matrix may influence the adsorption of enzymes because cations could neutralize the repulsive electrostatic potential of the negative charges of the protein and soil colloids forming a clay- or humus-cation-protein bridge in which the cation links the two negatively charged particles.

Frankenberger & Johanson (1982) concluded that the adherence of enzymes to the humic-clay fraction would allow some resistance to pH denaturation, since many

of the ionizable groups would then be less accessible to protonation and hydroxylation. Knowledge of the binding mechanisms between enzymes and the soil constituents on which they are stabilized may be essential to understanding the properties of soil hydrolases (Nannipieri *et al* 1982).

The pH of a constructed wetland soil may be manipulated successfully at a field scale in two main ways. Firstly, the pH of the effluent entering the wetland may be changed, by addition of acid or alkali, so that the soil pH changes as the wetland becomes saturated with the effluent. This method may not always be possible or suitable, for example the pH of effluent with a high buffering capacity is likely to need considerable addition of hydrogen or hydroxyl ions to effect a change. In addition, acidifying an effluent may cause solubilization of metals which would otherwise precipitate from solution. A second method by which the wetland soil pH may be manipulated is by the direct addition to the soil surface of acid (e.g. nitrate) or alkaline (e.g. lime) compounds.

Despite the clear influence of pH on the six hydrolases and phenol oxidase studied in these experiments, there was no similarly clear correlation between pH and enzyme activity in the fieldwork data (Chapter III). Other factors may have had a greater influence in field conditions, masking the contribution of pH. The different responses of enzymes in the soils of different wetlands preclude general statements about manipulating enzymes to optimize particular nutrient cycles. Before deciding whether, and how, to manipulate pH, it would be important to examine the pH response of the specific enzymes which are most important for water quality improvement at a particular wetland. All of the enzymes investigated in this experiment showed potential for manipulation via pH changes.

5.3 Soil temperature

Temperature is a primary control in many decomposition processes (Linkins *et al* 1984), and although climatic temperature is outside the control of wetland managers, there are possibilities for altering the temperature at which wastewater

is treated in wetlands. Latitude is a key determinant of annual temperature cycles. The temperature functions of enzymes are useful for estimating the potential enzyme activity at ambient temperatures in the field (McClaugherty & Linkins 1990).

In these experiments two temperature gradient bars (Dowrick 1998) were used to investigate the responses of β -glucosidase, N-acetylglucosaminidase and phosphatase to temperature, in soils from the Centre for Alternative Technology and Pelenna wetlands. The response of nitrous oxide, methane and carbon dioxide emissions were also monitored.

a. Methods

Soil collected in September 1997 from 2 cm below the wetland surface was thoroughly mixed by hand and placed in a 2 cm layer in a temperature bar on clingfilm. The clingfilm was wrapped over the soil to prevent moisture loss by evaporation, then a further layer of clingfilm and a double-glazed covering panel were placed on the soil surface. Both temperature bars were set to maintain a temperature gradient from 0 °C to 20 °C, covering the range of annual temperatures expected in British soils. The bars were kept in a constant temperature room at 10 °C; this was necessary as the bars have no thermostatic temperature control.

For each enzyme assay, (β -glucosidase, N-acetylglucosaminidase and phosphatase, and methylumbelliferone free acid), two 1 cm³ replicate soil samples were removed from each bar at 5, 10, 15 and 20 °C, and assayed using the MUF fluorescent substrate method (Chapter III). Each sample was incubated at the temperature it was collected from, using either a cabinet incubator or a chilled water bath. Seven ml of substrate was added to each soil sample, at 400 $\mu\text{M l}^{-1}$ for β -glucosidase and N-acetylglucosaminidase, and 200 $\mu\text{M l}^{-1}$ for phosphatase.

For gas sampling, 25 ml plastic bottles with screw cap lids and the bottoms removed were pressed 1 cm deep into the soil. The lid of each bottle had a gas sampling port to which a gas-tight syringe was attached at the start of sampling. An initial series of measurements were made to check that gas emissions were linear over time. Five ml gas samples were collected from four points on each temperature bar, where the temperature was 5, 10, 15 and 20 °C, and analysed using an Ai Cambridge gas chromatograph (see Chapter III for specification).

b. Results

All three investigated enzymes showed increasing activity over the temperature range used, in both wetland soils (Figure 5.2 a, b). Q_{10} values for the enzymes varied from 1.39 to 4.28 (Table 5.3), and for gases from 0.045 to 25.39 (Table 5.4).

Table 5.3

Q₁₀ values for enzymes in CAT and Pelenna wetland soils

Enzyme	CAT	Pelenna
β -glucosidase	3.03	1.99
N-acetylglucosaminidase	3.36	4.28
Phosphatase	3.79	1.39

Table 5.4

Q₁₀ values for gas fluxes from CAT and Pelenna wetland soils

Gas	CAT	Pelenna
Nitrous oxide	0.045	1.018
Methane	0.975	
Carbon dioxide	3.15	25.39

Figure 5.2

Response of β -glucosidase (\blacklozenge), phosphatase (\square) and sulphatase (\bullet) activity to changing temperature in soil from Centre for Alternative Technology (a) and Pelenna (b) wetlands. (mean, $n = 3$, \pm s.e.).

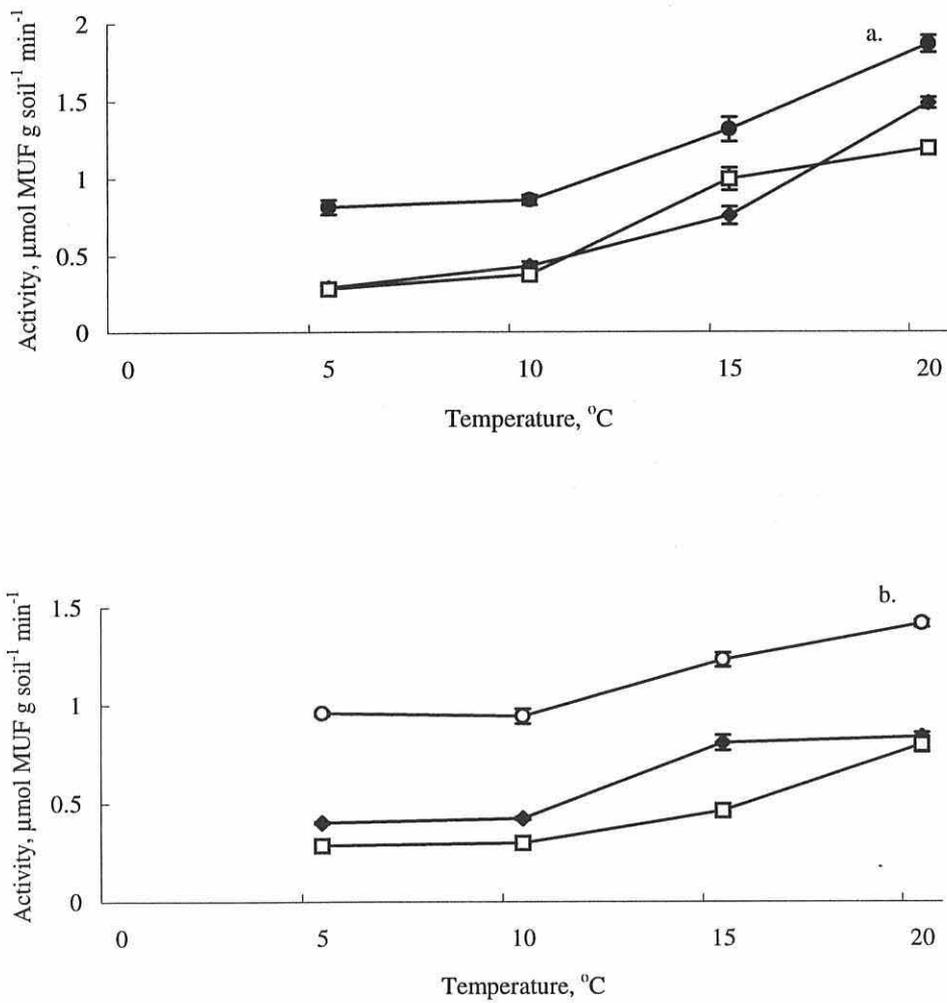
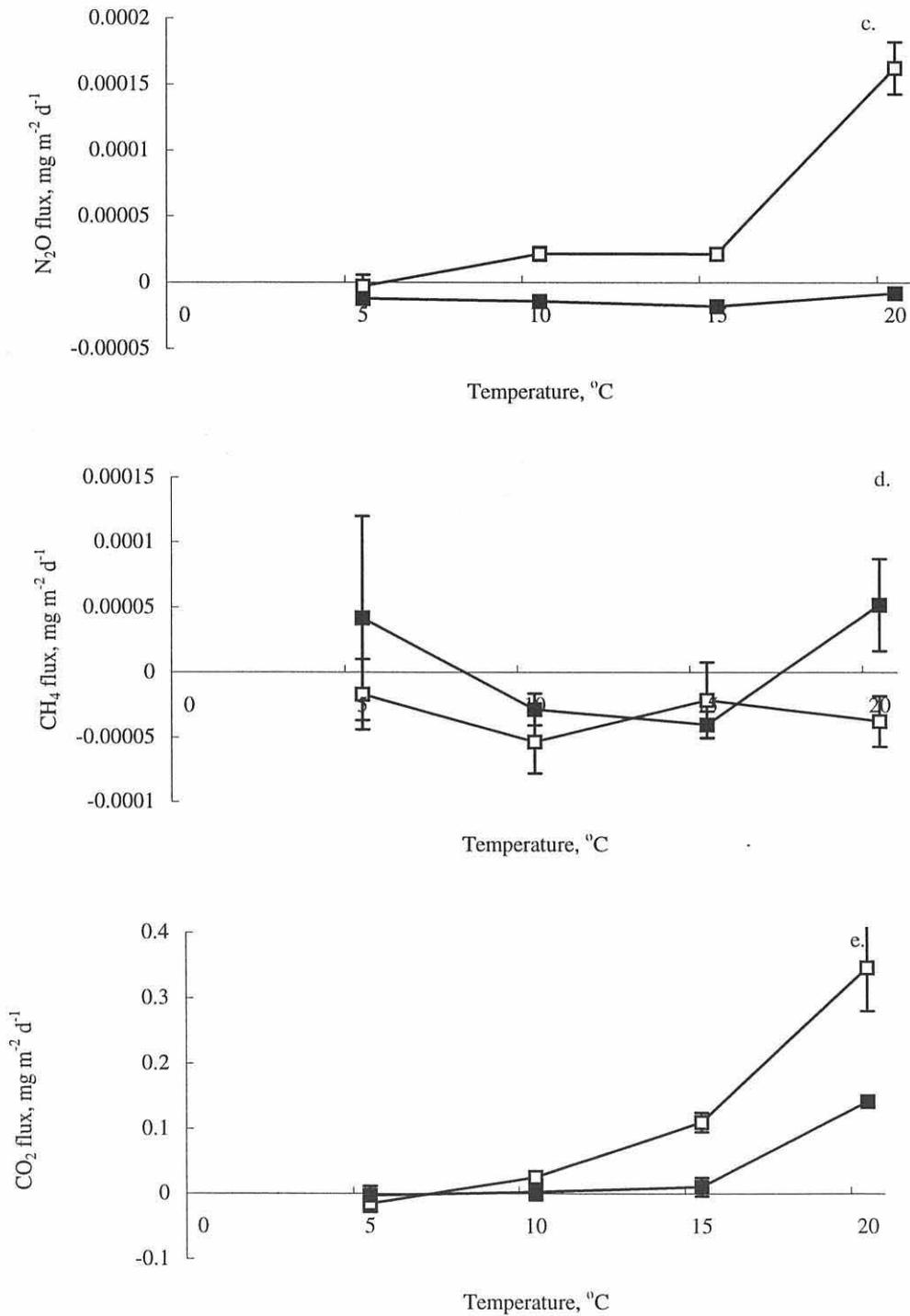


Figure 5.2 contd.

Response of nitrous oxide (c), methane (d) and carbon dioxide (e) emission to changing temperature in soil from Centre for Alternative Technology (□) and Pelenna (■) wetlands. (mean, $n = 3$, \pm s.e.).



c. Discussion

Although it would be surprising to find an enzyme which showed no response to temperature, it is the Q_{10} values which are the most interesting result of this experiment. Those for β -glucosidase, N-acetylglucosaminidase and phosphatase in CAT wetland soil are very similar, whereas those for the same enzymes in Pelenna soil vary widely, and are very different from the CAT values. It is apparent that the behaviour of these enzymes in different soils is disparate, and may indicate that different isoenzymes are involved, or that different stabilization mechanisms are at work in the two wetlands, which are inhibiting, inactivating or facilitating the enzymes differently. McClaugherty & Linkins (1990) reported that the type of binding involved in stabilizing an enzyme in soil may affect its response to temperature, and that the type of binding may in turn depend on the type of organic matter or mineral material available. Substrates in the CAT and Pelenna wetlands certainly differ in their organic matter and mineral contents; the CAT substrate consists of fine sand with some accumulation of organic matter, whereas the Pelenna substrate is bark chippings (high organic matter) with little mineral content.

Q_{10} values indicate the response of enzymes to increases in temperature; in the case of enzymes in constructed wetland soil, this is of interest in considering the seasonal changes in enzyme activity due to soil temperature. If soil temperature increases, for example in spring, then an increase in decomposition rates can be expected solely on the basis of that increase and irrespective of microbial growth or enzyme production (McClaugherty & Linkins 1990). Similarly, cooling in autumn would decrease enzyme activity without necessarily a decline in microbial biomass. The field studies (Chapter III) suggested evidence for these hypotheses, with strong correlations between temperature and carbon cycling enzyme activity at the CAT wetland; however as microbial biomass measurements were not part of the study, no definite conclusions can be drawn.

The manipulation of pH has already demonstrated that enzyme characteristics in the two wetlands (CAT and Pelenna) are different, and these temperature experiments confirm this.

Nitrous oxide emissions from the CAT wetland soil increased dramatically between 15 and 20 °C, which must be due to temperature-induced stimulation of denitrifying bacteria, or increased diffusion of nitrate to the sites of denitrification. Results from the field study showed a significant correlation between temperature and nitrous oxide emission at the CAT wetland, which are confirmed by this experiment. In Pelenna wetland soil there was a much smaller increase in nitrous oxide emission over the same temperature change; there may be fewer denitrifiers in this soil, and the field study did not reveal a significant correlation between temperature and nitrous oxide emission. The calculated Q_{10} values for these two sites appear to contradict these facts (Q_{10} of nitrous oxide at CAT = 0.045, at Pelenna = 1.018), but are distorted by the decline in emission from CAT soil between 5 and 15 °C.

Soil from the Centre for Alternative Technology wetland consumed methane at a similar rate at all the temperatures examined. Methanotrophic micro-organisms therefore appear to be more dominant in this soil than methanogens; the Q_{10} value is not meaningful. In Pelenna soil, methane oxidation increased with increase in temperature from 5-15 °C, then became positive flux above 15 °C. Increasing the temperature may have stimulated methanotrophic bacteria which were using methane as an electron acceptor, until at 15 °C methanogenic metabolism became dominant. The field study did not show a significant correlation between temperature and methane emission at any site. Hulzen *et al* (1999) reported Q_{10} values for methane production from natural wetlands of 2.9-3.6, and Bridgham & Richardson (1992) a value of 3.0. These are considerably larger than those calculated from the constructed wetland soil used in the temperature bar experiment.

Carbon dioxide emissions rose predictably with temperature, reflecting the increase in microbial activity at higher temperatures. The greater emission per hour from CAT soil may reveal the greater microbial population of this soil compared to Pelenna soil. Values of Q_{10} are slightly misleading as they suggest that the Pelenna soil will produce more carbon dioxide per temperature rise; this anomaly is a result of the slow start in carbon dioxide production with increasing temperature. Bridgham & Richardson (1992) report a Q_{10} of 2.0 for carbon dioxide production from anaerobic peat, which they report is similar to values reported for upland aerobic soil respiration (e.g. Schlesinger 1977). The temperature bar results agree well with those from the field studies, where temperature and carbon dioxide emission correlated significantly at the CAT and Waun-y-Cwrt sites (Chapter III).

5.4 Redox

Just as pH is a measure of hydrogen ion concentration, redox potential is a measure of electron activity (Ingold 1980). In soils, the redox potential may change as a result of changes in gas or moisture, and through the incorporation of carbonaceous wastes which have a high oxygen demand (Kralova *et al* 1992). The decomposition of such organic matter depends on the capacity of the soil to supply electron acceptors (which depends on soil redox potential), and on bacterial efficiency (Reddy *et al* 1986).

Although the main influence of redox potential may be on the nature of electron acceptors available to micro-organisms for metabolic oxidation of substrates, soil redox potential may also influence extra-cellular enzyme activity because electrons are essential reactants in biochemical as well as inorganic and organic reactions (Bohn 1971). Redox potential affects the oxidation state of hydrogen, carbon, nitrogen, oxygen, sulphur, iron, manganese, cobalt and copper in aqueous systems (Bohn 1971).

Redox gradients in wetlands are affected by hydrologic fluctuations, the presence of electron acceptors (such as oxygen, nitrate and sulphate) and transport of oxygen by plants into the rhizosphere (Reddy & D'Angelo 1997). Redox gradients may also be set up by buffering of alternate electron acceptors used during anaerobic respiration, and thus may be used as an indicator of potential nitrification-denitrification reactions, iron-oxide regulated precipitation of phosphorous, and oxidation of methane and sulphides, and breakdown of toxic organic compounds (Reddy & D'Angelo 1997). McLatchey & Reddy (1998) successfully buffered soil redox potentials at a range of values between -220 mV and 620 mV using the electron acceptors O_2 , NO_3^- , SO_4^{2-} and HCO_3^- in separate reactors. However, the addition of these different electron acceptors changed many physico-chemical properties of the soil, including the pH, available nutrients and dissolved organic carbon (McLatchey & Reddy 1998).

The simplest way to change the redox potential of a soil is to waterlog it. This retards gas exchange between the soil and atmosphere, changes the microbial population, decreases the redox potential, increases the pH, and causes electrochemical and chemical changes (Pulford & Tabatabai 1988). When oxygen is unavailable, micro-organisms use oxidized substrates such as nitrate, manganese oxide, iron hydroxide and sulphate as electron acceptors in respiration.

Soil redox potential determines the type of microbial respiration which dominates. Above $+300$ mV, aerobic respiration (including nitrification) occurs; at less than $+300$ mV, and above 0 mV, facultative anaerobes function (including denitrifiers), usually in deeper soil layers; at redox potentials below 0 mV, all respiration is by obligate anaerobes (Reddy *et al* 1986). The production of gases by soil is similarly related to redox potential, and nitrogenous gases are produced between $+100$ and $+400$ mV, during denitrification.

a. Methods

Following the method of Zehnder & Wuhrman (1976), titanium (III) citrate was used as a non-toxic oxidation-reduction buffering system for soil; Zehnder & Wuhrman originally used this buffer for the culture of obligate anaerobes. Pulford & Tabatabai (1988) changed soil redox potential by purging soils with nitrogen and tightly capping the bottles, but this did not produce satisfactory results for this experiment.

Preliminary experiments showed that titanium (III) citrate is stable at redox potentials around -500 to -300 mV, but that once purged with air to -200 mV it becomes unstable and quickly continues to oxidize, reaching +80 mV within 60 minutes without further purging. This made it difficult to obtain redox potentials in the range -200 to 0 mV, but the problem was later improved by purging the buffer with air to the required redox potential and then immediately purging with oxygen-free nitrogen to remove oxygen, which was causing the continued oxidation, from the solution.

A compound sample of soil from the Centre for Alternative Technology was collected in February 1998. One cm³ portions of this soil were mixed with 4 ml titanium (III) citrate redox buffer, prepared according to the method of Zehnder & Wuhrman (1976) by adding 5 ml of 15 % titanium (III) chloride to 50 ml of 0.2 M sodium citrate solution and neutralizing with a saturated solution of sodium carbonate (200 g l⁻¹). At pH 7, the equilibrium redox potential of titanium (III) citrate buffer should be -480 mV.

Eight sets of 18 1 cm³ soil samples were prepared, in order to assay the activity of seven enzymes and methylumbelliferone free acid (two replicates for each), and check pH (total 144 soil samples). At the start of the experiment 4 ml of freshly prepared titanium (III) citrate (redox potential -480 mV) was added to each of the first 18 samples and these were capped tightly; the remaining titanium (III) citrate was purged with compressed air for 10 minutes to raise the redox potential, after

which 4 ml was added to each of another 18 samples and these capped tightly. The titanium (III) citrate was then purged again with compressed air for 10 minutes and a third batch of 18 samples prepared; this procedure was repeated for all eight sets of 18 samples. pH check of the titanium (III) citrate buffer at different redox potentials showed no variation.

Two soil samples at each redox potential were then assayed for each enzyme activity (cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase and xylosidase) and quench in the usual way (Chapter III), adding 7 ml of substrate to each bottle.

The effect of redox potential on phenol oxidase activity could not be investigated using the L-dopa method as formation of a bright yellow colour on mixing the titanium (III) citrate and L-dopa solution interfered with spectrophotometric measurement.

b. Results

Despite not obtaining results for enzyme activities between redox potentials of -250 and -50 mV, some trends were obvious. Regression analysis (Minitab ver. 12) showed significant relationships between redox potential and enzyme activity for cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase and xylosidase.

The carbon-cycling enzymes cellobiohydrolase (Figure 5.3 a) and β -glucosidase (Figure 5.3 b) had higher activity at redox potentials between -100 and $+100$ mV. Activity of the third carbon-cycling enzymes, xylosidase (Figure 5.3 f), declined at these values, being slightly higher at -400 mV. N-acetylglucosaminidase (Figure 5.3 c), involved in the nitrogen cycle, was most active at -200 mV, with activity declining sharply above and below this potential. Phosphatase activity (Figure 5.3 d) showed highest activity at each end of the range of redox potentials

investigated, with very low activity at -200 mV; at -400 and $+100$ mV phosphatase activity was highest. Sulphatase activities (Figure 5.3 e) were scattered, but declined a little at $+100$ mV compared to -400 mV.

After standardization the curves for all six enzymes were compared statistically, and were significantly different ($F_{15,90} = 3.86$, $p < 0.001$). Of the six enzymes, cellobiohydrolase and β -glucosidase showed similar responses to change in redox potential (a linear increase), as did N-acetylglucosaminidase, sulphatase and xylosidase (a quadratic response).

Figure 5.3

Response of (a) cellobiohydrolase, (b) β -glucosidase and (c) N-acetylglucosaminidase to changing redox potential in soil from the Centre for Alternative Technology wetland.

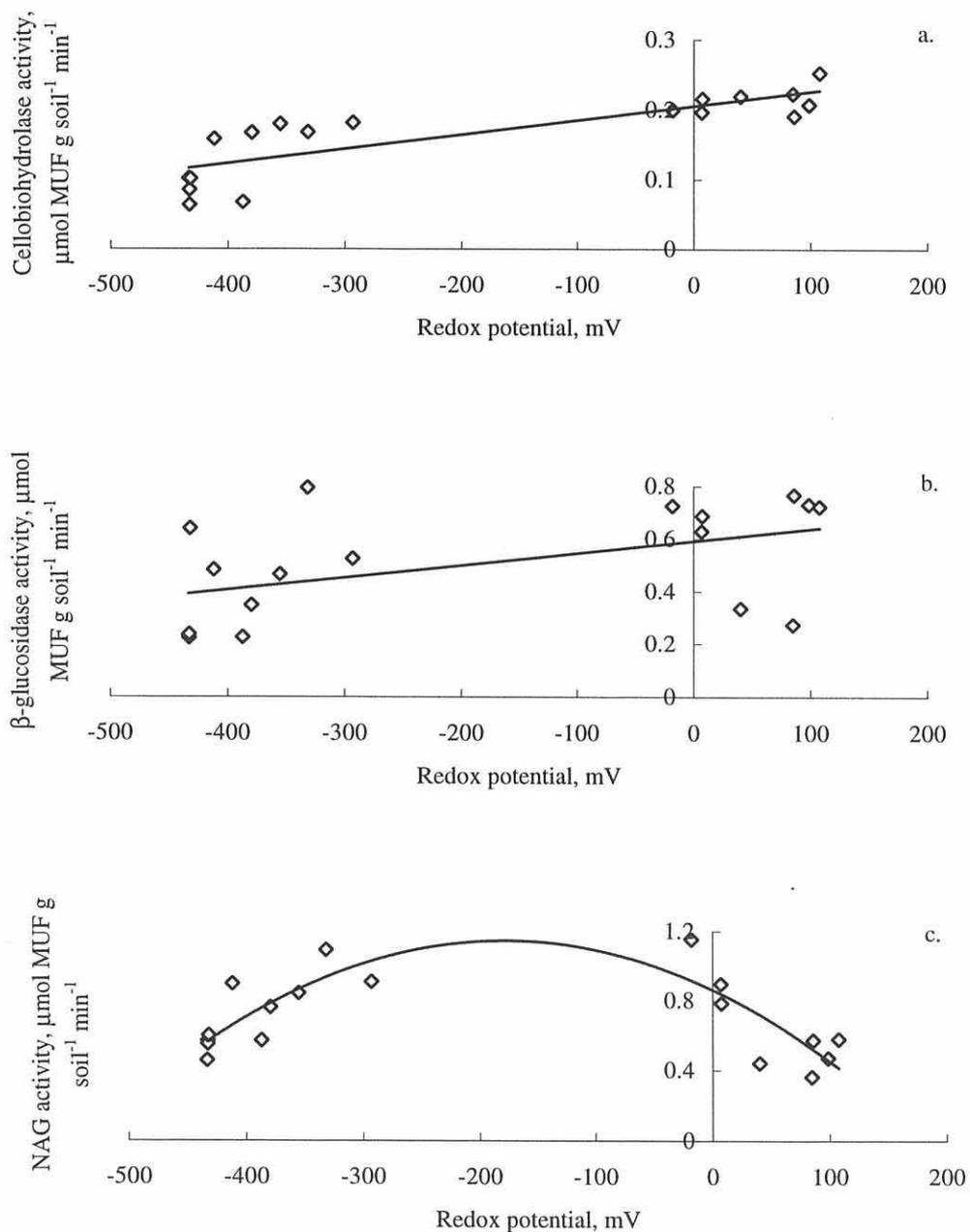
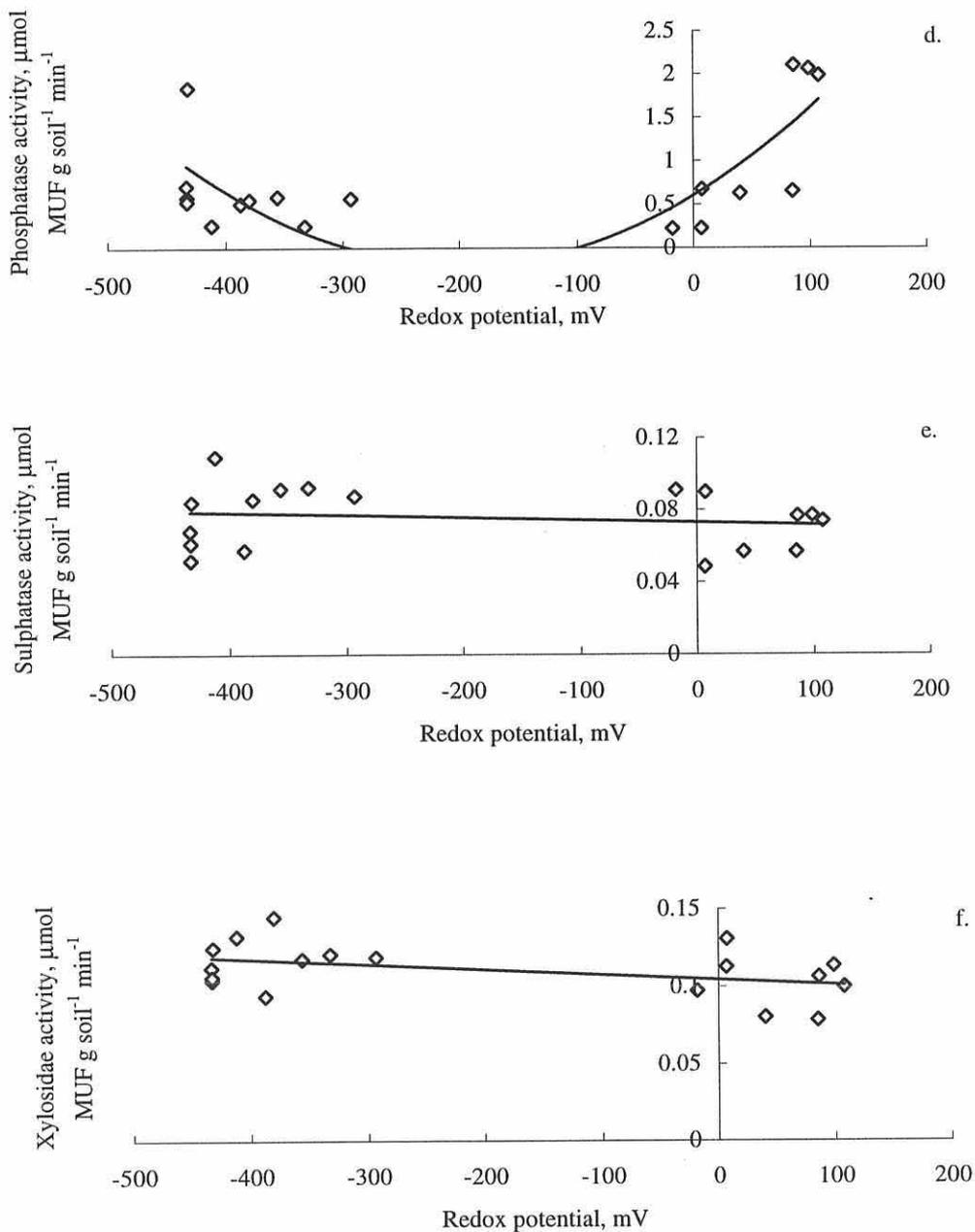


Figure 5.3 contd.

Response of (d) phosphatase, (e) sulphatase and (f) xylosidase to changing redox potential in soil from the Centre for Alternative Technology wetland.



c. Discussion

Altering the redox potential of soil is possible, but fine control of the actual potential obtained is difficult to achieve and maintain. Flooding is the easiest method, but cannot be used to control redox potential on a fine scale. Changes in soil redox potential affect enzyme activity directly by changing the availability of reduced metal ions which may inhibit or activate enzymes, and indirectly (over a longer time-period) by changing the microbial population and therefore *de novo* synthesis of enzymes (Pulford & Tabatabai 1988).

Pulford & Tabatabai (1988) reported the response of a range of enzymes to changes in soil redox potential. They noted mostly decreases (of up to 60 %) in activity as redox potential became more negative, particularly in acid and alkaline phosphatase, and in sulphatase, which they attributed to inhibition by reduced metal ions produced by the waterlogging method which they used to lower redox potential. β -glucosidase activity was found to follow hyperbolic curves. Results from this experiment for phosphatase showed a decline as redox potential approached zero, but for other enzymes the change in activity in this range was either negligible or showed an increase. Pulford & Tabatabai incubated soils for up to ten days to achieve the reduction in redox potential, but did not use microbial inhibitors to prevent *de novo* synthesis of enzymes by anaerobes.

As reduced metal ions are more soluble than their oxidized forms, Pulford & Tabatabai (1988) suggest that declines in enzyme activity which occur when soil is waterlogged are due to inhibition by these reduced metal ions.

The difficulty in poisoning the redox buffer at potentials between -250 mV and -50 mV meant measurements of enzyme activity at these potentials could not be made. An alternative method of changing redox potential is that employed by McLatchey & Reddy (1998), in which oxygen, nitrate, sulphate and hydrogen carbonate were added to soil to obtain redox potentials of approximately 620, 310, 100 and -220 mV respectively; whilst this method lacks the continuous variation

possible using Zehnder & Wuhrman's titanium (III) citrate buffer, it may result in more stable redox potentials. More controls would be required to account for changes in enzyme activity which were due to the added ions rather than the changed redox potential. McLatchey & Reddy (1998) found that microbial biomass and the mineralization rates of carbon, nitrogen and phosphorous decreased as soils became more reduced, and concluded that organic matter turnover and nutrient cycling were strongly correlated with redox conditions in wetland soils. The data obtained in this experiment for cellobiohydrolase, β -glucosidase and N-acetylglucosaminidase agree with this. Soil dehydrogenase activity (a reasonable measure of soil microbial activity) was found by Brzezinska *et al* (1998) to decline with decreasing redox potential.

5.5 Oxygenation

In oxidized environments, low concentrations of redox couples reduce the stability, reproducibility and general usefulness of redox potential measurements (Bohn 1971). In such environments, measurements of dissolved oxygen concentration are more stable and more useful. Pearsall & Mortimer (1939) and Mortimer (1941) (both cited in Armstrong 1967) report the change from oxidizing to reducing conditions in natural water systems at 8 % oxygen. The effect of different levels of oxygen on enzyme activity in soil from the Centre for Alternative Technology wetland was measured using soil slurries bubbled with air, oxygen-free nitrogen or a mixture of the two.

a. Methods

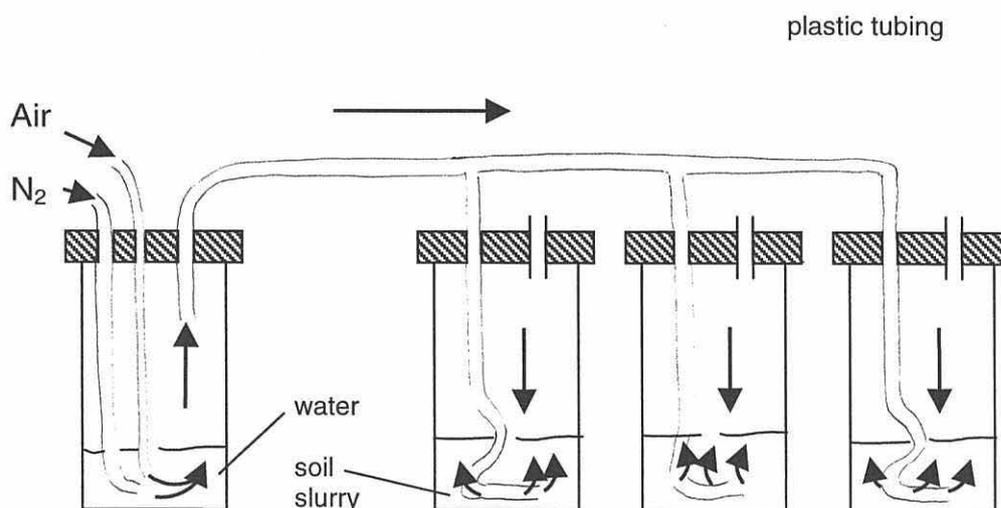
Three replicate slurries were prepared and assayed together for each enzyme at each oxygen concentration. Each slurry was prepared from 4 cm³ CAT soil and 16 ml deionized water, maintaining the 1:4 ratio of soil : water of previous laboratory work. MUF substrates were prepared as before (Chapter III) and bubbled with the appropriate gas mixture before use until they had reached the desired oxygen concentration. The three replicate slurries were then bubbled until they too were at the desired oxygen concentration, and then 28 ml (to maintain previous ratios of

slurry to substrate) of MUF substrate was added while bubbling continued. Timing of the incubation period began immediately on substrate addition.

Oxygen concentrations used were 0 % (bubbled with oxygen-free nitrogen), 50 % (bubbled with a mixture of oxygen-free nitrogen and air), and 100 % (bubbled with air). Gases used for bubbling were directed through water in a mixing chamber before passing to the experimental chambers, using the method described by Ostle (1994) (Figure 5.4a). During incubation each experimental chamber was loosely covered with Parafilm to preserve the required atmosphere above the slurry. During the 60 minute incubation, oxygen concentration was monitored continuously in all three slurries. After 60 minutes, two analytical replicates of 1.5 ml of slurry were removed and centrifuged at 10,000 rpm for 5 minutes. Fluorescence was measured as described in Chapter III.

Figure 5.4

Oxygenation experiment mixing chambers (after Ostle 1994)



The effect of oxygenation on phenol oxidase was investigated using soil slurries made with 1 cm³ of CAT wetland soil and 5 ml of deionized water. L-dopa substrate was made at 10 mM l⁻¹, and both slurry and substrate were bubbled with

a mixture of air and nitrogen to achieve the desired oxygen concentration. When this was reached, 6 ml of L-dopa was added to the slurry and timing began. After 1 min, two 1.5 ml aliquots were centrifuged and the absorbance of diqc produced was measured at 460 nm; two minutes later this was repeated to give a measurement at 3 minutes' incubation. Each measurement was carried out in triplicate, at three oxygenation levels (0, 50 and 100%).

Data for activity of each enzyme at each level of oxygenation were compared using one-way ANOVA (Minitab ver. 12), after testing for normality with Anderson-Darling tests.

b. Results

Activity varied differently between the three oxygenation levels between different enzymes (Figure 5.5). Cellobiohydrolase, β -glucosidase and xylosidase all showed lower activity at 50% oxygenation than at either 0% or 100% oxygenation, but this difference was only significant for cellobiohydrolase ($F_{2,6} = 7.89$, $p = 0.021$), where activities at 50 and 100% were significantly different, and β -glucosidase ($F_{2,6} = 19.67$, $p = 0.002$), where activities at 0 and 50%, and 50 and 100% were significantly different.

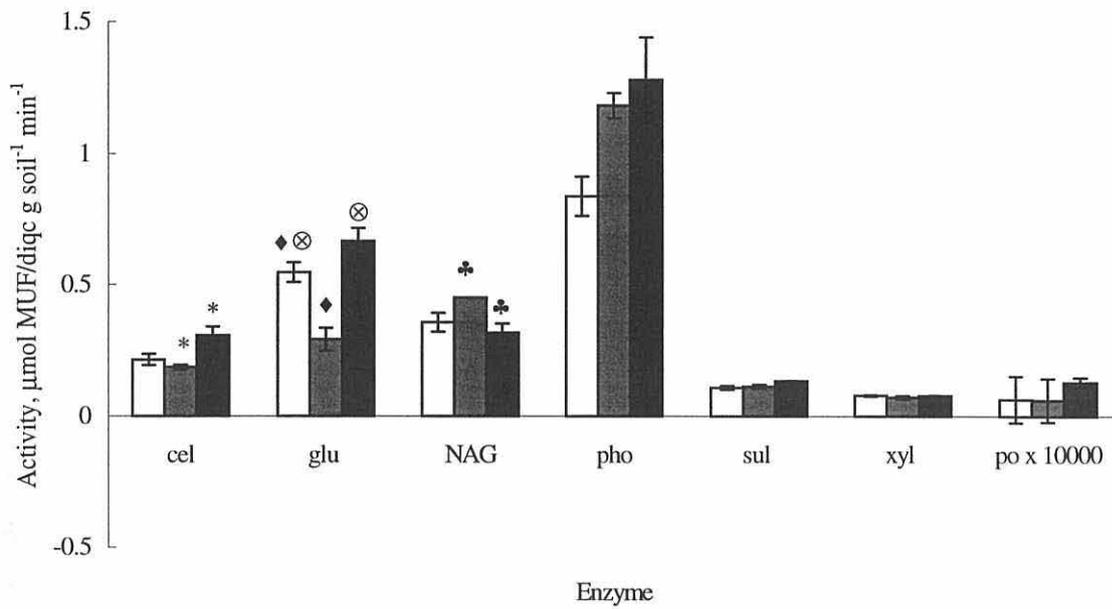
N-acetylglucosaminidase showed highest activity at 50% oxygenation ($F_{2,6} = 6.11$, $p = 0.036$), with activities at 50 and 100% being significantly different.

Phosphatase activity increased with increasing oxygenation, but there were no significant differences ($p > 0.05$) between activities. Sulphatase and xylosidase showed no significant response to increase in oxygenation.

The differences in phenol oxidase activity at the three different oxygenation levels were not significantly different, which was unexpected.

Figure 5.5

Response of cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase, xylosidase and phenol oxidase to oxygenation of 0 (□), 50 (▨) and 100 (■) %. Symbols indicate results with significant differences. (Mean, $n = 3, \pm$ s.e.).



c. Discussion

Oxygenation is largely controlled by the degree of saturation of the wetland soil, and there is also substantial published evidence that wetland plants release oxygen to the rhizosphere by internal gas transport and diffusion across an oxygen gradient (Brix 1994, Sorrell & Armstrong 1994). If this is so, then enzyme activities may be different in the rhizosphere, compared to the bulk soil, due to oxygenation. Very little information has been published regarding the effect of either oxygen level or molecular oxygen on enzyme activities; even microbiological studies have so far provided limited information. An anaerobic rumen microbe, *Prevotella ruminicola*, was found by Gasparic *et al* (1995) to produce a xylanase with considerable sensitivity to oxygen, and the same authors also describe an *E. coli* xylosidase with high oxygen sensitivity. Similarly Buchanan & Mitchell (1992) found cellulolytic enzymes in bovine and ovine rumens showed instability in the presence of oxygen, and it seems reasonable to assume that enzymes produced by anaerobic bacteria will be adapted to anaerobic functioning and therefore show sub-optimal and/or unstable activity in the presence of oxygen. Oxygen sensitivity can result from disulphide bond formation (Gasparic *et al* 1995), denaturing the enzyme protein. Oxygen can be the source of powerful free radicals which attack and potentially inactivate or destroy proteins such as enzymes.

The results of these experiments indicate that some enzymes are affected by oxygen concentration more than others, and most did not show a linear response to increasing oxygen concentration. Since mineralization is an oxidative process, resulting in production of a variety of oxides (carbon dioxide, phosphate, sulphate, nitrate, nitrous oxide), the enzymes which facilitate it would be expected to be adapted to higher oxygen levels. Hydrolysis requires hydrogen and oxygen for completion, so would be slowed or prevented by a lack of oxygen in the surrounding soil. Whether or not differences in activity were significant between oxygenation levels, all enzymes except N-acetylglucosaminidase and xylosidase had higher activity at 100% (atmospheric) oxygenation than at 0% oxygenation.

This suggests that soil nutrient cycling in constructed wetlands may proceed faster under aerobic conditions than under anaerobic (i.e. waterlogged) conditions, which agrees with evidence from the literature (e.g. Reddy & Patrick 1975, McLatchey & Reddy 1998). However as nitrogen cycling requires alternate aerobic and anaerobic processes for completion of mineralization, an infinite increase in oxygenation would not infinitely increase nitrogen cycling. N-acetylglucosaminidase and xylosidase are involved in the degradation of complex carbon-based substrates, and their rate of activity towards these substrates may be sufficiently slow as to be unaffected by the brief lack of oxygen encountered in this experiment. The results of kinetic analysis, described in Chapter II, showed that both N-acetylglucosaminidase and xylosidase had substrate affinities which were higher, and maximum velocities which were lower, than other carbon-cycling enzymes, lengthening the turnover times of their respective substrates.

The breakdown of humic compounds, as well as the formation of humus in soil, is controlled by enzymes which include phenol oxidase (Sarkar & Burns 1984). The absence of significant differences in phenol oxidase activity with oxygenation level in this experiment was surprising, as phenol oxidase requires oxygen in order to oxidize its substrates. If the (non-significant) trend of increasing activity at higher levels of available oxygen was confirmed it may produce a variety of effects in constructed wetlands. Firstly, higher phenol oxidase activity in aerated soil may degrade humic matter, resulting in fewer protective sites for extracellular enzymes; the enzymes thus released by breakdown of humic material around them may be subject to protease attack, and the overall nutrient cycling capacity of the soil would decline. Alternatively, if increased phenol oxidase activity meant increased humic matter formation (polymerisation of humic acids to form humus is oxidative, and catalysed by phenolase and peroxidase enzymes (Hartley & Whitehead 1985)), then more protective sites would potentially be available and greater nutrient cycling would be possible. The situation pertaining in any particular wetland will depend on whether phenol oxidase enzymes are primarily creating or destroying humic material, and this may be influenced by the

availability of other nutrients in the soil. For example Carreiro *et al* (in press) found that adding inorganic nitrogen could stimulate or reduce phenol oxidase activity depending on the lignin content of soil litter, while Nasyrov & Almamedova (in press) reported higher accumulation of humus with higher phenol oxidase activity.

The degree of oxygenation within the soil of a constructed wetland may be increased by using perforated down-tubes installed in the soil, to allow ingress of air to the deeper layers. This approach has been used experimentally at the Centre for Alternative Technology wetland. Similarly, denser planting of macrophytes with aerenchyma tissue may increase soil oxygenation in the rhizosphere. Thirdly, aerating the effluent (preferably by using a cascade rather than a motorized pump or stirrer) would allow the wastewater itself to carry oxygen to the deeper soil layers.

5.6 Phenolics

Phenolics are widely reported to influence enzyme activity (Ladd 1985, Wetzel 1992, Appel 1993, Wetzel 1993), and are of importance in soil because they form a substantial, and often largely recalcitrant, part of the soil organic matter (Killham 1996, Burns 1983). Most soil phenolics originate from the microbial degradation of aromatic compounds such as lignin, although a small amount is thought to be contributed by *de novo* microbial and chemical synthesis (Burns 1983); such processes result in the formation of soil humus, consisting of humic acids, fulvic acids and humins (Killham 1996). Humic materials in soils are only slowly degraded due to their large size and complex structure, and so turnover times are long. An equilibrium between humus mineralization and formation is established (Burns 1983), so that other soil components are constantly influenced by the presence of humic material.

Because of its predominantly anionic nature (Burns 1983), humic material is bound to the cationic clay particles within soil by covalent bonds; at low pH

values, fulvic acids may also be adsorbed within some clays (Burchill *et al* 1981). In addition, organic substrates are also likely to be associated with humus, which will reduce their accessibility (Burns 1983). Amongst these are enzymes, which form strong hydrogen bonds with phenolic soil constituents (Burns 1983), and are thought to be thus protected from protease attack. However, association with humic acids has also been proposed to partially or completely inactivate these extracellular enzymes (Wetzel 1993).

To investigate the possible role of natural phenolic compounds in the inhibition of wetland soil enzymes, soil was incubated with phenolic-rich effluent from a saw mill (British Soft Woods, Llandridnod Wells, Wales) and then assayed for enzyme activity. The effluent was known to contain a high concentration of wood-derived organic compounds. The influence of purified tannic acid on activity of the same enzymes was also investigated.

a. Methods

Slurries of soil from the Centre for Alternative Technology wetland were prepared using 1 cm³ soil and 4 ml of sawmill effluent. Before use the effluent was filtered to remove organic particulate material (which might interfere with measurements of phenolic content) but leave dissolved phenolics; this was done using Whatman number 1 filter paper twice, followed by Whatman GF/F filter paper, then a Millipore GS 0.2 µm filter membrane.

Five different dilutions of phenolic effluent were prepared by diluting with deionized water. For each dilution, a total of 17 slurries were prepared (two distilled water controls, two for each of six enzymes, two free acid controls and one pH control). Slurries were incubated for 90 mins prior to enzyme assays to allow interaction of phenolics with soil enzymes.

Enzyme assays for cellobiohydrolase, β -glucosidase, N-acetyl glucosaminidase, phosphatase, sulphatase and xylosidase were carried out using MUF substrates as described in Chapter III. Seven ml of substrate ($400 \mu\text{M l}^{-1}$, except MUF phosphate, $200 \mu\text{M l}^{-1}$) was added at the start of the assay; reaction termination and fluorescence measurement were carried out as before.

For investigation of the effect of phenolics on phenol oxidase assay, slurries were prepared using 6 cm^3 of wetland soil and 30 ml of diluted effluent. The assays were carried out as described in Chapter III, using L-dopa as a substrate. Experiments using tannic acid followed identical methods to the above experiments, using a range of concentrations of tannic acid. Tannic acid was prepared at 1000 mg l^{-1} carbon equivalent, using 1.865 g l^{-1} , and dilutions of 10, 20, 40, 80, 100, 120, 140, 160, 180 and 200 mg l^{-1} carbon equivalent were prepared from this stock solution. Slurries were prepared as described above, and assays for cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase, xylosidase and phenol oxidase were carried out.

Phenolic concentration in each dilution of the effluent was determined using Box assays as described in Chapter III. pH of control slurries was measured with an Orion glass pH electrode; pH ranged from 5.8 (deionized water) to 6.2 ($30\text{-}50 \text{ mg l}^{-1}$ phenolics) in saw-mill effluent slurries, and from 5.2 (30 mg l^{-1} phenolics) to 5.8 (190 mg l^{-1} phenolics).

Results were analysed using regression fitted line plots (Minitab v.12).

b. Results

Enzyme assays of soil mixed with saw-mill effluent gave mixed results; cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase and phenol oxidase (Figure 5.6 a) did not show significant changes in activity with increasing phenolic addition. However phosphatase, sulphatase and xylosidase showed

declines in activity with increasing phenolic additions which fitted linear regression models (Figures 5.6 b and c).

Incubation of soil with tannic acid solutions showed similar results; cellobiohydrolase, β -glucosidase and N-acetylglucosaminidase showed no differences in activity with increase in tannic acid concentration (Figure 5.5 d), but phosphatase, sulphatase, xylosidase and phenol oxidase did show declines in activity with increasing tannic acid addition (Figures 5.6 e and f). Table 5.5 lists the F and p values for phenolic and tannic acid addition regression results.

Table 5.5

Results of regression analysis for phenolic and tannic acid addition (F = regression coefficient, p = probability).

Enzyme	Phenolic addition		Tannic acid addition	
	F	p	F	p
Phosphatase	7.45	0.052	4.63	0.057
Sulphatase	6.03	0.070	7.27	0.022
Xylosidase	4.55	0.10	6.78	0.026
Phenol oxidase			14.91	0.012

Figure 5.6

Response of (a) cellobiohydrolase (\square), β -glucosidase (\blacksquare) and N-acetylglucosaminidase (\bullet), (b) phosphatase (\times $^{1/10}$) (\circ), sulphatase (\blacklozenge) and xylosidase (\blacklozenge), and (c) phenol oxidase to increasing phenolic concentration, in soil from Centre for Alternative Technology wetland. (Mean, $n = 5$, \pm s.e.).

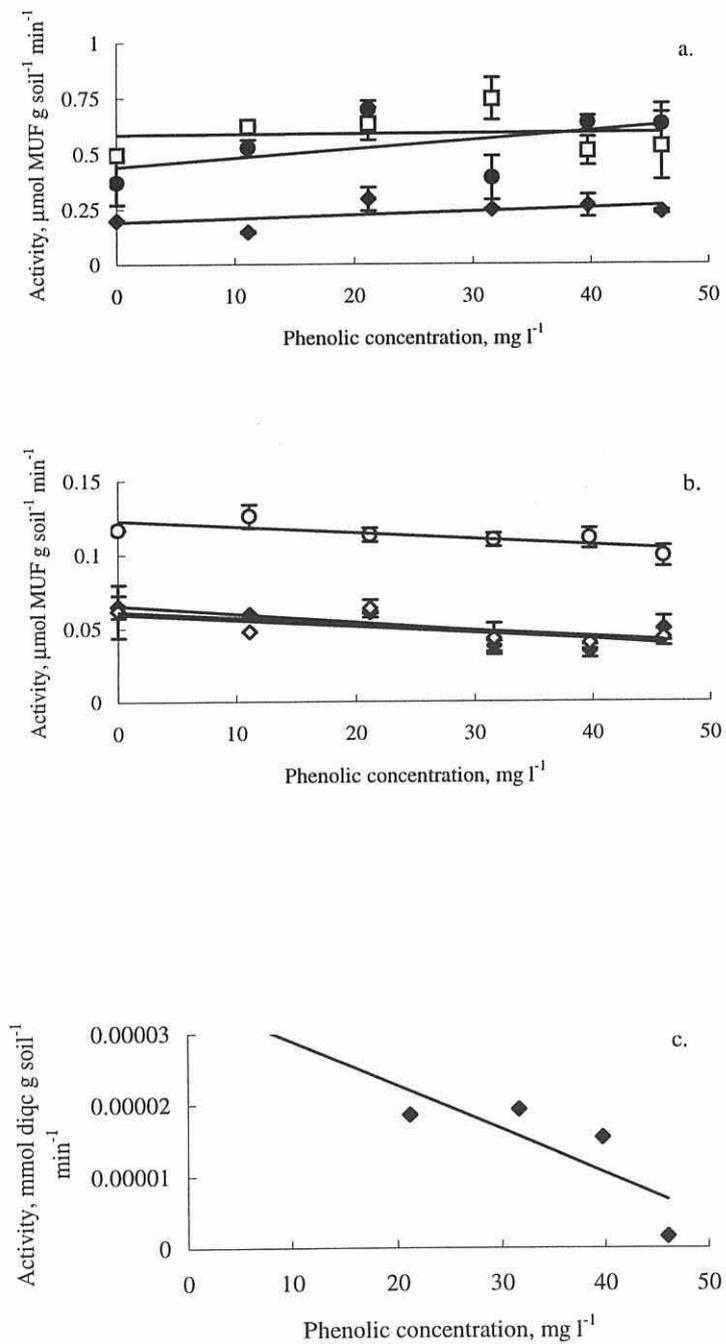
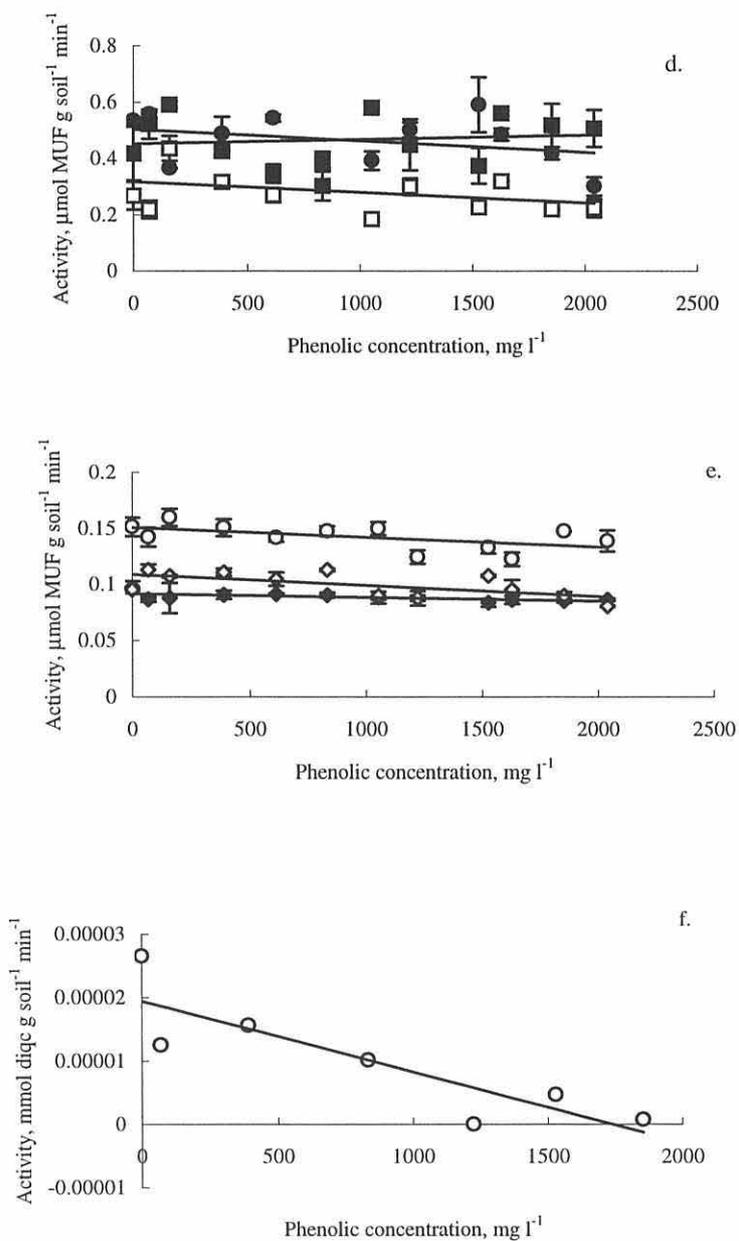


Figure 5.6 contd.

Response of (d) cellobiohydrolase (\square), β -glucosidase (\blacksquare) and N-acetylglucosaminidase (\bullet), (e) phosphatase ($\times 1/10$) (\circ), sulphatase (\blacklozenge) and xylosidase (\blacklozenge), and (f) phenol oxidase to increasing tannic acid concentration, in soil from Centre for Alternative Technology wetland. (Mean, $n = 5, \pm$ s.e.).



c. Discussion

Phenolic acids in soil include hydroxybenzoic, vanillic, coumaric, ferulic, caffeic and syringic acids (Hartley & Whitehead 1985), originating from the decomposition of plant residues, and from microbial synthesis. On average they constitute less than 0.1 % of soil total organic matter (Sparling *et al* 1981). Most are intermediates in humus formation, being polymerized to form relatively stable humic substances, or may be decomposed to yield carbon dioxide and water. In most soils the microbial decomposition of humic acids is primarily aerobic (Hartley & Whitehead 1985), involving the formation of a dihydroxyphenol before the benzene ring can be cleaved enzymatically. Under anaerobic conditions degradation is by photometabolism, nitrate respiration or methanogenic fermentation (Hartley & Whitehead 1985). Simple aromatic compounds can be used as the sole carbon source by many fungi and bacteria (Hartley & Whitehead 1985).

Slurry pH increased towards neutral at stronger concentrations of both phenolic effluent and tannic acid, whereas it might have been expected to become more acidic. The general lack of effect on enzyme activity of adding phenolic material to soil suggests that such enzymes as are already present in the wetland soil are well protected and therefore not susceptible to interaction with additional phenolics. The exception to this was phenol oxidase, and the decline in activity with increasing phenolic or tannic concentration of the slurry may be due to end-product inhibition.

The greater decline in phenol oxidase activity on addition of phenolic effluent, compared to addition of tannic acid, may be due to the phenolic effluent containing a mixture of phenolic acids, one of which is more inhibitory than tannic acid. It is also possible that the observed enzyme behaviour is due to end product inhibition.

The more significant relationships between phenolic or tannic acid concentration and decline in activity of phosphatase, sulphatase and xylosidase suggests that these hydrolases are less well protected by association with organic matter than are cellobiohydrolase, β -glucosidase and N-acetylglucosaminidase. Physical characteristics of these proteins, or weak initial binding to soil supports, may leave them vulnerable to complexation (and subsequent inactivation) by phenolic materials. It is possible that the reduced activity of xylosidase is a form of substrate inhibition, since xylosidase is involved in lignin degradation which may release aromatic compounds.

5.7 General discussion

These laboratory experiments have shown that the potential for manipulating enzyme activities in constructed wetlands, by altering physico-chemical factors, exists. Temperature, pH, redox potential and oxygenation had noticeable effects on the activity of some or all of the enzymes studied, while phenolic concentration appeared to have a lesser influence on enzyme activity.

The manipulation of enzyme activity for improving water quality amelioration must be considered in the context of the large-scale changes which would be required in a constructed wetland. Changing the wetland temperature would probably be costly and inappropriate for example, since the soil temperature is usually governed by prevailing climatic conditions unless the wastewater inflow contains heat from its source. Manipulation of pH may be much simpler, although the cost-benefit of different means of changing enzyme activity will depend upon the wastewater under consideration. Wetland treatment of some effluents may be so much more favourable than conventional treatment that relatively expensive manipulations of the nutrient cycling would be worthwhile.

The temperature manipulation experiments serve to demonstrate the dependence of enzyme activity and gas fluxes on seasonal variations in temperature. By using the calculated relationships it is possible to investigate the exact contribution of

temperature to enzyme activity and gas flux each month; this hypothesis is fully explored in Chapter IX.

Manipulating soil pH effectively altered the activity of all seven enzymes, although in slightly different ways, and for most revealed an optimum pH. Changing the pH of wetland soil would be relatively straightforward, but would alter the activity of different enzymes in different ways; from this point of view it would be important to consider the treatment system as a whole before altering its balance.

Redox potential and the level of soil oxygenation could potentially be adjusted by altering the wetland's hydrology, for example by intermittently dosing the beds with effluent instead of continuously flooding them. For processes such as denitrification an anaerobic/aerobic mosaic is essential, and intermittent wetting and drying cycles have been shown to be most effective in promoting denitrification (Groffman & Hanson 1997) although it is difficult to quantify the microscale changes in redox potential which contribute to this (Killham 1996). Organic matter decomposition also tends to proceed faster under aerobic conditions than anaerobic (McLatchey & Reddy 1998). However given the close association of redox potential with pH, a complex interaction of effects is likely to arise; redox potential may be either independent of or dependent on the pH value (Ingold 1980), so attempting to adjust redox potential may have wider effects than those anticipated. Changing the water table of a wetland, for example to alter the redox potential, increases the available phosphorous (Ponnamperuma 1955, cited in Patrick 1964) and could therefore alter the balance of microbial activity not only by favouring aerobes or anaerobes (depending on the direction of the change), but also by stimulating metabolism as a whole or result in greater releases of nutrients (e.g. phosphate). Addition of organic matter may lower the redox potential of soil in a constructed wetland, as oxygen is consumed during the decomposition.

Conclusions

- Temperature, pH, redox potential and oxygenation had noticeable effects on the activity of some or all of the enzymes studied.
- Concentration of phenolic materials had a less obvious influence on enzyme activity.
- Altering soil conditions such as pH and the degree of saturation would be simple methods by which the activity of extracellular enzymes in constructed wetlands could be manipulated to tailor enzyme activity to specific effluents.

Carbon supply and enzyme activity regulation in constructed wetlands

5.8 Introduction

During the treatment of wastewaters (such as sewage) by constructed wetlands, high molecular weight organic pollutants are degraded to low molecular weight nutrients, utilizable by microorganisms. Current knowledge of soil biochemistry suggests this mineralization is achieved via the metabolism of microorganisms (Brix & Schierup 1989) and the enzymic activity of the soil (Martens *et al* 1992, Kang *et al* 1998b, McLatchey & Reddy 1998). Soil enzyme activity includes enzymes excreted by microorganisms as part of extracellular metabolism, and enzymes immobilized on soil colloids and humic materials (Burns 1978), and is sensitive to changes in soil (micro-environmental) conditions such as temperature and pH (demonstrated in Chapter V a), and plant exudates and soil water chemistry.

Soil enzyme activity has been proposed to be an important determinant of water quality improvement in wetland systems (Kang *et al* 1998a, Freeman *et al* 1997). Therefore, manipulating enzyme activity may enable increases in wetland treatment efficiency. Enzyme activity in soil may increase either because suitable microbial substrates are at a premium (so micro-organisms produce enzymes to liberate more low molecular weight compounds), or because growth of the microbial population as a whole is stimulated, or because enzymes already present in the soil become activated. The presence of a specific substrate will prompt enzyme production in the case of inducible enzymes. In a constructed wetland, changed levels of nutrient input could be continuously available.

Methods on how soil enzyme manipulation could be achieved have been proposed in earlier studies of natural soil systems (Martens *et al*, 1992; Sparling *et al*, 1981), and investigated in Chapter V a. Soil amended with sources of organic

carbon such as poultry manure, sewage sludge, barley straw or fresh alfalfa showed doubled or quadrupled enzyme activity for a year after the addition (Martens *et al* 1992). Moreover, an increased enzyme activity in soil amended with glucose, in proportion to the amount of glucose applied, was also observed (Sparling *et al* 1981). According to the nature of the pollution under treatment (organic or inorganic), high or low enzyme activities may be desirable in constructed wetlands. Breakdown of organic components in wastewater, resulting in mineralization, is essential.

The aim of this study was to investigate the contribution of one possible regulator (carbon supply) to rates of activity of three enzymes involved in organic matter decomposition. The importance of organic carbon in relation to soil extracellular enzyme activities was highlighted by the field work described in Chapter III, where both dissolved organic carbon and phenolic materials correlated significantly with a variety of enzyme activities. The hypothesis investigated in this study is based on the essential role of carbon in microbial processes, namely (i) as substrates for microbial enzyme synthesis, (ii) as substrates for enzyme activity, and (iii) as products of enzyme activity. The composition and quantity of dissolved organic carbon entering a constructed wetland may significantly affect the level of enzyme activity in it. The objectives were to determine the effect on β -glucosidase, phosphatase and arylsulphatase activities of adding different amounts of high and low molecular weight carbon to wetland soil.

Soil samples from a constructed wetland were supplied with different forms of carbon at different concentrations, and with carbon free water or sewage effluent. The activities of β -D-glucosidase, phosphatase and arylsulphatase extracellular enzymes, as indicators of activity in the carbon, phosphorous and sulphur cycles respectively, were monitored over a 54-day period using a fluorescent substrate method.

5.9 Methods

Soil samples were taken from the constructed wetland at the Centre for Alternative Technology (CAT), mid-Wales (described in Chapter III). This reed bed receives on average 290,000 litres of domestic sewage per year.

a. Wetland soil core preparation and treatment

Thirty soil cores, 100 mm high x 110 mm diameter were extracted from one cell of the vertical flow reed bed and inserted into wide-mouth plastic tubs with drainage tubes at the base. The cores were maintained in a controlled temperature room at 12°C ($\pm 3^\circ\text{C}$) throughout the experiment. Soil cores were supplied with carbon of different molecular weight and in different concentrations, and the activities of β -glucosidase, phosphatase and sulphatase enzymes were monitored as described below over a 54-day period. The cores were divided into six groups of five replicates, and six different treatments were applied as detailed in Table 5.6.

Table 5.6

Treatments applied to wetland soil cores

	Treatment	Composition	Code
1	Control	Milli-Q ultrapure water	MQ
2	Low labile carbon	10 mg l ⁻¹ D-glucose	G 10
3	High labile carbon	100 mg l ⁻¹ D-glucose	G 100
4	Low complex carbon	10 mg l ⁻¹ carboxymethylcellulose	C 10
5	High complex carbon	100 mg l ⁻¹ carboxymethylcellulose	C 100
6	Sewage effluent	Inflow to system	E

b. Source of carbon

Carboxymethylcellulose was chosen as a soluble form of cellulose. Control and

organic carbon solutions were supplied from reservoirs via peristaltic pumps at a rate of 0.08 ml min^{-1} , on a "12 h on/12 h off" cycle. The supply rate was calculated using the average daily supply ($\text{litres min}^{-1} \text{ m}^{-2}$) to the CAT system.

The activities of β -glucosidase, phosphatase and arylsulphatase enzymes in the cores were measured on the day after collection (before any treatments were applied), and subsequently on days 5, 8, 12, 20, 22, 26, 33, 40, 47 and 54, using the fluorescent substrate method of Freeman *et al* (1995b), described in Chapter III. On each assay day, four 0.2 cm^3 samples of soil were taken from 3-5 cm depth in each core. These were used to assay β -glucosidase, phosphatase, arylsulphatase, and free acid quench in the soil of each core. One point four ml of enzyme substrate (MUF- β -D-glucoside $200 \mu\text{M l}^{-1}$, MUF-phosphate $200 \mu\text{M l}^{-1}$ or MUF-sulphate $400 \mu\text{M l}^{-1}$) or methylumbelliferone free acid ($50 \mu\text{M l}^{-1}$) was added to and thoroughly mixed with the soil. After incubation at 12°C for 60 min, the reaction was terminated by centrifuging at $10,000 \times g$ for 5 min, and 0.5 ml of supernatant was mixed with 2.5 ml of deionized water in a cuvette. The fluorescence of this was measured at 330 nm excitation, 450 nm absorbance (slit width 0.25 nm) using a Perkin Elmer LS50 fluorimeter. Enzyme activity was calculated as μmol methylumbelliferone released per gram of soil per minute using a standard curve and the free acid assay values to correct for soil quenching. Soil dry weight per cm^3 was calculated by drying at 105°C for 24 hours.

Enzyme activity was plotted cumulatively as μM MUF released per gram of soil per minute, against sampling day. Statistical analysis was carried out using Minitab version 12.1, using regression analysis to compare the line slopes after checking the residuals for normality (all conformed). Minitab 12.1 was also used to find the slope of each regression line.

5.10 Results

a. β -glucosidase

When plotted cumulatively, β -glucosidase activity in all cores (except G 100) proceeded linearly but at varying rates over the 54 day experimental period (Figure 4.7a). β -glucosidase activity increased over the course of the experiment by 127 - 161 % in response to cellulose addition, and decreased by 84 % after sewage effluent addition.

Adding 10 mg l⁻¹ or 100 mg l⁻¹ cellulose significantly ($p < 0.05$) increased β -glucosidase activity compared to control cores treated with Milli-Q water (slope (m) MQ = 0.526, mC10 = 0.841, mC100 = 0.645). Adding effluent significantly ($p < 0.05$) decreased β -glucosidase activity compared to control cores treated with Milli-Q water (mE = 0.433). Treatments of 10 mg l⁻¹ and 100 mg l⁻¹ glucose had no significant effect on β -glucosidase activity compared to control cores, although 100 mg l⁻¹ glucose cores showed a marked decline in β -glucosidase activity after day 26. The gradient of accumulation was 0.675 up to day 26 ($r^2 = 97.7\%$), dropping to 0.119 after day 33 ($r^2 = 99.2\%$). A Student's *t*-test comparing β -glucosidase activity data from before and after day 26 showed a significant % decrease in activity at $p < 0.05$.

In order of their relative effect on β -glucosidase activity, the treatments were ranked as follows (greatest significant effect first):

C 10 >> C 100 >> MQ >> E (where >> indicates a significant difference ($p < 0.05$) and > indicates no significant difference between treatments).

In addition to the significant differences between treatments and Milli-Q controls, there were also significant differences between G 10 cores and E, G 100, C 10 and C 100 cores, between C 100 cores and G 100, C 10 and E cores, and between C 10 cores and E and G 100 cores ($p < 0.05$). Table 5.7 summarises the effects of different treatments on β -glucosidase activity.

Table 5.7

Differences in β -D-glucosidase activity between treatments

	E	MQ	G 10	G 100	C 10	C 100
E		*	*		*	*
MQ					*	*
G 10				*	*	*
G 100					*	*
C 10						*
C 100						

- indicates a significant difference between treatments at $p < 0.05$

-

b. Phosphatase

Cumulative phosphatase activity showed linear accumulation (Figure 5.7 b), but there were no significant differences in phosphatase activity between control (Milli-Q) (mMQ = 0.533) and treatment cores (m of treatments ranged from 0.48 – 0.609). However, there were significant differences ($p < 0.05$) between C 10 and G 10, G 100 and C 100 cores. Table 5.8 summarises the effects of different treatments on phosphatase activity.

Figure 5.7

Cumulative β -glucosidase (a), phosphatase (b) and sulphatase (c) activity after addition of 10 mg l⁻¹ glucose (□), 100 mg l⁻¹ glucose (■), 10 mg l⁻¹ cellulose (○), 100 mg l⁻¹ cellulose (●), sewage effluent (△) or Milli-Q ultrapure water (control) (▲).

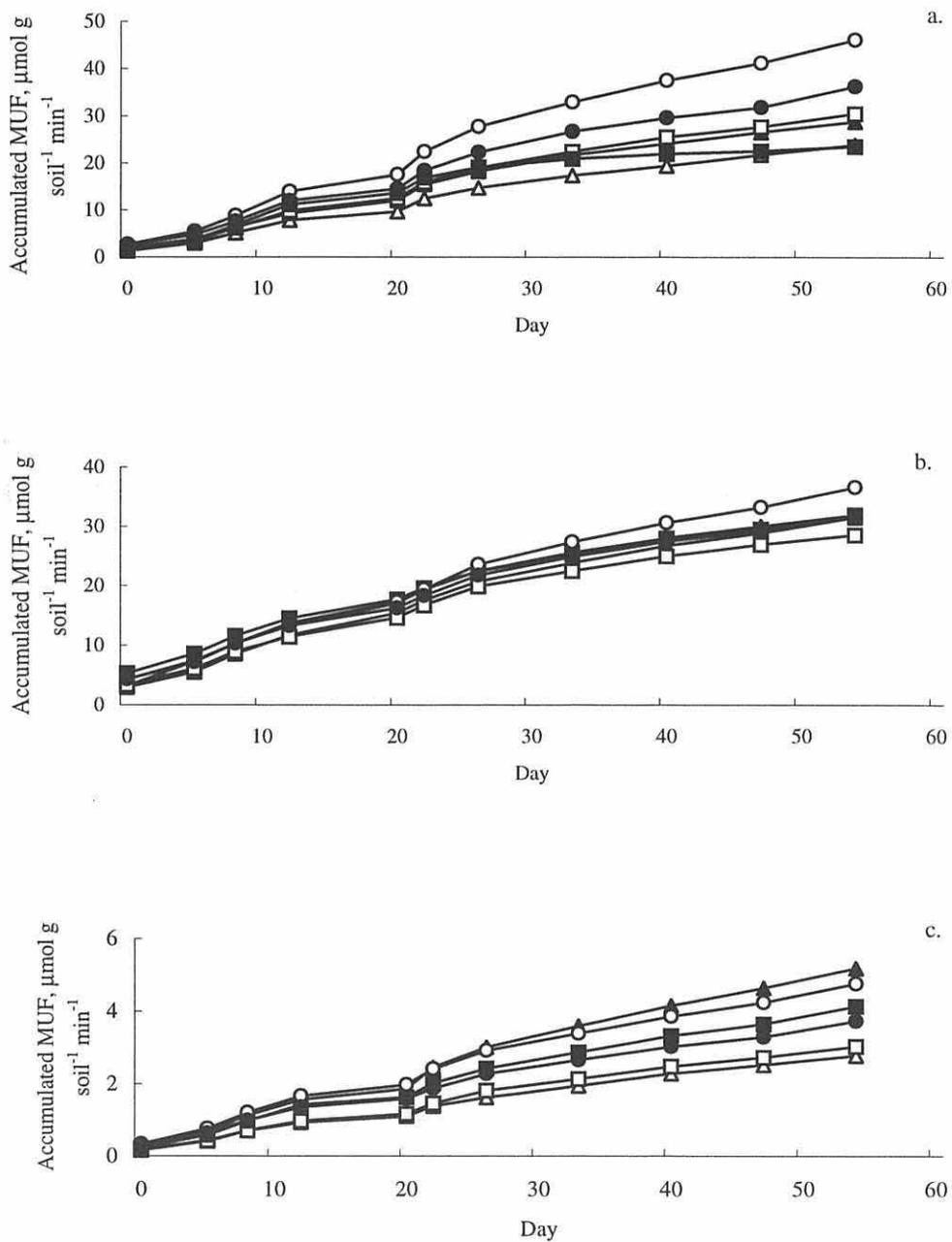


Table 5.8
Differences in phosphatase activity between treatments

	E	MQ	G 10	G 100	C 10	C 100
E						
MQ						
G 10					*	
G 100					*	
C 10						*
C 100						

* indicates a significant difference between treatments at $p < 0.05$

c. Sulphatase

Sulphatase activity in all cores was circa $1/10$ th of either β -glucosidase or phosphatase activity throughout the experimental period. In all cores, cumulative sulphatase activity was linear throughout the experiment (Figure 5.7 c). Sulphatase activities over the course of the experiment were significantly lowered by glucose, cellulose and sewage effluent addition, to between 53 % and 79 % of the activity in controls.

Compared to Milli-Q control cores (mMQ = 0.0929), 10 mg l⁻¹ glucose (mG10 = 0.0537), 100 mg l⁻¹ glucose (mG100 = 0.0719), 10 mg l⁻¹ cellulose (mC10 = 0.0817), 100 mg l⁻¹ cellulose (mC100 = 0.0625) and sewage effluent (mE = 0.0486) significantly ($p < 0.05$) decreased sulphatase activity.

In order of their relative effect on sulphatase activity, the treatments were ranked as follows (greatest significant effect first):

MQ >> C 10 > G 100 >> C 100 >> G 10 > E (where >> indicates a significant difference ($p < 0.05$) and > indicates no significant difference between treatments).

In addition to the significant differences between treatments and Milli-Q controls, there were also significant differences between E and G 100, C 10 and C 100 cores, between G 10 and G 100, C 10 and C 100 cores, and between C100 cores and G 100 and C 10 cores (all $p < 0.05$). Table 5.9 summarises the effects of different treatments on sulphatase activity. The results of the experiment are summarised in Table 5.10.

Table 5.9

Differences in sulphatase activity between treatments

	E	MQ	G 10	G 100	C 10	C 100
E		*		*	*	*
MQ			*	*	*	*
G 10				*	*	*
G 100						*
C 10						*
C 100						

* indicates a significant difference between treatments at $p < 0.05$

Table 5.10

Summary of results (\uparrow indicates an increase in activity with carbon addition, \downarrow indicates a decrease, and \circ no significant effect).

Treatment	β -glucosidase	Phosphatase	Sulphatase
G10	\circ	\circ	\downarrow
G100	\downarrow	\circ	\downarrow
C10	\uparrow	\circ	\downarrow
C100	\uparrow	\circ	\downarrow
E	\downarrow	\circ	\downarrow

5.11 Discussion

Adding carbon to soil (as cellulose or glucose in this experiment) changes the ratio of Carbon:Nitrogen:Phosphorous. Nitrogen and phosphorous may become more limiting factors to the growth of the microbial biomass when carbon is present in excess. However, if the excess carbon is of high molecular weight but not recalcitrant, enzyme activity will rapidly release labile carbon from it and so provide carbon to excess. Glucose, as a readily metabolized substrate, stimulates microbial metabolism if added to soil in sufficient quantity; the concentration needed to stimulate metabolism varies in different soils, and has been reported to range from 500 to 8000 mg l⁻¹ in mineral soils (Alef & Sparling 1995), while Anderson & Domsch (1975) used D-glucose at 800 mg l⁻¹ and higher to obtain maximum stimulation of respiration. In this experiment, carbon was added at two levels (10 mg l⁻¹ and 100 mg l⁻¹) which were considered to be low and high (respectively) compared to the inflow to the wetland (average circa 50 mg l⁻¹ DOC in 1998).

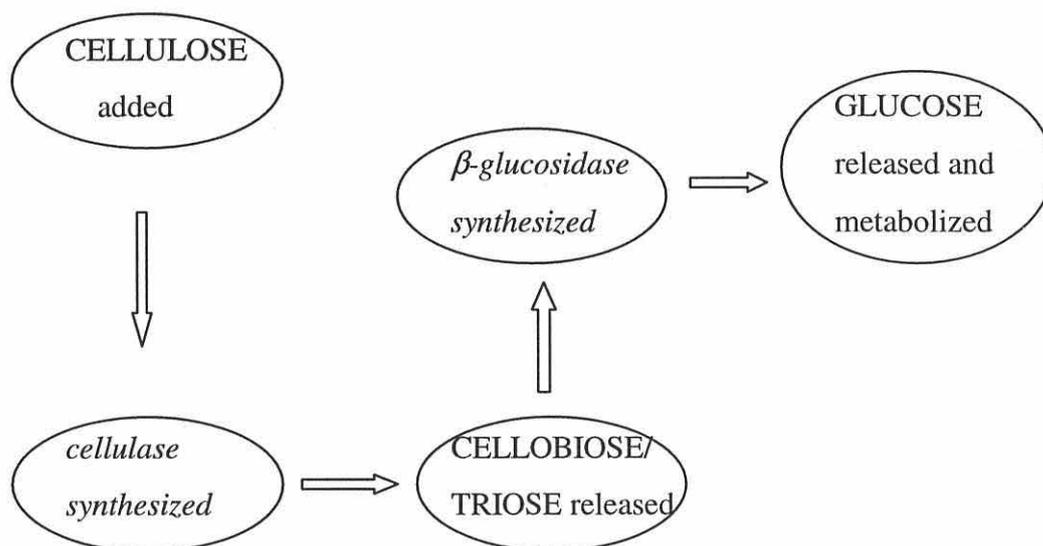
In water samples supplemented with D-glucose and amino acids, Chróst (1991) recorded drastically reduced β -glucosidase activity, which was presumed to be the result of enzyme synthesis repression by the added enzyme products. Chróst (1991) also reported greatly decreased affinity of β -glucosidase for d-glucose in the presence of excess D-glucose, by substrate inhibition. The results of the present experiment support Chróst's hypothesis that β -glucosidase activity is inhibited by excess substrate; cumulative activity in G 100 cores initially proceeded at a similar rate to β -glucosidase activity in G10, C100 and MQ cores, but after day 26 declined to less than 20% of the initial rate.

Recent studies have suggested that increased levels of β -glucosidase activity may be explained in terms of enzyme activation or induction (Killham 1996, Chróst 1991). The synthesis of cellulase enzymes is induced by the presence of cellulose

in soil, and the activity of these induced enzymes hydrolyses subunits such as cellobiose, cellotriose, cellotetraose and cellopentaose from large polymers after initial conditioning of the molecules (Killham 1996). Chróst (1991) reported that cellobiose in particular strongly induces the synthesis of β -glucosidase; which suggests an explanation for the increased activity of this enzyme in cores treated with 10 mg l^{-1} and 100 mg l^{-1} cellulose. The proposed sequence of events is summarized in Figure 5.8.

Figure 5.8

Effect on soil enzyme activity of adding cellulose to soil



Sewage effluent-treated cores showed significantly lower ($p < 0.05$) β -glucosidase activity than control (Milli-Q) cores, which may indicate de-activation due to an excess of low molecular weight carbon in the effluent. When such readily metabolized carbon is freely available, it has been suggested that there is no need for micro-organisms to acquire it enzymically (Chróst & Rai 1993).

Nannipieri *et al* (1979) reported an increase in phosphatase activity after a lag of two days, in soil amended with one initial treatment of glucose (3 mg g^{-1}), but after five days this decreased markedly. Increases in phosphatase activity were reported to coincide with an increase in bacterial biomass. Nannipieri *et al* (1979) and Ladd & Paul (1973) found that newly-synthesized phosphatase in glucose-amended soils was short-lived, decreasing after an initial increase. No evidence was found in this experiment for glucose-induced increases in phosphatase activity, perhaps because insufficient glucose was added. As 10 mg l^{-1} glucose did not produce any significant increase in phosphatase activity, cellulose hydrolysis to glucose could not be responsible for the increase seen in the C10 cores. There are many reports of an inverse relationship between phosphatase activity and inorganic phosphate availability (Siuda & Chróst 1987, Cotner & Wetzel 1991) and of a correlation between organic phosphorous mineralization and phosphatase activity. On this basis, Sinsabaugh & Moorhead (1994), suggest that increased activity is a response to low environmental availability of phosphorous, and Chróst (1991) suggests derepression of alkaline phosphatase when inorganic phosphate is limiting. As phosphatase activity did not change significantly in any of the treatments compared to the control, it may be concluded that either phosphorous was not limiting, or phosphatase activity is regulated by factors other than carbon availability.

Tabatabai & Bremner (1970) reported a significant positive correlation between arylsulphatase activity and the organic carbon content of soil, possibly due to covalent binding of enzyme to macromolecular organic soil components, and Falih & Wainwright (1996) found an increase in arylsulphatase activity after adding organic carbon to soil. These studies do not agree with the results of this experiment, in which carbon addition resulted in decreased arylsulphatase activity. Falih & Wainwright (1996) also reported an increase in phosphatase after organic carbon addition which is not supported by this experiment; however as the source

of organic carbon in Falih & Wainwright's experiment was sugarbeet, which is likely to have contained organic phosphorous compounds (e.g. ATP), the two experiments are not directly comparable. The responses observed in the present study for sulphatase and phosphatase are difficult to interpret in terms of the existing literature but an insight into the underlying mechanisms may be proposed with reference to recent studies by Sinsabaugh & Moorhead (1997).

The microbial biomass and enzyme activities of a soil reflect the capacity of that soil to maintain these parameters (Nannipieri *et al* 1983), and biomass and enzymes in excess of this capacity are destroyed. Soil has a carrying capacity like any other environment, in which microbial communities are able to maintain steady conditions. The increases in β -glucosidase activity in this experiment after cellulose addition (which could be thought of as increased capacity) support this idea. A nutrient-limited microbial population may expand to the carrying capacity of the soil micro-environment while the nutrient supplementation continues (Nannipieri *et al* 1983). However to understand the decrease in arylsulphatase activity which carbon supplementation brought about, the balance between availability of different nutrients must be considered.

Sinsabaugh and Moorhead (1997) proposed a model (MARCIE) of plant litter decomposition based on extracellular enzyme activity; in this, the extracellular enzymic degradation of large molecules was proposed to be a rate-limiting step in microbial production. In turn, the synthesis of extracellular enzymes was said to be regulated by induction and repression/derepression mechanisms, which were linked to nutrient availability in the surrounding environment. Thus, if carbon becomes unlimited not all microbial process will accelerate, but only those for which there are substrates available but for which energy (derived from carbon) was lacking.

The MARCIE model groups soil enzymes into three categories of carbon acquisition, nitrogen acquisition and phosphorous acquisition, in each of which any single enzyme can be assumed to be an indicator of the others. In this experiment the three enzymes monitored (β -glucosidase, phosphatase, sulphatase) are taken as indicators of carbon, phosphorous and sulphur cycling (respectively) in the wetland soil; Sinsabaugh & Linkins (1988) described β -glucosidase as a mediator of cellulose degradation, phosphatase as involved in the acquisition of phosphorous from organic sources (Sinsabaugh *et al* (1993), and sulphatase as releasing sulphate from organic ester sulphates (Sinsabaugh *et al* 1993).

The significantly lower levels of sulphatase activity in all treatment cores compared to Milli-Q (control) cores point towards a reallocation of resources after carbon additions. Increasing the supply of organic carbon (as either glucose or cellulose) appeared to suppress sulphatase activity compared to cores receiving no carbon (MQ).

When more microbial metabolic effort is directed to nitrogen acquisition, less is available for carbon acquisition (Sinsabaugh *et al* 1993); by the same reasoning we propose that when wetland soil is supplemented with complex carbon, microbial metabolic resources are directed away from sulphur acquisition, to carbon acquisition. The lack of a similar suppression of phosphorous acquisition enzymes is puzzling, especially given the essential role phosphorous plays in cell growth and maintenance. It may be attributable to a different regulatory system for phosphatase; phosphate is widely reported to be present in most soils as a large immobilized pool, with phosphatase activities regulated by edaphic factors (Spier & Ross 1978). Sulphatase on the other hand may be under a much greater degree of microbial regulation, including through *de novo* synthesis.

Of the twelve significant relationships between enzyme activity and dissolved

organic carbon or phenolic materials, which were found in data from the field study of constructed wetlands (Chapter III), half referred to enzymes investigated in this study. The directions (direct (+) or inverse (-)) of these correlations are summarized in Table 5.11.

Table 5.11

Significant relationships between enzyme activity and dissolved organic carbon or phenolic materials (from Chapter III).

	β-glucosidase	Phosphatase	Sulphatase
DOC	+ (Pelenna, Tollesbury)	- (CAT)	+ (Pelenna)
Phenolics	+ (CAT)	+ (Waun-y-Cwrt)	

These relationships partly support those revealed in this study. The positive relationship between carbon substrates and β -glucosidase (from fieldwork) is the same as that found in this study between carboxymethylcellulose and β -glucosidase. In this study phosphatase did not show any significant changes with changes in carbon supply, which reflects the mixed results of the fieldwork. However sulphatase showed a positive correlation with dissolved organic carbon in the fieldwork, but in this study sulphatase activity was consistently decreased by carbon addition.

In this experiment, different enzymes have been shown to be differently affected by the addition of labile and complex carbon sources; whilst phosphatase activity was unaffected, β -glucosidase increased in some cases and sulphatase activity decreased in all cases. This has implications for the optimization of wetland treatment efficiency, because different nutrient cycles may be affected in differing ways. Organic carbon is sometimes added to wetland treatment systems to provide a source of electrons for decomposition processes, but it also increases the surface area available for microbial colonization and enzyme immobilization. The

desirability of this practice will depend on the contaminants in the waste water. In some wetlands, e.g. sewage treatment, it would be beneficial to increase complex carbon availability as the results of this study indicate that this may increase organic pollutant decomposition. In contrast, the decline in sulphatase activity after carbon addition may be beneficial in acid mine drainage treatment wetlands as it would prevent re-release of organically bound sulphate. However, stimulating carbon-cycling could compromise metal removal, as organic matter degradation would potentially release heavy metals associated with it. Careful consideration should be given to the required outcome of wetland treatment before adding organic matter, but clearly the addition of exogenous organic substrates may be beneficial in highly contrasting systems.

Conclusions

- After carbon addition, changes in β -glucosidase activity were similar to activity changes reported in other, non-saturated, soil after carbon addition; however phosphatase and sulphatase activities responded differently after carbon addition than has previously been reported.
- Phosphatase activity was not significantly altered by the addition of labile and complex carbon sources, whilst β -glucosidase was increased by some forms and concentrations of carbon, and sulphatase activity was decreased by all forms and concentrations.
- Decreased sulphatase activity after carbon addition may prevent re-release of organically bound sulphate during acid mine drainage treatment. However, stimulating organic matter decomposition by the same addition could compromise metal removal.

Chapter VI

Investigation of the springtime decline
in enzyme activity

Possible causes of the springtime decline in enzyme activity

6.1 Introduction

Enzyme activities measured in the field (Chapter III) showed some significant correlations with the annual temperature trend. However a noticeable decline in the activity of most enzymes in late spring did not correlate with temperature or with any other measured variable. Distinct reductions in the activity of cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase and xylosidase were recorded in May 1997 at the CAT wetland, and for all hydrolases except sulphatase in June 1998 at the CAT wetland. At the Waun-y-Cwrt and Pelenna wetlands all six hydrolase activities showed marked declines in activity in May 1997. Phenol oxidase activity declined in April 1997 at the CAT and Tollesbury wetlands, in May 1997 at the Waun-y-Cwrt and Pelenna sites, and in April 1998 at the CAT wetland. These declines did not appear in the autumn as temperature fell again through the same range. Temperature is an important factor in the control of many decomposition processes (Linkins *et al* 1984), but the absence of temperature correlations in this case suggests a different cause for the declining activity.

A similar phenomenon was recorded by Kang (1999), working on a variety of natural vegetated wetlands. In these (a bog, a fen and a swamp), some marked declines in the activity of cellobiohydrolase, β -glucosidase, N-acetylglucosaminidase, phosphatase, sulphatase and xylosidase were seen in May of the field sampling year (1996), and occasionally in April or June. Kang postulated that the dip may be caused by competition between plants and microbes for soil nutrients during rapid growth in the spring; microorganisms are able to respond quickly to increasing soil temperature due to their smaller size and higher turnover, while plants take longer to respond. Thus warming of the soil in spring at first causes an increase in enzyme activity through increased synthesis from the greater microbial population, and later, as plant growth begins, a decline

in enzyme activity because plant uptake of nutrients outcompetes microorganisms. Declines in the activity of freshwater epilithic and epiphytic β -glucosidase, and epiphytic xylosidase in May of their sampling year were also recorded (but not discussed) by Chappell & Goulder (1994a, 1994b).

Possible causes of the springtime decline in enzyme activity include:

- 1 temperature
- 2 rapid plant growth in spring
- 3 a reduction in microbial activity

Of these, temperature may be eliminated, as the trends in soil temperature during the spring period at the wetlands studied in Chapter III do not correspond to the sudden dips in extracellular enzyme activity which were recorded. Freeman *et al* (1998) showed that plants may outcompete microbes for inorganic nutrients during active growth. Rapid changes in microscale soil conditions may occur at the onset of plant growth in spring, as root exudation of carbon substrates changes during growth (Grayston *et al* 1996). Rhizodeposition is an important source of carbon for bacteria in the soil (Whipps & Lynch 1983), because soil microbial growth tends to be carbon limited (Grayston *et al* 1996). Therefore rapid spring plant growth may result in less extracellular enzyme activity due to competition between plants and micro-organisms for nutrients.

To clarify whether plant growth, or microbial activity, or an interaction of both factors, were responsible for the spring decline in enzyme activities, a series of laboratory experiments using wetland cores and soil samples were carried out. The aim of the experiments was to investigate the hypothesis that microbial activity had a greater influence on extracellular enzyme activity than did plant growth.

To fulfill this aim the following experiments were carried out:

- (1) changes in soil enzyme activity were monitored during stimulated plant growth

- (2) soil enzyme activity was monitored during a period of reduced microbial activity.

6.2 Simulation of spring

To investigate changes in soil enzyme activity during spring plant growth, cores from a constructed wetland, including early reed shoots, were collected before spring. The plants were stimulated to grow in half the cores by keeping them warmer than the field temperature, while a parallel group of cores were kept as dormant as possible. The temperature difference between the two groups of cores was more extreme than that encountered during the change from winter to spring, to induce strong growth.

a. Methods

The experiment was carried out using cores collected from the CAT wetland at the beginning of March 1999, before spring growth had begun. Ten cores (110 mm high x 100 mm diameter) which included very young reed shoots were collected and randomly allocated to two groups. The first group were wrapped completely in foil and maintained at 12 °C (close to field temperature) to simulate continued winter conditions. The second group were maintained in continuous artificial light at 24 °C to simulate spring warming and stimulate growth.

Soil from the top 5 cm of each core was sampled at intervals over a 23 day period and assayed for β -glucosidase, phosphatase and sulphatase activity. Enzyme assays were carried out using 1 cm³ of soil and 7 ml of MUF substrate, and the method described in Chapter III.

b. Results and discussion

Of the three enzymes studied, only β -glucosidase showed a different response in warm conditions compared to cold conditions; activity of β -glucosidase dropped significantly on day 11 ($T = -2.81$, $p = 0.023$), and rose again to the previous level

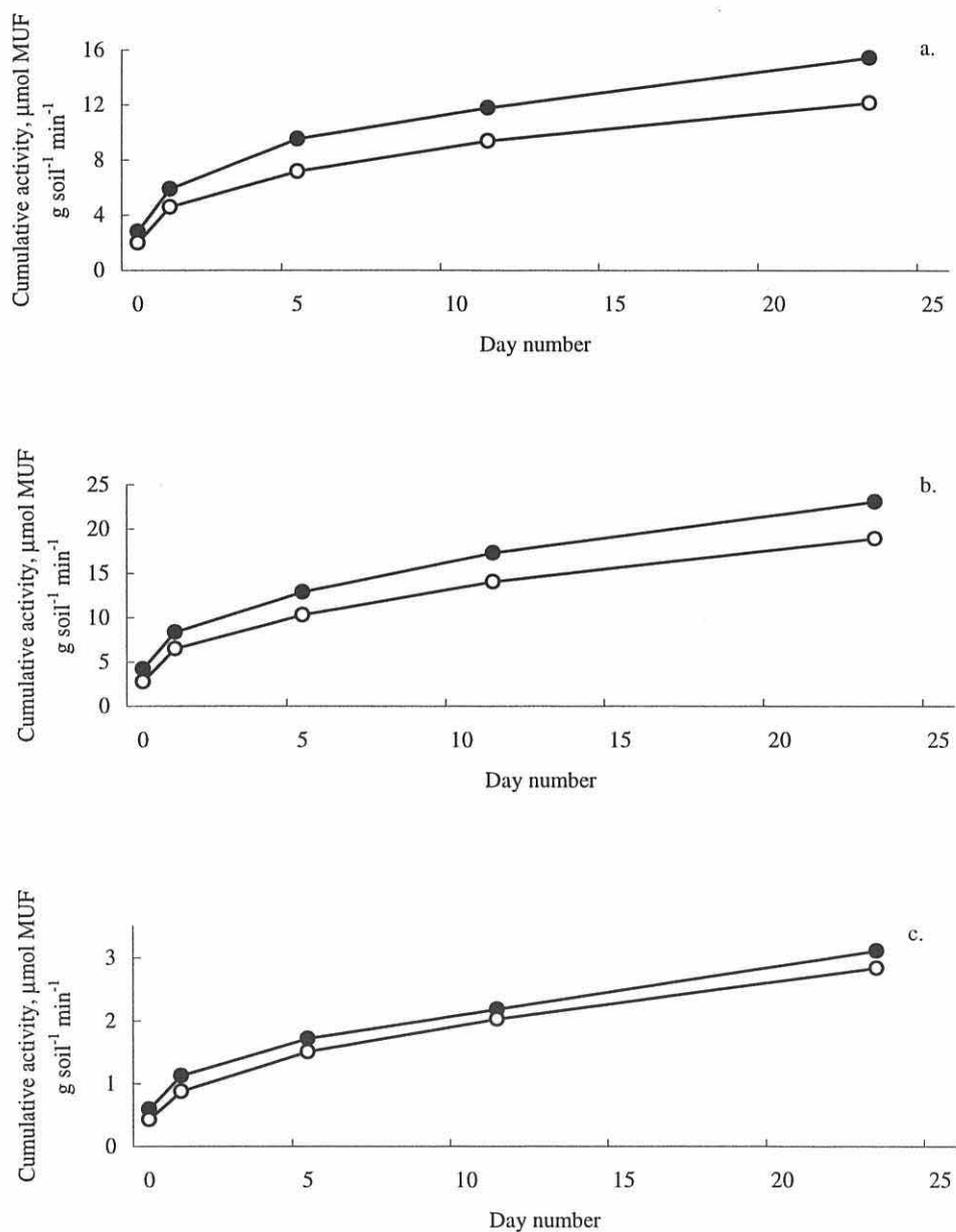
by day 23 (Figure 6.1 a). Phosphatase and sulphatase activities were consistently higher in warm conditions than in cold, but were not significantly different between conditions (Figure 6.1 b and c). All three enzymes showed significant increases in activity during the experiment, in both warm and cold conditions ($F_{4,40} = 3.9$, $p = 0.009$ for β -glucosidase, $F_{4,40} = 6.14$, $p = 0.001$ for phosphatase and $F_{4,40} = 4.53$, $p = 0.004$ for sulphatase).

Rather than looking at the differences in enzyme activity between the cold and warm conditions, it may be more instructive to consider the potential enzyme activity which might have been expected when transferring the wetland soil from an early spring field situation to a warm laboratory. According to the Q_{10} values calculated from temperature experiments in Chapter V a, a rise in temperature of 10 °C should produce at least a three-fold increase in β -glucosidase and phosphatase activity ($Q_{10} = 3.03$ for β -glucosidase, 3.79 for phosphatase; sulphatase activity was not included in the experiment). The actual increase in activity during plant growth in the spring simulation experiment was very much lower than this, suggesting that the plant growth did inhibit enzyme activity.

Evidence for a change in microbial activity was sought from the field data collected in Chapter III. Springtime declines in carbon dioxide flux, without corresponding declines in soil temperature, were found at all four wetland sites; at CAT in April 1997, at Waun-y-Cwrt in June 1997, at Pelenna in May 1997 and at Tollesbury in April and May 1997. At three wetlands there were also significant correlations between annual carbon dioxide flux and enzyme activity (β -glucosidase at CAT, β -glucosidase and N-acetylglucosaminidase at Pelenna, and phenol oxidase at Tollesbury). However data describing enzyme activity and carbon dioxide emissions from February – August (a six month interval straddling the springtime decline and rise of enzyme activity) did not correlate significantly, partly because the carbon dioxide declines tended to occur in the month before the enzyme activity declines.

Figure 6.1

Cumulative β -glucosidase (a), phosphatase (b) and sulphatase (c) activity in soil cores kept dormant (○) or stimulated to grow (●) from Centre for Alternative Technology wetland (means, $n = 5$).



In spring, rapid plant growth may lead to increased root exudation; growing roots are a significant source of carbon for the microbial biomass (Whipps & Lynch 1983, Wilson *et al* 1989), and in the rhizosphere root exudates have the greatest stimulatory effect on microbial growth and activity because are readily assimilated (Krafczyk *et al* 1984, Lynch & Whipps 1990). Hexose sugars, aliphatic acids and amino acids are frequently released as root exudates (Grayston *et al* 1996), and these labile, low molecular weight substrates may reduce extracellular enzyme activity by end product inhibition. Root exudates enhance nutrient availability because they represent carbon substrates which can be metabolized and so increase microbial growth and activity in the rhizosphere (Grayston *et al* 1996). During periods of increased root exudation, there may therefore be less requirement for extracellular enzyme activity to make microbial substrates such as glucose available.

Lack of oxygen has been reported to enhance root exudation (Whipps & Lynch 1986), due to increased cell membrane permeability (Grayston *et al* 1996). An increase in soil dissolved organic carbon in the spring may lead to increased levels of microbial activity (stimulated by the increased carbon supply), and so cause a lower soil oxygen tension. At the Centre for Alternative Technology wetland a springtime increase in DOC was recorded (Chapter III) in May 1997 and May 1998, but the same pattern was not seen at the Waun-y-Cwrt or Pelenna wetlands.

6.3 Microbial inhibition

The contribution of micro-organisms to the springtime declines in enzyme activity was suspected because carbon dioxide emissions also declined in the month prior to enzyme activity decline at the CAT, Waun-y-Cwrt and Pelenna wetlands. To investigate the possibility that reduced microbial activity in spring led to the declines in enzyme activity, a laboratory experiment with microbially inhibited soil was used.

Bacterial and fungal metabolic inhibitors have largely been used for differentiation by selective inhibition in the study of microbial community structure, and to estimate the relative contributions of bacterial and fungal populations to soil respiration (e.g. Anderson & Domsch 1975). This technique was extended to achieve total inhibition of the soil microbial population. Anderson & Domsch (1975) used streptomycin sulphate (bactericide) and cycloheximide (fungicide) at concentrations of 500-3000 mg l⁻¹ (streptomycin sulphate) and 500-4000 mg l⁻¹ (cycloheximide). Both of these antibiotics work by blocking protein synthesis, and are therefore most active against metabolically active micro-organisms.

By inhibiting microbial activity it was possible to observe changes in enzyme activity which were dependent on it. Gas flux measurements were used to show how effective the inhibition was.

a. Methods

A 120 g sample of soil from the Centre for Alternative Technology sewage treatment wetland was sieved (1.18 mm mesh), divided into six 20 g sub-samples and placed in sterile jars with screw top lids with self-resealing silicon septa. These were equilibrated to 12 °C by incubating (uncapped) for 60 minutes. After equilibration the jars were capped and incubation was continued for a further 60 minutes; after this time a 5 ml sample of headspace gas was collected from each jar, using a gas-tight syringe and sampling needle inserted through the silicon septum.

Five ml of a solution containing both bacterial and fungal metabolic inhibitors (3000 mg l⁻¹ streptomycin sulphate, 4000 mg l⁻¹ cycloheximide) was added to three of the jars, and 5 ml deionized water to the remaining three jars. All six jars were left for three hours before being capped again. Two samples of background gas were collected at the time of capping to monitor microbial activity, and 60

minutes after capping the headspace gas was re-sampled using the same method as before.

The activities of cellobiohydrolase, β -glucosidase and xylosidase enzymes were measured before the first and second cappings by taking 0.5 ml of soil from each jar and mixing with 3.5 ml of the relevant substrate (MUF β -D-cellobiopyranoside, MUF- β -D-glucoside and MUF-xyloside respectively) according to the method described in Chapter III.

b. Results

Gases

There were no significant differences in nitrous oxide, methane or carbon dioxide emission between control and experimental soils before the addition of inhibitors.

Nitrous oxide (Figure 5.3 a) One hour after the addition of deionized water/inhibitor solution, nitrous oxide emission from control soil had increased, and that from inhibited soil decreased, but not significantly compared to emissions before addition. However nitrous oxide emission from the control soil was significantly greater than from inhibited soil ($T = -6.07$, $p = 0.009$) after the additions. After 15 hrs nitrous oxide emissions from the inhibited soil were significantly higher than before or immediately after the water addition ($F_{2,12} = 13.38$, $p = 0.006$), but were not significantly different to those from the control soil.

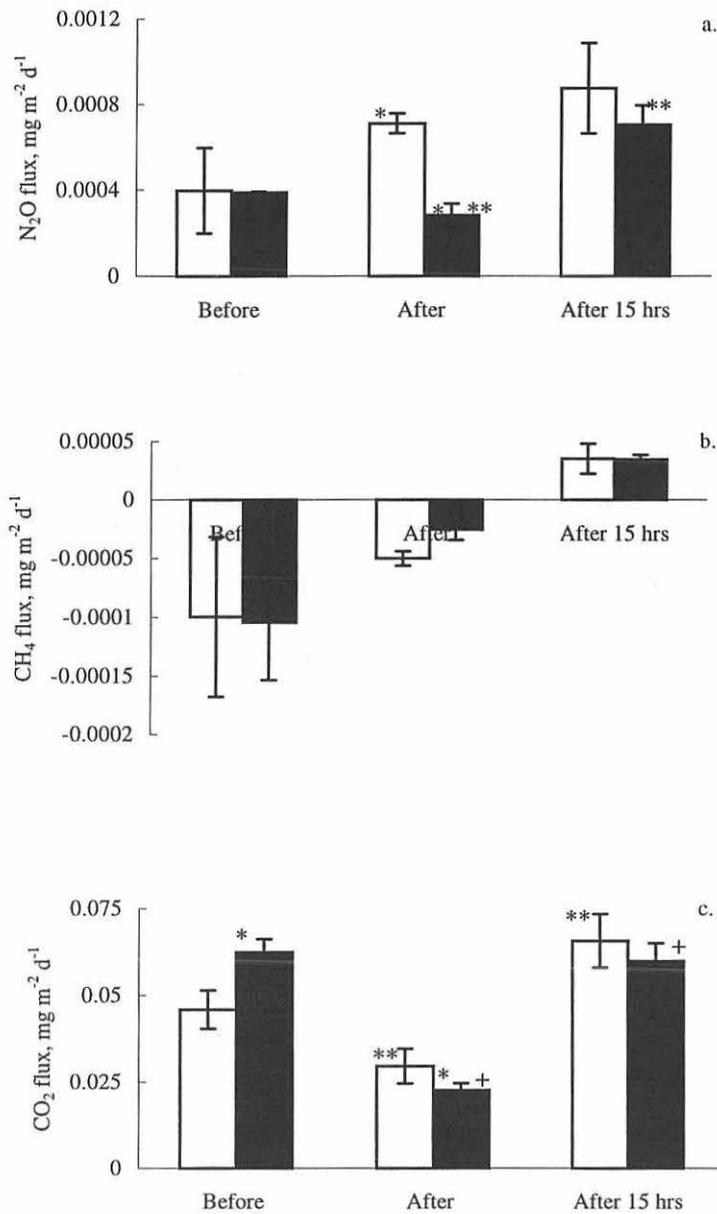
Methane (Figure 5.3 b) One hour after addition of deionized water or inhibitor solution, there were no significant differences in emission between control and inhibited soils; neither were there 15 hrs after addition.

Carbon dioxide (Figure 5.3 c) One hour after the deionized water addition there was a significant reduction in carbon dioxide emission from the inhibited soil ($T = 9.47$, $p = 0.0025$), but no significant change in the control soil. After 15 hrs carbon

dioxide emissions from both control and experimental soils had increased significantly compared to immediately after water/inhibitor addition ($T = -6.74$, $p = 0.021$ for control soil, $T = -3.92$, $p = 0.03$ for experimental soil).

Figure 6.2

Nitrous oxide (a), methane (b) and carbon dioxide (c) emission from control (□) and experimental (■) soil before, immediately after and 15 hours after inhibition by antibiotics. Symbols (*, ** and +) indicate significant differences as described in the text. (Mean, $n = 5$, \pm s.e.).



Enzymes

(Figure 6.3) There was no significant difference in the activity of cellobiohydrolase, β -glucosidase or xylosidase in control and experimental soils before the addition of water/inhibitors. After the additions, cellobiohydrolase activity decreased significantly in inhibited soil ($T = 5.24$, $p = 0.014$), and β -glucosidase activity decreased significantly in control soil ($T = 7.45$, $p = 0.018$).

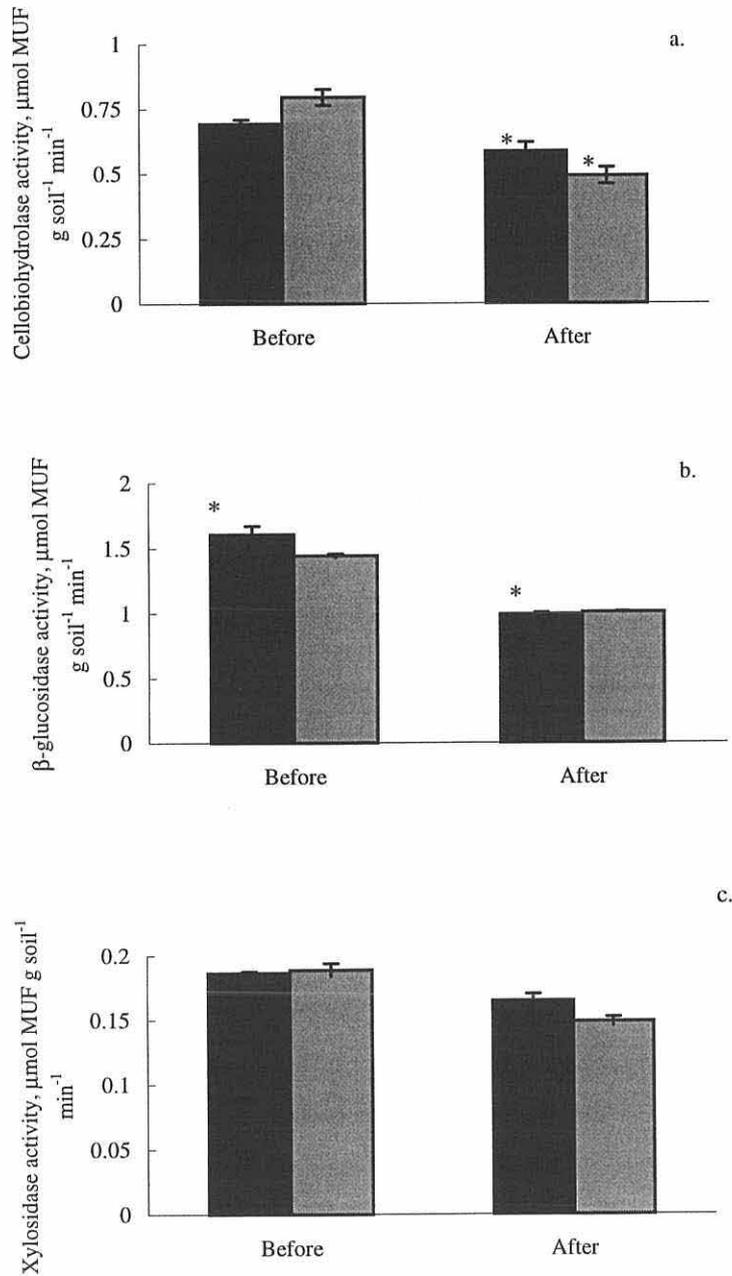
c. Discussion

Of the three gas fluxes investigated, only nitrous oxide and carbon dioxide showed significant decreases after the addition of antibiotic and antifungal inhibitors to the soil. Nitrous oxide emissions were significantly decreased (compared to controls) after addition of the inhibitors, suggesting that nitrifying or denitrifying metabolism was partly prevented by one or both inhibitors. Without labelling experiments, it is impossible to know whether the nitrous oxide was evolved from nitrification or denitrification, or both.

Methane fluxes showed no significant changes after addition of the inhibitors; in both control and inhibited soil methane oxidation was occurring at the start of the experiment, and this gave way to methane emission at 1 and 15 hours (respectively) after the inhibitor addition. This suggests that the small quantities of soil used became more anaerobic after the water/inhibitor solution additions, which is not unexpected due to the lower solubility of oxygen in water than in air. As a result, methane emission increased after the methanotrophic population (and therefore consumption of methane) had declined. The inhibitors did not appear to affect the methanotrophic or methanogenic microbial populations as there were no significant differences between control and inhibited soil samples.

Figure 6.3

Cellobiohydrolase (a), β -glucosidase (b) and xylosidase (c) activity before (■) and after (▨) inhibition by antibiotics. Symbols (*) indicate significant differences as described in the text. (Mean, $n = 5$, \pm s.e.).



Carbon dioxide emissions were initially reduced after the inhibitor addition, but after 15 hours had rebounded to the levels recorded before inhibition. This suggests that the aerobic microbial population was initially inhibited, but not irreversibly. To obtain longer term inhibition in this soil it may be necessary to use higher concentrations of inhibitors. The only difference in enzyme activities between controls and treatments was in cellobiohydrolase activity, suggesting that different carbon enzymes may be controlled by different factors. The decrease in cellobiohydrolase activity after microbial inhibition may indicate the importance of continuous microbial synthesis for maintenance of cellobiohydrolase activity; contrastingly, β -glucosidase and xylosidase activities may be independent of microbial enzyme synthesis, and be controlled more by end product inhibition.

6.4 General discussion

Given the absence of correlations from the field data (Chapter III) between carbon dioxide emissions (used as a measure of microbial activity) and extracellular enzyme activity, and the results of this experiment, which indicate that inhibiting microbial activity only affects the activity of some enzymes, it seems most likely that the springtime decline in enzyme activity is attributable to a combination of plant growth and microbial activity changes. However the frequency of field sampling did not allow detailed analysis of changes in microbial respiration; it is possible that declining microbial activity causes a delayed response in enzyme activity which would not have been detected in the inhibition experiment described here.

Enhanced plant growth may potentially have a greater influence than decreased microbial activity on enzyme activity, perhaps via root exudation. When the microbial population was inhibited there was a small effect on extracellular enzyme activity, but when plant growth was stimulated the actual temperature-related increase in activity fell very short of the potential increase.

Conclusions

- Enzyme activity appeared to be suppressed in soil in which plants were subjected to forced growth.
- Soil in which the microbial population was inhibited showed little change in extracellular enzyme activity.
- The springtime decline in enzyme activity may be attributable to a combination of plant growth and microbial activity changes.

Chapter VII

Microbial contribution to wetland gas emissions

The contribution of soil enzymes to wetland gas emissions

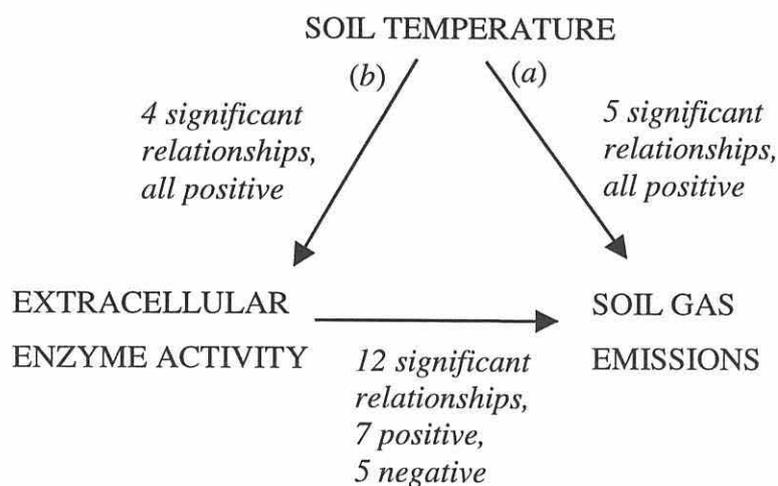
7.1 Introduction

Emissions of nitrous oxide, methane and carbon dioxide from soil are the metabolic products of fungi, bacteria, animals and plants living in the soil, and some of the ultimate end-products of organic matter degradation and transformation begun by extracellular enzymes (Bergstrom *et al* 1994, Williams & Crawford 1984, Bridgham & Richardson 1992).

Data from the field study of constructed wetlands showed that emissions of nitrous oxide, methane and carbon dioxide were all strongly correlated with both enzyme activity and soil temperature (Chapter III). These gases are radiatively active, and ideally their release from constructed wetlands would be minimal to avoid simply transferring a pollution problem from water to atmosphere (Freeman *et al* 1997). However it was not clear whether temperature influenced gas emissions directly (*a* in Figure 7.1), or via enzyme activity (*b* in Figure 7.1).

Figure 7.1

Inter-relationships between soil temperature, extracellular enzyme activity and soil gas emissions, from fieldwork (Chapter III, Tables 3.4, 3.8, 3.13 and 3.16).



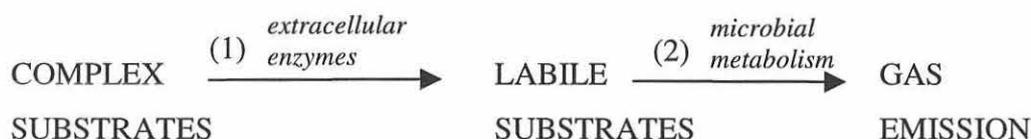
Non-biological production of nitrous oxide has also been reported from some soils (Nelson & Bremner 1970, Parkin & Codling 1988) via chemodenitrification catalysed by metallic cations.

To investigate whether temperature has a direct or indirect effect on wetland gas emissions, it would be necessary to investigate gas emissions in the absence of microorganisms. The response of the wetland soil would also indicate what might happen to gas fluxes if the microbial population of a constructed wetland was disrupted. Such disruption may occur if, for instance, wastewater containing toxic compounds was released to a wetland not designed or adapted to treat them.

Xenobiotics with this potential include some fertilizers and agrochemicals, including plant growth regulators, environmental contaminants such as industrial and domestic wastes and effluents, and by-products of biological processes such as fermentation, distillation and animal production (Goodman *et al* 1992).

Damage to microbial biofilms used in conventional sewage treatment, for example by cyanide, can result in the complete failure of treatment (J. Sutton, Environment Agency pers. comm.).

There are two possible ways in which temperature could increase gaseous outputs from soil, by changing the rate of (1) or (2) in the following model:



(1) is the rate at which extracellular enzymes transform organic matter into substrates utilizable by microorganisms.

(2) is the rate at which labile substrates are utilized by microorganisms and converted to gaseous products.

In this simplified model of the soil ecosystem, organic matter mineralization is a chain of events. A certain sized pool of labile (microbially available) substrates can sustain a certain level of microbial respiration and activity; that level of respiration and activity can produce a given level of gas emissions (nitrous oxide, methane and carbon dioxide). When temperature is increased, either (1) higher extracellular enzyme activity increases the supply of labile substrates (possibly leading to more microbial respiration and activity, and thus greater gas emissions), and/or (2) increased microbial respiration and activity result in higher gas emissions.

However if the soil microbial population is inhibited, it cannot increase when temperature rises, although the rate of extracellular enzyme reactions *can* still increase. Suppose that the microbial population was not substrate limited before inhibition, and was producing gases at as fast a rate as possible; a rise in temperature after inhibition will not then increase gas production, because the microbial population cannot grow to take advantage of the freely available substrates. Contrastingly, suppose that the microbial population *was* substrate-limited before inhibition; in this case, after inhibition an increase in temperature will allow an increase in extracellular enzyme activity, which will increase the supply of labile substrates. Subsequently, although the microbial population cannot grow, gas emissions may increase as a result of the increased supply of labile substrates, which were previously limiting gas production.

The aim of this study was therefore to investigate the following hypotheses:

- (1) that the supply of labile substrates to the soil microbial population limits gas production
- (2) that the rate of microbial respiration and activity limits gas production.

By conducting experiments employing microbial inhibition it should be possible to discriminate between the two processes. Both depend on soil conditions such as pH, water content and organic matter composition as well as temperature; if these are kept constant the contribution of microorganisms can be assessed by inhibition, and it should be possible to discover whether process (1) or (2), in the model described above, creates the bottleneck which limits gas production. The gases nitrous oxide, methane and carbon dioxide may behave differently with respect to control of their production by extracellular enzymes or microbial metabolism.

In addition, microbial inhibition would show whether chemodenitrification made a significant contribution to nitrous oxide emission from the soil of this constructed wetland.

For this experiment it was assumed that any disrupting agent would affect only the live microbial population, leaving extracellular enzymes intact; this would be a reasonable assumption of the effects of many organic pollutants. To avoid the possibility that parts of the microbial population might be able to metabolize (as a carbon source) an organic pollutant which was toxic to the majority, a method of inhibition which affected microorganisms but not soil enzymes was required. Sterilizing agents which specifically and exclusively affect respiring cells include antibiotic and antifungal metabolic inhibitor compounds, chemical sterilizing agents such as methyl bromide and ethylene oxide, and irradiation. Autoclaving is unsuitable because it indiscriminately denatures all soil protein; apart from the loss of extracellular enzyme activity which this causes, autoclaving also increases the concentration of organic carbon and soluble electrolytes in soil solution (Ramsay & Bawden, 1983). Microorganisms subsequently introduced to autoclaved soils may be poisoned by the release of toxic materials (Ramsay & Bawden, 1983). Chemical sterilization of soil can leave residues which are toxic

to microorganisms (Eno & Popenoe 1963) or which inactivate soil enzymes (ethylene oxide) or release cellular enzymes (toluene) (McLaren 1969).

Gamma-irradiation at doses > 2.5 Mrad has been reported to inactivate the microbial biomass whilst leaving intact the non-living protein in the soil (McLaren *et al* 1962). It is an effective method of sterilization when an ionizing event occurs inside, or close to, a microorganism, preventing its subsequent division or causing death by releasing chemical entities (Jackson *et al* 1967). During irradiation, water reacts to form peroxides which contribute to the breakdown of organic matter (Salonius *et al* 1967).

Extracellular enzymes are more resistant to radiation than are microorganisms (Stotzky & Mortensen 1959), and irradiation has a greater inhibitory effect on enzyme synthesis than on the activity of existing enzymes (Peterson 1962). Jackson *et al* (1967) report that gamma irradiation is a "rapid and effective" method of sterilizing small soil samples (circa 30 g); van Cleemput & Patrick (1974), Brown (1981) and Lensi *et al* (1991), amongst others, also report good sterilization of soil after varying doses of gamma irradiation. Cobalt-60 is the most popular isotope for soil irradiation (Brown 1981), as the radiation it produces is barely attenuated by passing through water, yet there is not sufficient energy to make samples radioactive.

Rather than complete sterilization, irradiation renders most microorganisms unable to grow, divide or reproduce (McLaren 1969); thus they retain their metabolic capabilities until they die within a few weeks. It was therefore interesting and important to measure gas flux immediately after irradiation treatment and later when nearly all metabolic activity should have ceased, leaving only immobilized enzymes active.

7.2 Methods

a. Inhibition by gamma irradiation

A 600 g sample of soil from the Centre for Alternative Technology sewage treatment wetland was sieved (1.18 mm mesh), divided into thirty 20 g sub-samples, and placed in sterile 50 ml glass jars with screw top lids with self-sealing silicon septa.

Fifteen of the soil samples received a minimum 25 kGray dose (equivalent to 2.5 Mrad, Brown 1981) of γ -radiation from a Cobalt 60 source (Isotron plc, Swindon), at 6 kGrays h⁻¹; the remaining fifteen samples acted as controls, and were taken to the irradiation facility at the same time as the irradiated samples. After irradiation the lids of all jars were tightened for transport back to the laboratory.

b. Gas sampling

Before measurement of the gas fluxes, all the jars were placed in a sterile lamina flow cabinet, uncapped, and the air inside flushed with sterile air. Samples of air from the lamina flow cabinet were collected as background, using air-tight gas syringes, for later reference. With the lids tightly replaced (in the flow cabinet) the jars were distributed between four controlled temperature rooms (4, 12, 24 and 37 °C); in each, three control and three irradiated samples were left to incubate for 150 minutes.

After this period, 5 ml of head-space gas was collected from each jar using gas-tight syringes, and analysed (with the background samples collected earlier) for nitrous oxide, methane and carbon dioxide using an Ai Cambridge model 92 gas chromatograph (specification and operating conditions are described in Chapter III).

c. Enzyme activity assay

The activity of β -glucosidase in control and irradiated samples was compared using the MUF-substrate technique described in Chapter III; 1 g of soil from each of the jars incubated at 24 °C was mixed with 7 ml of 400 $\mu\text{mol l}^{-1}$ MUF- β -D-glucoside and incubated for 60 mins at 24 °C.

The electron transport system (dehydrogenase) activity of control and irradiated samples was compared using the soil incubated at 24 °C, and the INT assay. One g of soil was mixed with 7 ml of 0.2 % (w/v in methanol) INT solution, incubated at 24 °C for 60 minutes and then centrifuged at 10,000 g for 5 minutes. The supernatant was then discarded and 5 ml methanol added to dissolve the pellet. The Formazan produced was extracted for 15 minutes on ice, and after a further centrifugation at 10,000 g (5 minutes), the absorbance of the supernatant was measured at 480 nm, using methanol as a blank. A calibration curve was prepared using Formazan (dissolved in methanol) at 0.03, 0.015, 0.0075, 0.00375 and 0.00187 g l⁻¹.

7.3 Results

a. Enzyme activity

Twenty four hours after the irradiation treatment, there was no significant difference ($p > 0.05$) in β -glucosidase activity between control and irradiated soil at 24 °C. There was also no significant difference ($p > 0.05$) in dehydrogenase activity, measured using the INT assay, between control and irradiated soil at 24 °C.

b. Gases

Twenty four hours after irradiation treatment:

Nitrous oxide (Figure 7.2 a) There were significant increases in gas emissions between the four incubation temperatures ($F_{3,16} = 5.93$, $p = 0.006$), and between the two treatments (control soil and irradiated soil)

($F_{1,16} = 20.24$, $p < 0.001$). Nitrous oxide production increased by 533 %, 235 %, 253 % and 124 % in soil incubated at 4, 12, 24 and 37 °C respectively after irradiation. There was no significant interaction ($P > 0.05$) between temperature and treatment. Tukey post-hoc tests showed significant differences in carbon dioxide emission between 4 and 37 °C and between 12 and 37 °C. Nitrous oxide emission and incubation temperature correlated well in control soil ($r = 0.844$, $p = 0.001$), but there was no correlation in irradiated soil ($r = 0.479$, $p = 0.115$).

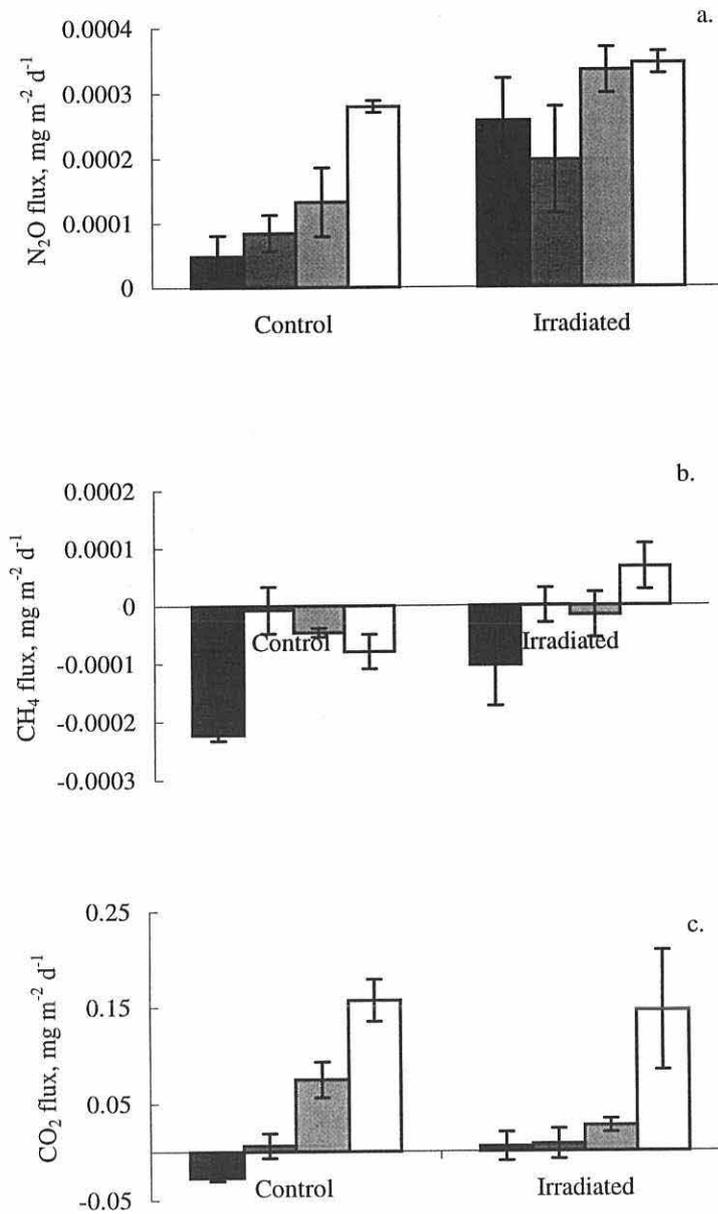
Methane (Figure 7.2 b) There were significant differences in methane emission between the four incubation temperatures ($F_{3,16} = 7.86$, $p = 0.002$) and between the two treatments ($F_{1,16} = 7.90$, $p = 0.013$); there was no significant interaction ($p > 0.05$) between temperature and treatment. At 4 and 24 °C, methane oxidation after irradiation was 45 % and 37 % of that before. At 12 and 37 °C, methane production after irradiation was 2 % and 84 %, respectively, of oxidation before irradiation. Tukey post-hoc tests showed significant differences in emission between 4 and 12 °C, 4 and 24 °C, and 4 and 37 °C. Methane emission and incubation temperature were uncorrelated in control soil ($r^2 = 0.41$, $p = 0.186$), but were correlated in irradiated soil ($r = 0.594$, $p = 0.042$).

Carbon dioxide (Figure 7.2 c) There were significant differences in gas emission between the four incubation temperatures ($F_{3,16} = 15.77$, $p < 0.001$), but not between treatments. At 4 °C, production of carbon dioxide after irradiation was 20 % of consumption before. At 12, 24 and 37 °C, carbon dioxide production increased by 126 %, 36 % and 93 %, respectively, after irradiation. There was no significant interaction ($P > 0.05$) between temperature and treatment. Tukey post-hoc tests indicated significant differences in carbon dioxide emission between 4 and 37 °C, 12 and 37 °C, and 24 and 37 °C. Carbon dioxide emission and

incubation temperature were well correlated in both control and irradiated soil ($r = 0.951$, $p < 0.001$ in control soil, $r = 0.694$, $p = 0.012$ in irradiated soil).

Figure 7.2

Nitrous oxide (a), methane (b) and carbon dioxide (c) emission in control and irradiated soil incubated at 4 (■), 12 (▒), 24 (▓) and 37 (□) °C. (Mean, $n = 3$, \pm s.e.)



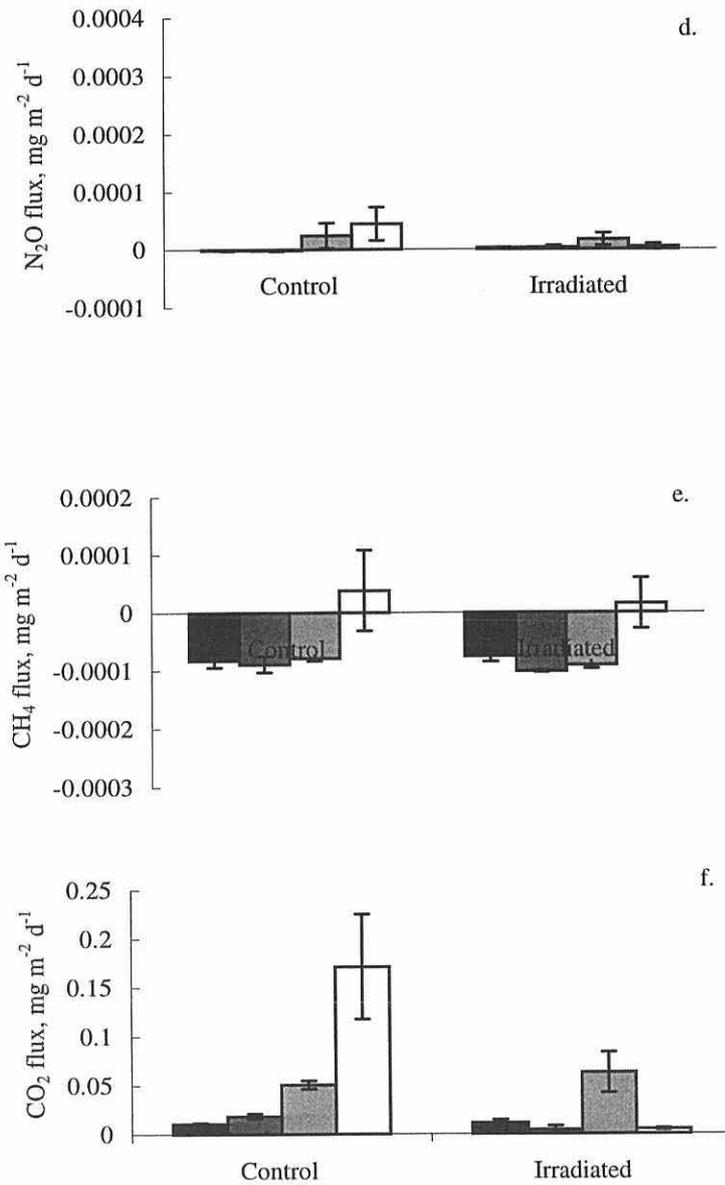
A week later:

Nitrous oxide (Figure 7.2 d) A week after irradiation, nitrous oxide emission was reduced to about $1/7^{\text{th}}$ of the original level in control soil ($T = -3.74$, $p = 0.0025$), and to about $1/10^{\text{th}}$ in irradiated soil ($T = -9.15$, $p < 0.001$), although there were no significant differences at different temperatures, or between the two treatments. There was no interaction ($P > 0.05$) between temperature and treatment. Control soil showed negative nitrous oxide flux at 4 °C and 12 °C, while irradiated soil showed no discernible pattern of nitrous oxide emission. Nitrous oxide emissions were again correlated with temperature in control soil ($r = 0.579$, $p = 0.049$), but not in irradiated soil ($r = 0.183$, $p = 0.57$).

Methane (Figure 7.2 e) A week after irradiation, methane oxidation at 4 °C in control soil was about half of the original level, and at other temperatures had increased. In irradiated soil methane oxidation increased at 12 and 24 °C. None of these changes was statistically significant. Methane oxidation had increased overall in both the control and irradiated soils. There was a significant difference in gas emission between temperatures ($F_{3,16} = 7.24$, $p = 0.003$), but not between treatments. There was no interaction between temperature and treatment ($p > 0.05$), so it can be concluded that gas emissions from both treatments are responding similarly to temperature. Tukey post-hoc tests revealed a significant difference between methane emissions at 4 and 37 °C, at 12 and 37 °C, and at 24 and 37 °C. Methane emissions showed no correlation with incubation temperature.

Figure 7.2 contd.

Nitrous oxide (d), methane (e) and carbon dioxide (f) emission a week after inhibition by irradiation, in soil incubated at 4 (■), 12 (■), 24 (▨) and 37 (□) °C. (Mean, n = 3, ± s.e.).



Carbon dioxide (Figure 7.2 f) After a week, carbon dioxide emission patterns were the same in the control as at the beginning of the experiment; in irradiated soil however, the high production seen at 37 °C had virtually disappeared. There were significant differences in gas emission between the four incubation temperatures ($F_{3,16} = 6.66$, $p = 0.004$), and between the two treatments ($F_{1,16} = 8.31$, $p = 0.011$). There was a significant interaction between temperature and treatment ($F_{3,16} = 8.28$, $p = 0.001$) which Tukey post-hoc tests showed originated mainly from the control soil; carbon dioxide emissions from control soil were significantly different at 4 and 37 °C, 12 and 37 °C, and 24 and 37 °C. Carbon dioxide emissions and incubation temperature were correlated in control soil ($r = 0.797$, $p = 0.002$), but not in irradiated soil ($r = 0.125$, $p = 0.699$).

7.4 Discussion

The irradiation treatment did not significantly alter β -glucosidase enzyme activity, and there was also no significant reduction in dehydrogenase activity, which is consistent with the way in which gamma irradiation acts to prevent cell proliferation rather than killing cells directly (McLaren 1969). If higher temperatures increase microbial metabolism, leading to increased gas emissions, then a week after irradiation we would expect to see greatly reduced gas emissions from irradiated compared to non-irradiated soil because live but non-proliferating cells will have died. If nitrous oxide emissions are not reduced by the irradiation treatment, there is evidence to support non-biological gas production (e.g. chemo-denitrification), or production by the direct activity of enzymes immobilized extracellularly.

Nitrous oxide

Gas emissions monitored immediately after irradiation indicated that the irradiation treatment had disrupted the normal control of nitrous oxide emission by temperature; emissions were much higher than usual at a lower temperature. In control soil the Q_{10} of nitrous oxide emission was 2.75, which was comparable

with that determined from the field data in Chapter III ($Q_{10} = 1.96$). It is possible that a bottle-neck in the nitrification/denitrification process was removed by the radiation treatment, allowing faster mineralization of nitrogenous substrates in the soil; this might be substantiated by the lower emissions after a week (when substrates had become depleted).

Because sterilization by irradiation theoretically does not disrupt soil or cell structure, soil treated with this method might be expected to show the same nitrous oxide emissions as non-irradiated soil, i.e. an increase in nitrous oxide emission with increased temperature, as higher temperatures enable higher metabolic rates in soil bacteria, up to the point of enzyme denaturation. The first results (obtained on the day following irradiation) of this experiment certainly support those of van Cleemput & Patrick (1974) and Lensi *et al* (1991); nitrous oxide emissions from irradiated soil were equal to or greater than those from non-irradiated soil at all four experimental temperatures. However, the emissions did not show the correlation with temperature which those from control soil did, suggesting support for hypothesis 2, that the microbial population is not limited by substrates for nitrous oxide production. The overall increase in nitrous oxide production, at all incubation temperatures, may be due to the slow release of nitrous oxide formed by ionization during the irradiation.

Previous workers have found that denitrification was still functional after irradiation sterilization (van Cleemput & Patrick 1974, Lensi *et al* 1991). After the irradiation treatment, nitrous oxide emissions from irradiated soil were not significantly different at different temperatures, and did not show a linear increase with temperature, which may indicate that the denitrifying enzymes have been released from cells, or that a temperature-dependent rate-limiting step in the reduction of nitrate/nitrite to nitrous oxide has been removed by the irradiation. Lensi *et al* (1991) found that nitrous

oxide production was immediately decreased by gamma irradiation, and concluded that although denitrifying enzymes remained active, they were probably still contained in non-proliferating cells because the reactions were too complex to be supported extracellularly. Nitrous oxide emissions from the soil irradiated in this experiment increased at lower temperatures after the irradiation, but after a week had virtually ceased, which supports the theory that nitrous oxide production after irradiation is carried out by non-proliferating cells. After a week these are dead, and have not been replaced due to irradiation damage.

The experimental results did not show evidence of chemo-denitrification occurring in this soil. A week after the irradiation treatment there were not significant differences in nitrous oxide emission between the treated and control soils. Control soil might be expected to show greater nitrous oxide production than irradiated soil a week after treatment if chemo-denitrification was a significant source of the gas, because the control soil also has a residual biological production. According to Nelson & Bremner (1970), it is unlikely that metallic cations are involved in chemodenitrification because under aerobic conditions soils do not contain sufficient iron, tin or copper ions; however Moraghan and Buresh (1977) reported low levels of nitrous oxide production even in the absence of copper ions.

Methane

Irradiation appeared to reduce methane oxidation in this soil, and promoted production instead of oxidation at 37 °C. The reduction in methane oxidation suggests that the methanotrophic population has been reduced by the irradiation treatment, and possibly that at 37 °C methanogens have become more active. Methanogens may be more resistant to irradiation than methanotrophs. Methane production at different temperatures did not correlate with temperature before

irradiation, but did (significantly) afterwards. The results weakly support hypothesis 1, that the methanogenic population is substrate limited in this soil. However the soil may not be sufficiently anaerobic to obtain reliable results for methane production. The lack of correlation between methane flux and temperature in the control soil was also seen in the field study (Chapter III) and was later supported by a laboratory experiment (Chapter V a).

Carbon dioxide

Irradiation had little immediate effect on carbon dioxide emission, which was not significantly different in controls compared to experimental soils, and in both showed an increase with increasing temperature. This suggests that the soil microbial population were still respiring and able to respond to an increase in temperature with an increase in activity, which is consistent with previous reports that irradiation prevents reproduction but not respiration (McLaren 1969). Net uptake at 4 °C became net production after irradiation. Because carbon dioxide is a metabolic product, an increase in its emission would be expected with an increase in temperature. Also, cells which have been irradiated in soil will cease to produce carbon dioxide only if the radiation dose has killed them, and not if it has only prevented their proliferation. A week after irradiation there was indeed significantly less carbon dioxide from irradiated soil at 37 °C, probably reflecting the faster turnover of cells at this temperature which results in the earlier decline and death of the microbial population because reproduction is prevented.

Carbon dioxide emissions correlated with temperature both before and after irradiation, but were slightly (although not significantly) reduced afterwards. These results suggest that the irradiation treatment did not immediately affect microbial respiration, and support hypothesis 1, that for carbon dioxide production the microbial population is substrate limited.

Popenoe & Eno (1962) reported carbon dioxide emissions after radiation which were initially reduced but later equalled or exceeded those of control samples, and Stotzky & Mortensen (1959) found that carbon dioxide evolution by irradiated soil was characteristic of non-irradiated soil, and concluded that there were therefore no significant alterations to substrate oxidative processes following irradiation. However in this experiment carbon dioxide emissions a week after irradiation were greatly reduced in irradiated soil at 37 °C, but had hardly changed in control soil. Lensi *et al* (1991) reported decreased carbon dioxide emission from soil immediately after gamma irradiation, but noted that the persistence of production indicated a radioresistant enzymatic pool. Carbon dioxide evolution after irradiation could also result from radiolytic decarboxylation of soil organic matter (Lensi *et al* 1991).

Soil enzymes

Lensi *et al* (1991) also found that enzyme activities were resistant to irradiation, and continued to result in carbon dioxide production, β -glucosidic bond lysis and deamination of nitrogenous organic compounds; he suggested that denitrifying enzymes remained active within cells which could no longer proliferate, and that any loss of denitrifying activity was the result of cell lysis. Cawse (1968) found that *Nitrobacter* and *Nitrosomonas* retained their ability to oxidize ammonium to nitrate after irradiation at 10 kGrays, although they failed to proliferate; this is confirmed by Lensi *et al* (1991) who report active denitrifying enzymes after irradiation sterilization, and by van Cleemput & Patrick (1974) who demonstrated that nitrate and nitrite reduction can be achieved by radiation-resistant enzymes from non-proliferating cells of denitrifiers. Given a long enough time interval between irradiation and nitrous oxide emission measurement, it may therefore be possible to eliminate production of nitrous oxide completely once the active (but non-proliferating) cells had died. However in field situations, particularly in

wetlands with a continuous inflow, this seems unlikely because recolonization of soil by denitrifiers may be rapid and continuous.

Sterilization by irradiation causes minimum heat production, and although van Cleemput & Patrick (1974) believe the physical and chemical effects on soil are mild and often negligible, there are many reports of an increase in nutrient release (N, P, S) following irradiation (Eno & Popenoe 1964, Stotzky & Mortensen 1959, Cawse & Cornfield 1971). Cawse & Cornfield (1969) showed that nitrite accumulation in soil after irradiation was the result of nitrate reduction rather than ammonia oxidation, possible due to the activity of radio-resistant enzyme systems. Irradiation can also cause chemical changes in the soil, for example increases in ammonium concentration (Cawse 1968), and in concentration of soluble organic matter (Ramsay & Bawden 1983). Total extractable nitrogen and phosphorous can be increased by gamma-irradiation in some soils (Eno & Popenoe 1963), from killed organisms and from the effect of radiation on organic compounds in the soil.

The effect of irradiation for sterilization depends on the initial population of microorganisms (van Cleemput & Patrick, 1974). The levels of radiation previously used for soil sterilization have ranged from 10 kGray for fungal elimination (Jackson *et al* 1967), to 40 kGray (Brown 1981). Bacteria and actinomycetes are reported to need a higher radiation dose than fungi for effective sterilization (Jackson *et al* 1967, Stotzky & Mortensen 1959). In this experiment soil received a minimum 25 kGray dose, which Brown (1981) reported to be sufficient to prevent reproduction of all microorganisms; McLaren (1969) cites evidence that a 25 kGray dose of irradiation does not change soil pH or affect the redox potential of humus.

It is clear that soil enzyme activity remains after doses of gamma irradiation which prevent reproduction of all microorganisms (Cawse 1968, van Cleemput & Patrick 1974, Brown 1981, Lensi *et al* 1991). The diversity and intensity of this

activity however seems variable, and almost certainly depends on the type of soil and its water content during the irradiation treatment. It has even been proposed (McLaren *et al* 1962) that enzyme activity could increase after irradiation if the permeability of dormant and dead cells increased. There is some consensus that enzymes in wet or peaty soil are more sensitive to gamma irradiation (Brown 1981, Lensi *et al* 1991) than those in dry and/or mineral soils; this is probably a consequence of free radicals, generated by irradiation of soil water, reacting with amino acids and nucleic acids (Okada 1958, Scholes & Weiss 1960).

Additionally, because enzymes are in humic-colloid complexes in peat soils rather than the more stable adsorption to clay minerals in mineral soils, they may be more sensitive (Brown 1981), and it is also true that soil enzymes are more sensitive at higher moisture contents to a range of factors (Ramirez-Martinez & McLaren 1966, Burns 1978) including gamma irradiation (Salonius *et al* 1967).

Although extracellular enzymes are important, especially for breakdown of carbonaceous organic matter such as cellulose, these experiments have shown that the microbial biomass is intrinsic to soil gas emissions. Nitrous oxide, methane and carbon dioxide are produced under differing soil conditions, and by different metabolic processes; this is probably why they show different responses to microbial inhibition. Nitrous oxide production continued after irradiation, but the subsequent decline of the microbial population (in the absence of proliferation) prevented indefinite denitrification. Methanogenic bacteria on the other hand may be relatively insensitive to irradiation. Applying toxicants to a wetland could be very destructive, if only in the short term. Depressions in soil microbial activity are likely to recover if they are comparable in magnitude with those found in natural situations, where the doubling time for microbial cells is approximately ten days, and a 90% depression will take about 30 days to recover (Goodman *et al* 1992). Xenobiotics which entered a wetland in wastewater and depressed the soil microbial population may considerably extend this recovery period, for example to 60 days (Goodman *et al* 1992).

Whilst reduced rates of denitrification may be beneficial in terms of reduced nitrous oxide emissions, the potentially higher outflow of nitrate from a constructed wetland with an inhibited microbial population could be extremely damaging to sensitive water courses. It is therefore essential that inadvertent microbial inhibition (e.g. inhibition by allowing toxins to enter the wetland) is avoided, and that constructed wetlands are not operated at their treatment capacity. This ensures that a safety margin is kept in case treatment processes are ever slowed by microbial inhibition.

Conclusions

- Immediately after irradiation, temperature control of nitrous oxide production was lost; the microbial population involved in nitrous oxide production is probably not substrate limited in this soil.
- There was no evidence of chemo-denitrification in this soil
- Methane production was decreased by irradiation; methanotrophic bacteria may be more inhibited than methanogenic bacteria. Methanogenic bacteria are probably substrate limited in this soil.
- Micro-organisms producing carbon dioxide in this soil may be substrate limited. Irradiation caused a delayed decline in microbial respiration.

Chapter VIII

Persistence of enzymes added to wetland soil

Persistence of enzymes added to wetland soil

8.1 Introduction

Extracellular enzymes in soil are assumed to remain active for as long as they are protected from degradation by any biological, chemical or physical means; protection from biological (e.g. protease) or chemical (e.g. acid) attack is reportedly conferred by association with soil constituents, in particular adsorption to clay minerals or immobilization on humic colloids (Burns 1982). Protection from physical denaturation is extremely difficult.

Sarkar & Burns (1984) remark that soluble enzymes are rapidly denatured, degraded or otherwise inactivated in soil. The mechanisms of attachment and stabilization between enzymes and soil humates probably include ion exchange, entrapment within three-dimensional micelles, lipophilic reactions and hydrogen and covalent bonding (Sarkar & Burns 1984). Some humic components may protect enzymes associated with them because they inhibit potentially degradative micro-organisms. Garzillo *et al* (1996) immobilized acid phosphatase by creating a phosphatase-polyresorcinol complex which modelled the humic-enzyme complexes believed to exist in soil; this improved enzyme stability and protease (trypsin and chymotrypsin) resistance, and retained the original activity, but when added to soil had no such advantages over the free enzyme. Garzillo *et al* concluded that this was probably due to adsorption of the free phosphatase onto soil clay-humic colloids, which was stronger than that occurring in the immobilization process.

Natural immobilization may also be on organic, non-humic material excreted by plant roots (Floyd & Ohlrogge 1970) and forming a mucigel in the rhizosphere. Ciurli *et al* (1996) immobilized urease on polygalacturonic acid, a constituent of root mucilage, which was found to be a very efficient carrier for soil bacterial urease (Ciurli *et al* 1996). Further, Ciurli *et al*'s work suggested that urease may

actually be more efficient in the rhizosphere due to polygalacturonic immobilization, and so supply plants with higher levels of ammonium ions than they would otherwise have available.

Similarly, studies by Garzillo *et al* (1996) showed that immobilizing acid phosphatase on polyresorcinol gave the enzyme a higher stability towards pH, temperature and proteases compared to free enzyme *in vitro*, but not in soil. The half-life of immobilized phosphatase was improved in soil. Marzadori *et al* (1998) immobilized potato acid phosphatase on calcium polygalacturonate, a compound with similar composition and morphology to root mucigel, and found that doing so increased enzyme stability but also increased thermal and proteolytic inactivation.

Supplementing wetlands with extracellular enzymes in addition to those which naturally accumulate from microbial activity has been proposed as a way to increase the rate and efficiency of waste treatment (Aitken 1993). However, enzymes added to soil in a free form would normally be rapidly degraded by proteases such as pronase and trypsin (Ciurli *et al* 1996) because they constitute a labile source of protein carbon. If enzymes added to a wetland are not quickly or previously immobilized, and so protected from microbial attack as sources of protein, then supplementation becomes an expensive and redundant exercise.

To date the literature contains only scarce references to enzyme additions to soil, and much of the work has concentrated on immobilized enzymes. Cervelli & Perret (1998) report on the addition of immobilized acid phosphatase and of β -glucosidase to soil with the eventual aim of decontaminating soil. They used partially and completely sterilized soil, and added enzymes immobilized on sintered clay; the results suggested that such immobilization could restore at least part of the soil enzyme activity lost by sterilization, and after 14 days activity had declined by 8 % and 12 % for β -glucosidase and phosphatase respectively.

To investigate the persistence of the activity of extracellular enzymes added to wetland soil, commercially produced enzymes were added to soil from the Centre for Alternative Technology sewage treatment wetland in batch experiments. The activity of soil extracellular enzymes was subsequently monitored over a period of up to 60 days. Enzymes were also added to soil from the Centre for Alternative Technology (sewage treatment) and Pelenna (mine drainage treatment) wetlands in continuous flow experiments to test the hypothesis that inorganic material release would increase, and organic material removal would improve.

8.2 Batch experiments

a. Methods

Soil from the wetland was prepared by sieving through a 1.2 mm mesh; two treatments (enzyme solution or enzyme diluent) were applied to fresh soil and to autoclaved soil (120 °C, 1.2 bar to destroy existing microorganisms and denature soil proteins). Five replicate 20 g (fresh weight) samples of soil were prepared for each treatment, from both fresh and autoclaved soil, and placed in sterile (autoclaved) 100 ml glass jars.

Summary of treatments:

Soil	Treatment	Replicates
Fresh	Enzyme	5
Fresh	Diluent	5
Autoclaved	Enzyme	5
Autoclaved	Diluent	5

The experiment was carried out for cellulase in deionized water, cellulase added in acetate buffer (pH 5.3), cellulase added in water, Savinase added in acetate buffer, Savinase added in water and β -glucosidase added in water. Enzyme solution

concentrations and methylumbelliferyl (MUF) substrate concentrations are shown in Table 8.1.

Acetate buffer was used in some experiments to maintain the natural pH of the soil, while in others deionized water was used as a diluent in order to compare the effect on enzyme activity persistence when pH was controlled only by the natural buffering capacity of the soil. Acetate buffer has often been used in soil enzyme assays for pH stabilization (Martens *et al* 1992 (sulphatase), Garzillo *et al* 1996 (phosphatase), Klose *et al* 1999 (arylsulphatase)).

Five ml of enzyme solution or diluent (water or buffer) were added evenly to each soil sample and after 6-12 hours the enzyme activity was assayed again. Enzyme activity assays were then repeated at increasing time intervals up to 60 days.

Table 8.1

Enzyme solution and MUF substrate concentrations

Enzyme added	Diluent	Concentration of added enzyme solution	MUF substrate used for soil assay
Cellulase	Acetate buffer	1 g l ⁻¹	200 µmol l ⁻¹ MUF cellobiopyranoside
Cellulase	Deionized water	1 g l ⁻¹	200 µmol l ⁻¹ MUF cellobiopyranoside
Savinase	Acetate buffer	1 g l ⁻¹	0.5 mmol l ⁻¹ Leucine amino methylcoumarin
Savinase	Deionized water	1 g l ⁻¹	0.5 mmol l ⁻¹ Leucine amino methylcoumarin
β-glucosidase	Deionized water	2 g l ⁻¹ (24 units/5 ml)	200 µmol l ⁻¹ MUF-β-D-glucoside
Alcalase	Deionized water	1 g l ⁻¹	200 µmol l ⁻¹ MUF β-D-glucoside, MUF phosphate, MUF sulphate

The initial enzyme activity of each replicate was measured before the treatments were applied, using MUF substrates; 0.5 g of soil from each replicate was mixed with 3.5 ml of MUF substrate (see Table 8.1) and incubated for 60 mins. Reaction termination and fluorescence measurement was as described in Chapter III.

Quench correction assays with methylumbelliferone free acid (methylcoumarin for Savinase) were carried out in parallel.

b. Enzyme characteristics

Enzymes were obtained from the National Centre for Biotechnology Education, University of Reading (cellulase (E.C. 3.2.1.4), Savinase (E.C. 3.4.21.62) and alcalase (E.C. 3.4.21.62)), and Sigma chemicals (β-glucosidase (E.C. 3.2.1.21)); cellulase and proteases were supplied as granulated enzyme preparations under the commercial names Celluzyme, Savinase and Alcalase, and β-glucosidase as a lyophilized powder, from almonds.

Celluzyme is an endo-cellulase produced by submerged culture of *Humicola*. It is non-toxic and biodegradable. Savinase is a serine-type protease produced by submerged fermentation of genetically modified *Bacillus*; it is optimally active at c.50 °C (but retains activity down to 10 °C) and at pH 8-9. It is non-toxic and biodegradable. Alcalase is also a serine-type protease, produced by submerged fermentation of *Bacillus licheniformis*; it is optimally active at c.60 °C (but retains activity to below 10 °C) and at pH 7-9.

c. Results

For each experiment, mean enzyme activity (n=5) (as μmol of methylumbelliferone (MUF) or methylcoumarin (MC) released $\text{g soil}^{-1} \text{min}^{-1}$) for each treatment was plotted against days since enzyme addition.

Cellulase in acetate buffer

(Figure 8.1 a) Cellobiohydrolase activity in fresh soil increased by 173 % after the addition of cellulase, and continued to rise gradually (with some small decreases) until day seven. In fresh soil with no added cellulase, cellobiohydrolase activity decreased by 58 % after addition of acetate buffer and then remained stable.

Killed soil with added cellulase showed an initial rise in cellobiohydrolase activity after enzyme addition which was more than double that in fresh soil (375 %), and a subsequent gradual increase in activity at the same rate as that in fresh amended soil. Cellobiohydrolase activity in killed soil without added cellulase remained low and stable with no changes.

Cellulase in deionized water

(Figure 8.1b) Fresh soil with added cellulase showed an increase in cellobiohydrolase activity of 530 % after enzyme addition; this declined slightly

and became very stable at day seven. Cellobiohydrolase activity in fresh soil without added cellulase rose slightly after water addition, and subsequently remained stable.

In killed soil with added cellulase, cellobiohydrolase activity rose by 2048 % after enzyme addition (nearly four times the activity increase in fresh soil), then dipped and rose even higher to peak on day 15. After this, activity declined gradually. Killed soil without added enzymes showed no change in cellobiohydrolase activity during the course of the experiment.

Factorial ANOVA with Tukey post-hoc tests showed significant differences in cellobiohydrolase activity with time ($F_{7,128} = 21.29$, $p < 0.01$) and with treatment ($F_{3,128} = 153.09$, $p < 0.01$). There was significant interaction between time and treatment ($F_{21,128} = 8.44$, $p < 0.01$).

Savinase in acetate buffer

(Figure 8.1 c) Protease activity in all four soils (fresh amended and control, killed amended and control) was dramatically reduced after the addition of either savinase in acetate buffer (amended soils) or acetate buffer alone (controls). In fresh soil activity was reduced by 75 %, and in killed soil by 48 %.

Savinase in deionized water

When savinase was added in deionized water, there was no significant change in activity, in either fresh or killed soil.

β -glucosidase in deionized water

(Figure 8.1 d) Activity of β -glucosidase in fresh soil increased by 370 % after β -glucosidase was added, rose until day five and then declined a little and remained stable at 325 % of its original activity. Fresh soil with no added β -glucosidase

showed no significant change in β -glucosidase activity either after addition of water, or during the course of the experiment.

In killed soil, β -glucosidase activity increased by 4210 % after the addition of β -glucosidase (an increase eleven times greater than the activity increase in fresh soil), continued to rise slowly until day 11 and subsequently declined to reach the same level of activity as fresh soil with added enzyme. β -glucosidase activity in killed soil without added enzyme rose 1000 % between days 5 and 11, to the same level as fresh control soil, and then remained stable.

Electron transport system activity in fresh soil with added β -glucosidase showed a slight rise during the course of the experiment (Figure 8.1 e); activity in fresh soil without enzyme showed a similar pattern. In killed soil with added β -glucosidase, electron transport system activity rose steadily, approximately doubling every 10 days, but after day 31 began to decline again; electron transport system activity in killed soil without added enzyme showed a later and slower increase than supplemented soil, but eventually reached the same end-point.

Factorial ANOVA with Tukey post-hoc tests showed significant differences in β -glucosidase activity with time ($F_{7,128} = 77.04$, $p < 0.01$) and with treatment ($F_{3,128} = 437.93$, $p < 0.01$). There was significant interaction between time and treatment ($F_{21,128} = 30.60$, $p < 0.01$).

Alcalase in deionized water

The activities of β -glucosidase, phosphatase and sulphatase were not significantly altered after 11 days' incubation with alcalase (Figure 8.2 a-c).

Figure 8.1

Cellobiohydrolase activity with acetate buffer (a), with deionized water (b), and savinase activity with acetate buffer (c), in amended fresh soil (\square), control fresh soil (\blacksquare), amended killed soil (\circ) and control killed soil (\bullet). (mean, $n = 5, \pm$ s.e.).

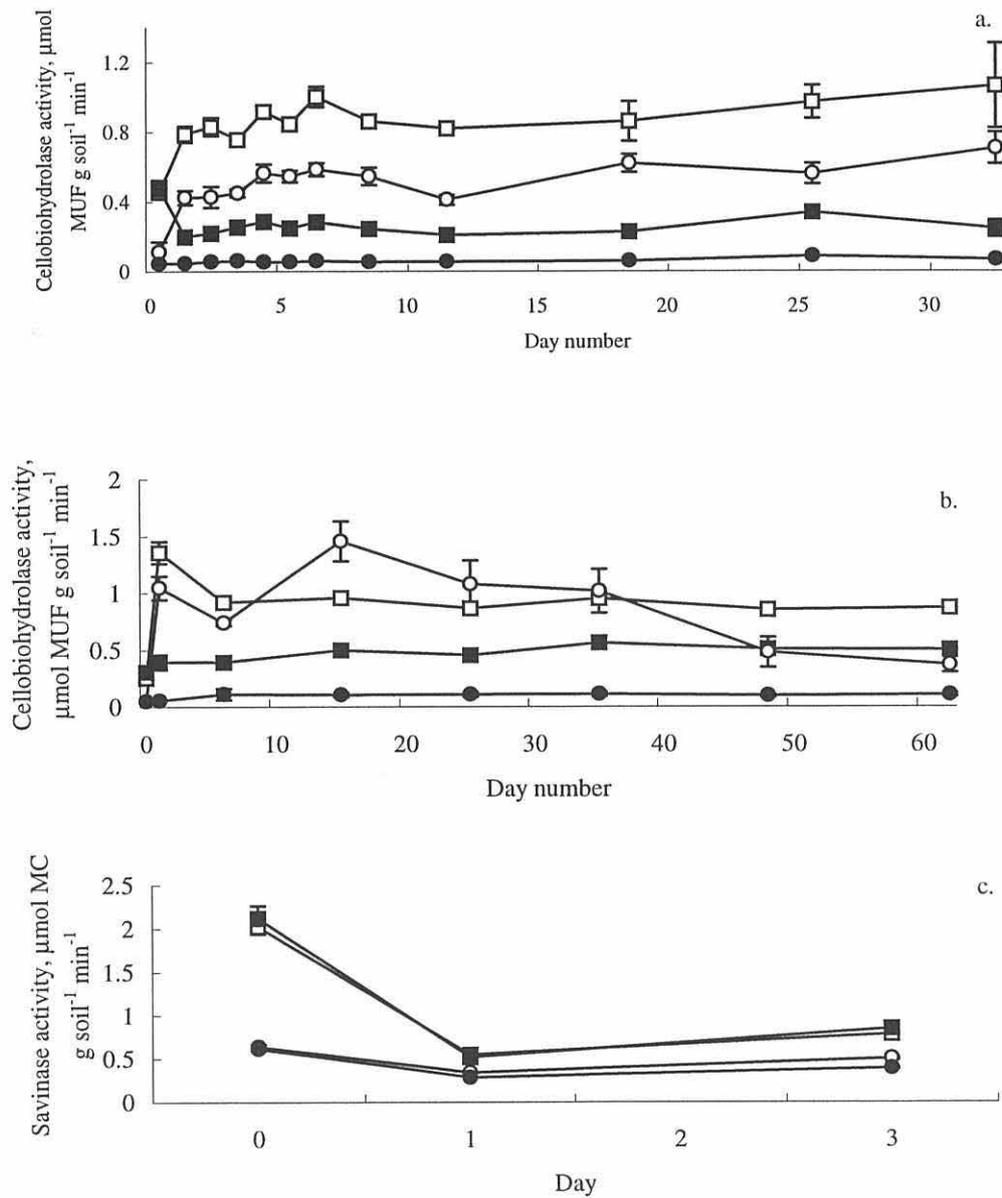


Figure 8.1 contd.

β -glucosidase activity with deionized water (d), and electron transport system activity in β -glucosidase addition experiment (e), in amended fresh soil (\square), control fresh soil (\blacksquare), amended killed soil (\circ) and control killed soil (\bullet). (mean, $n = 5, \pm$ s.e.).

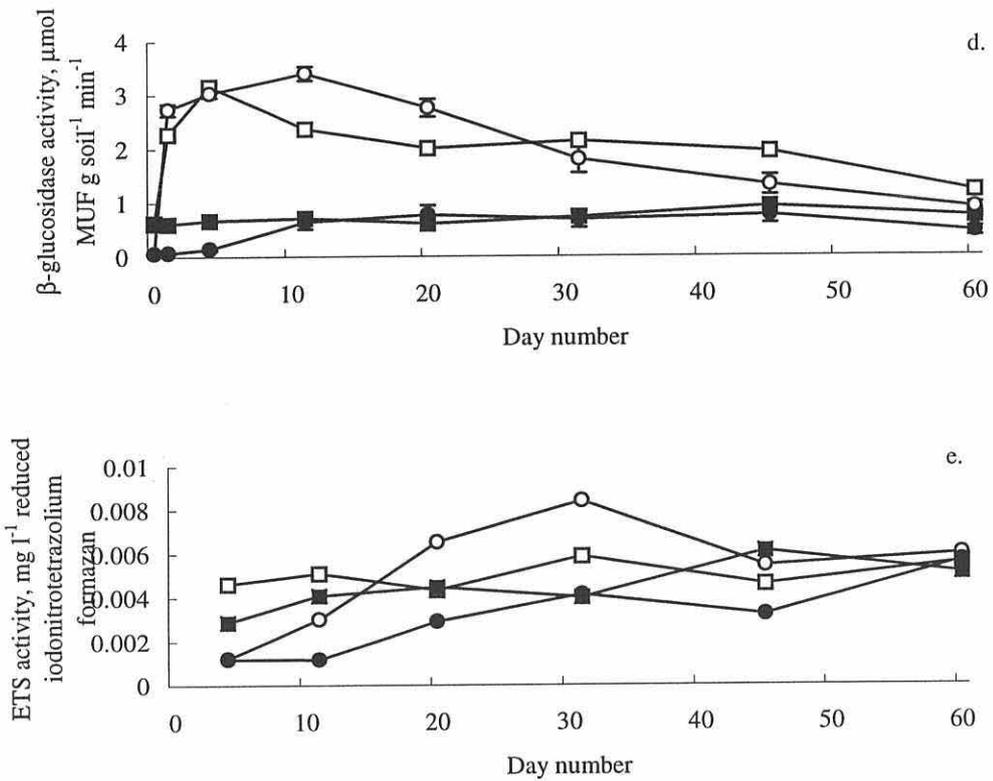
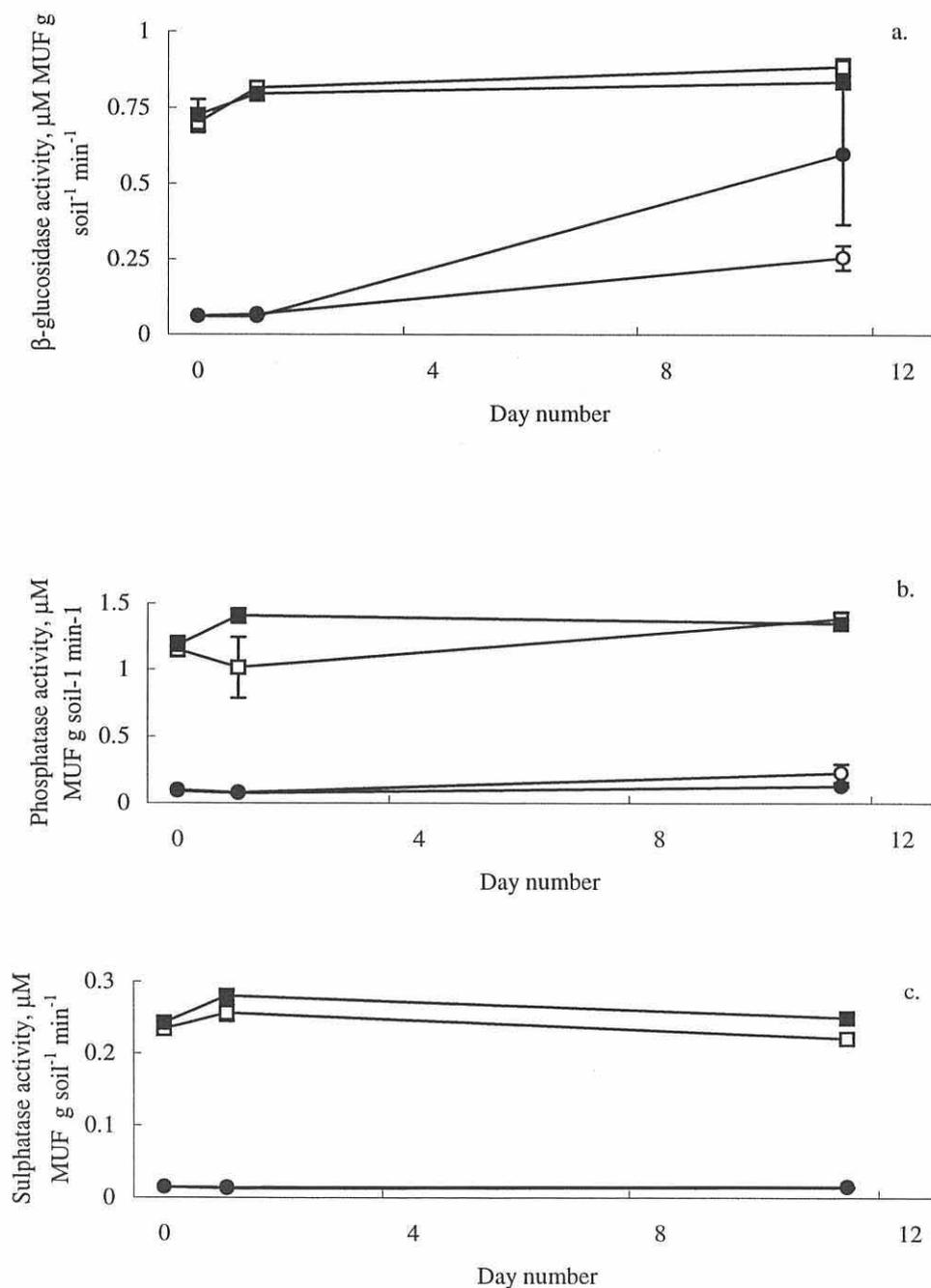


Figure 8.2

β -glucosidase (a), phosphatase (b) and sulphatase (c) activity after alcalase addition, in amended fresh soil (\square), control fresh soil (\blacksquare), amended killed soil (\circ) and control killed soil (\bullet). (mean, $n = 5$, \pm s.e.).



d. Discussion

The initial increases in enzyme activity on addition of enzyme solution are easily accounted for, but there was often a continued increase after the initial addition (Figure 8.1 a-c). This occurred in both fresh and autoclaved soil when cellulase in acetate buffer was added, to some extent when cellulase in deionized water was added, and when β -glucosidase in deionized water was added. Control experiments did not show a parallel rise in activity, so the changes in soil moisture affecting native soil enzyme activity are not the cause. The addition of commercial enzymes may have stimulated microbial production of the same enzymes, or if the enzymes have broad specificity, some products may have stimulated release of enzymes also detectable by the method used.

The activity of cellulase added in acetate buffer in fresh and autoclaved soil showed almost identical patterns of increase and sustained activity over the study period. When cellulase was added in deionized water, its activity showed a greater increase in both soils compared to the acetate buffer experiment, even though identical concentrations were added in both experiments. However activity in the deionized water experiment faded more rapidly than that in the acetate buffer experiment, indicating that although the buffer prevented maximal activity, it also in some way prevented the decline of cellulase activity. There are several possible ways in which this could have happened. If the products of the enzyme activity tend to alter the soil pH, and the soil is unbuffered, enzyme activity could be reduced. This would only happen if the products were not rapidly removed by further enzyme activity or by microbial uptake, which in natural situations would be unlikely (end products would also tend to inhibit enzyme activity). However if soil enzymes are supplemented with additional enzymes, a bottleneck may develop allowing a build-up of end products.

When added in water, cellulase activity declined much more rapidly in autoclaved soil than in fresh soil, even taking into account the different times over which the

experiments were conducted. This contradicts the intuitive conclusion that sterilized soil would support added enzyme activity for longer because of the (assumed) absence of either proteases or microbial activity.

When Drozdowicz (1971) added cellulase obtained from *Aspergillus niger* to soil, it was completely inactivated with 18-20 hours in fresh soil, and in autoclaved soil had lost 60 % of its activity after two days. The contrast between this work and the results of the above experiment suggest that cellulase enzymes from different sources have very different half-lives in soil. Drozdowicz suggested that the organic matter and clay content of the soil was an important factor in determining the persistence of enzyme activity, and this is supported by the later work of Burns (1983).

β -glucosidase activity in either fresh or autoclaved soil was less stable when added to soil than cellulase was. After the initial addition, followed by a slight increase in activity, β -glucosidase activity declined at different rates in fresh and autoclaved soil. Fresh soil showed less increase in activity after enzyme addition, with activity dropping from day four, whereas in autoclaved soil activity continued to increase until day 11 and then began to decline, more quickly than activity in fresh soil. The effect of autoclaving on soil can result in changes to the physical and chemical properties, including increases in soluble electrolytes, ammonium levels and organic matter (Ramsay & Bawden, 1983). These changes could potentially affect enzyme activity because of the amphoteric nature of enzyme structure.

In these experiments fresh soil was found to be just as able to support added enzymes as sterilized soil, and sometimes even more so. Nannipieri *et al* (1978) investigated the kinetics and stability of humus-urease preparations, and found that complexed enzymes had better resistance to thermal denaturation and

proteolysis than free urease. It would be of value to investigate the ability of a soil to sustain the activity of added enzymes, in relation to its humic content. This would allow constructed wetlands to be designed with the necessary capacity to immobilize additional enzymes, which do not originate from the naturally colonizing microorganisms, soil animals and plants. Immobilization on different soil extracts alters the K_m constant (substrate affinity) of an enzyme by varying amounts (Nannipieri *et al* 1978). It may be possible to supplement wetland soil with soil extracts which cause the least increase in K_m , thereby providing material for enzyme immobilization which optimizes the activity of added enzymes.

When the protease Alcalase was added to soil, there was no significant effect on β -glucosidase, phosphatase or sulphatase in fresh or autoclaved soil. This rather suggests that these enzymes are indeed protected from protease attack by soil components. However when a different protease, Savinase, was added to soil, it was rapidly deactivated, suggesting that it did not become protected in the same way that the added hydrolases did, and was therefore vulnerable to degradation. There may be fundamental differences in the structure of different enzyme proteins which determine their capacity for immobilization on entering the soil. Additionally, the humic content of a soil may be a very important factor in determining its capacity to stabilize added enzymes. Dick & Tabatabai (1983) and Hope & Burns (1987) attributed the inhibition of free enzymes added to soil to masking of the active sites as the enzymes became bound to soil particles.

8.3 Continuous flow experiments

Wetland cores which were receiving effluent (mine drainage or sewage liquid) were supplemented with the same enzymes as before and the persistence of enzyme activity was monitored at the same time as water chemistry (outflow) and gas emissions.

a. Methods

Pelenna

Fifteen wetland cores in plastic containers (100 mm diameter, 110 mm high, nominal volume 700 cm³) were collected from cell 4 of the Pelenna pilot wetland for mine drainage treatment (Tonmawr, Gwenfrwd Valley, Neath; described in Chapter III). Each core was collected by first inserting a plastic cylinder into the soil, then digging this out and transferring the soil core to a plastic container with a drainage tube fitted in the base. For transport back to the laboratory each core was sealed with a lid and wrapped in plastic. Approximately 40 litres of mine drainage flowing into the wetland system were collected at the same time as the soil cores, and returned to the laboratory.

The fifteen soil cores were randomly allocated to one of three groups, to receive (in addition to a controlled flow of the mine drainage) no treatment, added cellulase or added β -glucosidase (five replicates in each group). The cores were maintained at 12 °C (± 2 °C). Each core received acid mine drainage at 0.08 ml min⁻¹ via pump tubing and a peristaltic pump from a reservoir. The pumps operated on a “12 hours on/12 hours off” cycle, and the experiment was continuously lit. Outflow from the cores was restricted, and each was emptied of liquid every three days; this allowed samples of outflowing water to easily be extracted from the soil for analysis.

The experiment was set up and monitored for 7 days before addition of enzyme solutions to experimental soil cores, and deionized water to control cores, on day 8. Cellulase enzyme solution (source as before) was made up at 1 g l⁻¹ in deionized water, and 180 ml added to each of cores 6-10. β -glucosidase enzyme solution (source as before) was made up at 2 g l⁻¹ in deionized water, and 180 ml added to each of cores 11-15. 180 ml of deionized water was added to each of cores 1-5 which acted as experimental controls.

Water, gas and enzyme analysis

All soil cores were sampled for water, gas and soil enzyme activity periodically during the 34 day course of the experiment.

On days 0, 4, 9, 13, 18, 27 and 34, samples of inflowing and outflowing water, and soil for enzyme analysis were taken. Water samples (5 ml) were filtered (0.2 μm) and stored at 4 °C until analysis. Soil samples (0.2 cm^3 per assay) were placed in Eppendorf microcentrifuge tubes and assayed within two hours according to the method in Chapter III. Soil from cores 1-5 was assayed for cellobiohydrolase and β -glucosidase enzyme activity, that from cores 6-10 only for cellobiohydrolase activity, and that from cores 11-15 only for β -glucosidase activity. Soil from all 15 cores was assayed for quench correction on each sampling occasion. On days 0, 7, 13, 18, 27 and 34 gas samples were collected by capping the cores and using gas-tight syringes to collect a 5 ml sample of headspace gas after 60 minutes. Collected gas was analysed using a Cambridge Ai model 92 gas chromatograph for nitrous oxide, methane and carbon dioxide (see Chapter III for specification and operating conditions). Background gas samples were taken to allow calculation of gas flux.

Water samples were assayed for dissolved organic carbon using a Skalar autoanalyser (colorimetric method), for phenolic content using the Box assay (Chapter III) and for iron content using a Pye Unicam SP9 air/acetylene atomic absorption spectrometer, at 248.3 nm.

Centre for Alternative Technology

The same experiment was repeated using soil from the sewage treatment wetland at the Centre for Alternative Technology, Machynlleth (described in Chapter III). Fifteen soil cores were collected as described above, and approximately 40 litres

of inflowing sewage liquid were collected and returned to the laboratory. The cores were set up as before, randomly allocated to three groups to receive either no treatment, cellulase addition or β -glucosidase addition (five replicates per group).

After twelve days' stabilization, enzyme solutions were added as before (at the same volume and concentration) to the experimental cores, and deionized water added to the control cores. Water and soil samples were collected using the above methods on days 0, 7, 12, 18, 21, 25, 28 and 32, and gas samples on days 5, 19, 22, 26 and 29. The same analyses were carried out, with the exception of water samples; for the CAT samples, inflow and outflow water was analysed for nutrient ions instead of iron content (NO_3 , PO_4 , SO_4 and NH_4) using a Dionex DX-120 ion chromatograph (CS12 and AS4A-SC columns). Carbon in water samples was analysed as described in Chapter III.

b. Results

The results of these experiments were not only quite different to each other but also different to the laboratory experiments with enzyme supplementation.

Pelenna

(Figure 8.3 b) Factorial ANOVA showed a significant difference in cellobiohydrolase activity with time ($F_{6,56} = 3.76$, $p = 0.003$), but no significant difference in activity compared to controls ($p > 0.05$). There was no significant interaction between time and treatment.

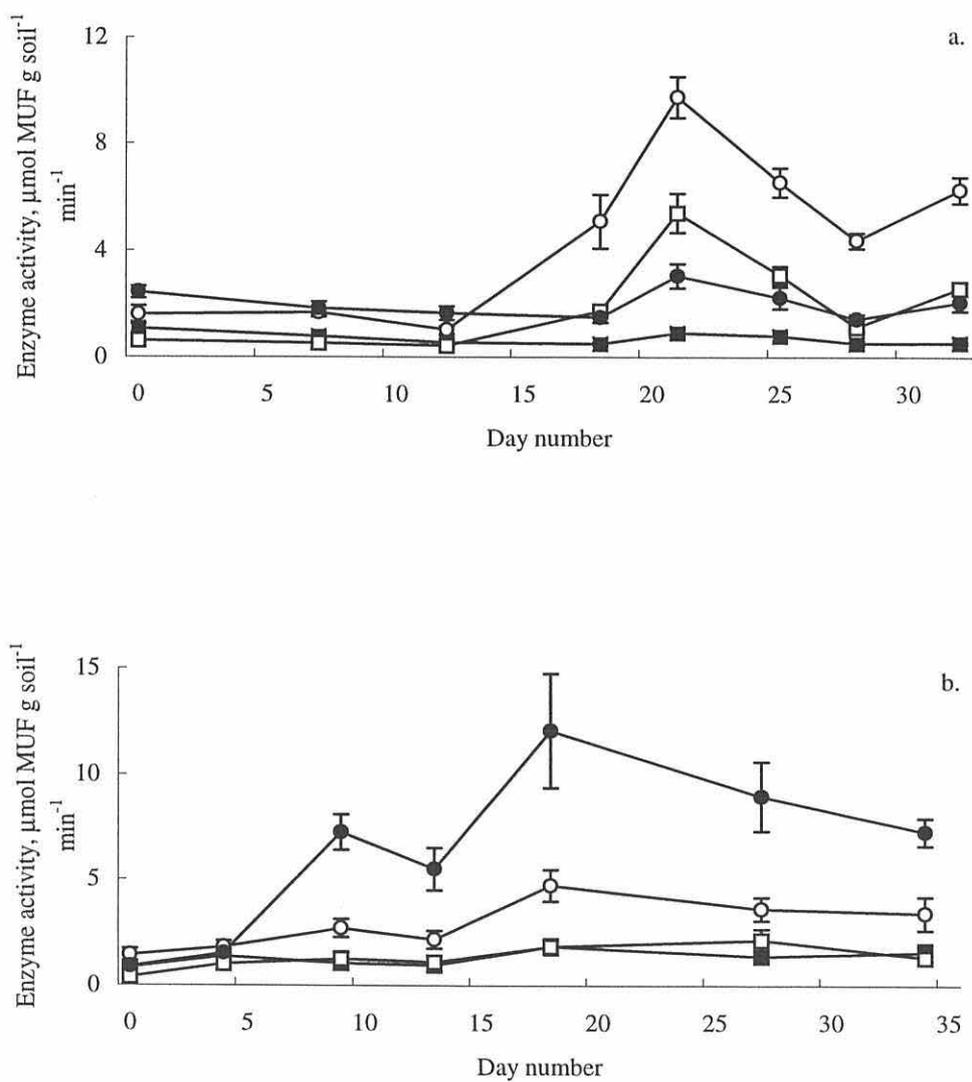
For β -glucosidase activity, factorial ANOVA showed significant differences with time ($F_{6,56} = 10.24$, $p < 0.001$) and compared to controls ($F_{1,56} = 31.21$, $p < 0.001$). There was also an interaction between time and treatment ($F_{6,56} = 3.28$, $p = 0.08$), which was significant at a lower level.

Nitrous oxide emission varied significantly over time ($F_{5,72} = 7.66$, $p < 0.001$), and between control, cellulase and β -glucosidase cores ($F_{2,72} = 8.06$, $p = 0.001$). There was significant interaction between time and treatment ($F_{10,72} = 4.53$, $p < 0.001$) (Figure 8.4 a). Methane emissions were significantly different over time ($F_{5,72} = 3.45$, $p = 0.007$), but not between treatments, and there was no interaction between time and treatment (Figure 8.4 b). The same was true of carbon dioxide emissions, which were only significantly different over time ($F_{5,72} = 29.45$, $p < 0.001$) (Figure 8.4 c).

The output of dissolved organic carbon did not vary significantly over time or between treatments and controls. Output of phenolics did show significant variation over time ($F_{6,73} = 7.66$, $p < 0.001$), and between treatments ($F_{2,73} = 4.04$, $p = 0.022$). There was no interaction between time and treatment (Figure 7.5 a-c).

Figure 8.3

Cellobiohydrolase activity in control (■) and cellulase-amended (□) soil cores, and β -glucosidase activity in control (○) and β -glucosidase-amended (●) soil cores from the Centre for Alternative Technology wetland (a) and Peledda wetland (b). (mean, $n = 5$, \pm s.e.).



CAT

(Figure 8.3 a) Factorial ANOVA showed significant differences in cellobiohydrolase activity with time ($F_{7,64} = 23.18$, $p < 0.001$) and compared to controls ($F_{1,64} = 89.59$, $p < 0.001$). There was significant interaction between time and treatment ($F_{7,64} = 20.90$, $p < 0.001$).

β -glucosidase activity also showed significant differences with time ($F_{7,64} = 23.00$, $p < 0.001$) and compared to controls ($F_{7,64} = 104.56$, $p < 0.001$). There was significant interaction between time and treatment ($F_{7,64} = 15.64$, $p < 0.001$).

Nitrous oxide emission varied significantly over time ($F_{4,60} = 17.27$, $p < 0.001$) but not between treatments. There was no significant interaction between time and treatment (Figure 8.4 d). Methane emissions were not significantly different over time nor between treatments, and there was no interaction between time and treatment (Figure 8.4 c). Carbon dioxide emissions were significantly different over time ($F_{4,60} = 6.42$, $p < 0.001$), and between treatments ($F_{2,60} = 4.67$, $p = 0.013$) (Figure 8.4 f).

Output of dissolved organic carbon increased significantly ($F_{7,84} = 6.65$, $p < 0.001$) with time from all three sets of cores, but there were no significant differences between treatments. There was no significant interaction between time and treatment. Phenolic output showed a very similar trend, with a significant increase over time ($F_{7,92} = 7.18$, $p < 0.001$) but no significant differences between treatments and controls, or interaction between time and treatment (Figure 8.6 a-c).

The nutrient ions ammonium, nitrate and phosphate did not show noticeable differences in outflow concentrations between treatments and controls after addition of enzymes (Figure 8.6 d-f). However sulphate concentration in outflowing water from cellulase supplemented cores dramatically increased after

addition of the enzyme; cores supplemented with β -glucosidase showed no increase in sulphate output.

Figure 8.4

Nitrous oxide (a), methane (b) and carbon dioxide (c) emission from control (■), cellulase-amended (□) and β -glucosidase-amended (●) soil cores from the Pelenna wetland. (mean, $n = 5$, \pm s.e.).

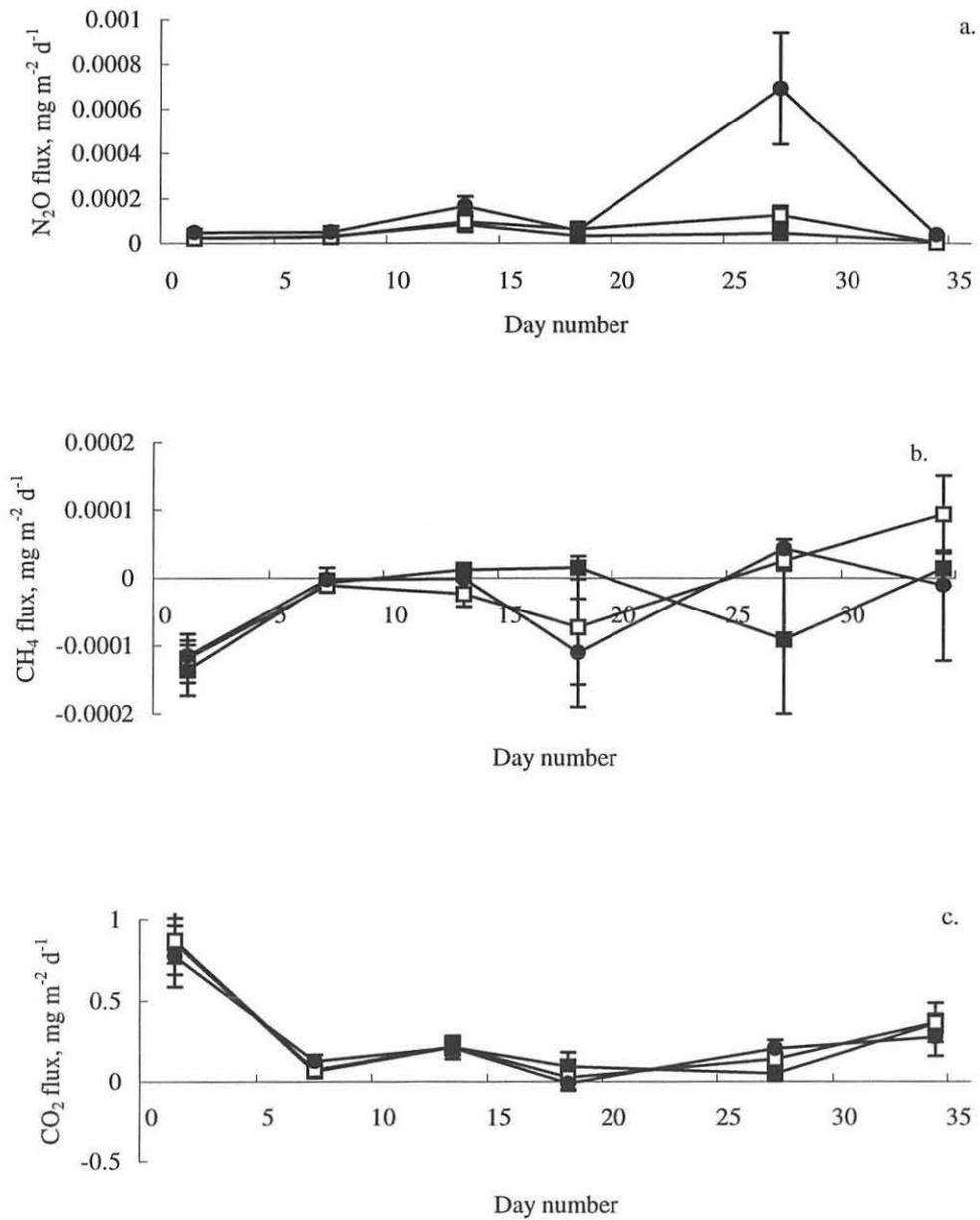


Figure 8.4 contd.

Nitrous oxide (d), methane (e) and carbon dioxide (f) emission from control (■), cellulase-amended (□) and β -glucosidase-amended (●) soil cores from the Centre for Alternative Technology wetland. (mean, $n = 5$, \pm s.e.).

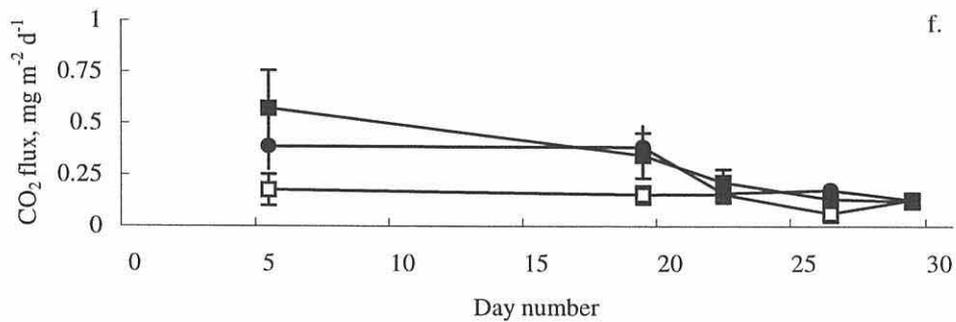
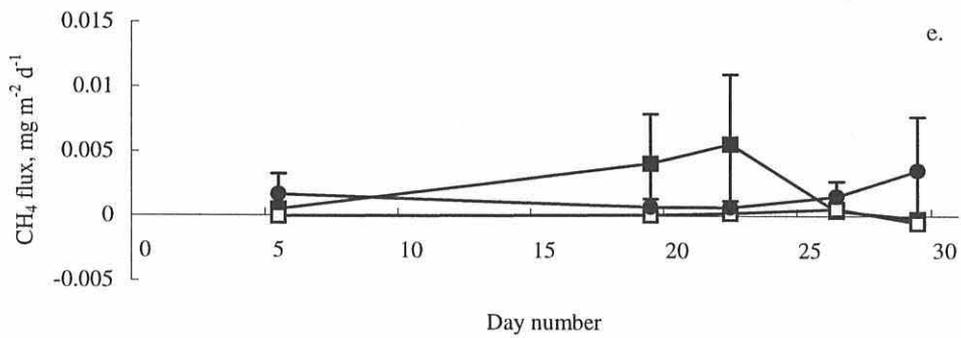
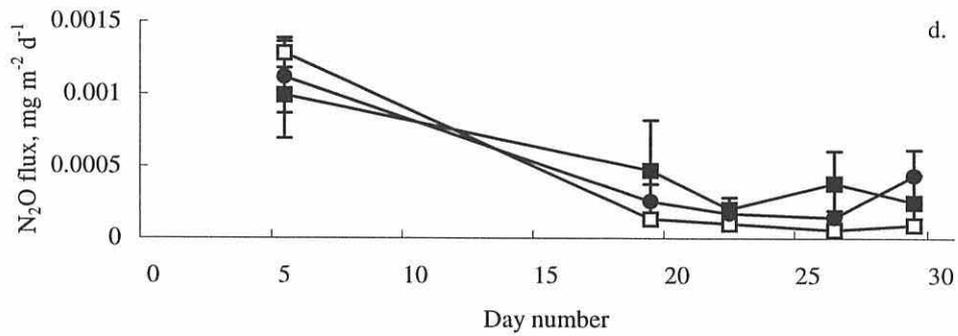


Figure 8.5

Dissolved organic carbon (a), phenolics (b) and iron (c) in outflow of control (■), cellulase-amended (□) and β -glucosidase-amended (●) soil cores from the Pelenna wetland. (mean, $n = 5$, \pm s.e.).

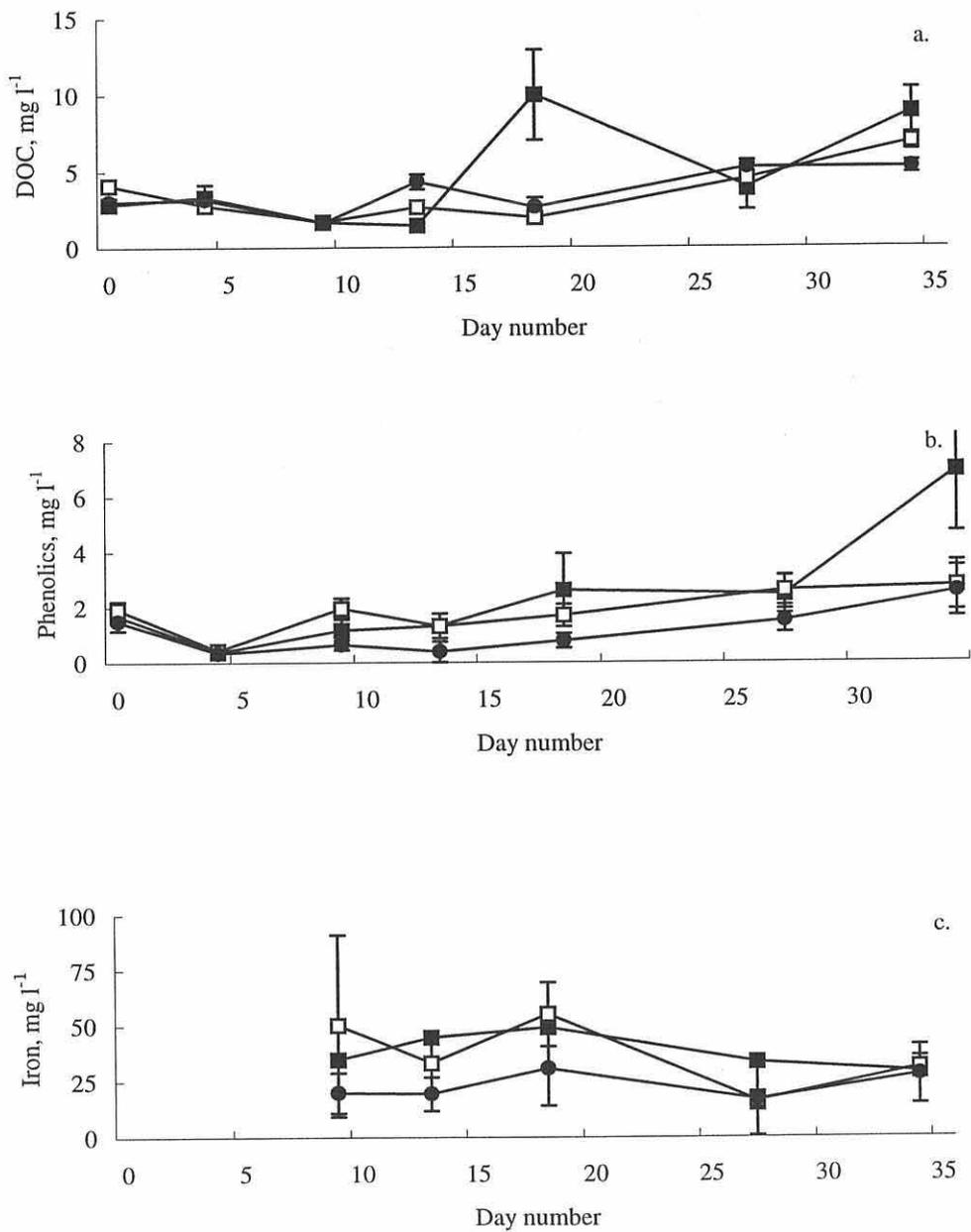


Figure 8.6

Dissolved organic carbon (a), phenolics (b) and nitrate (c) in outflow from control (■), cellulase-amended (□) and β -glucosidase-amended (●) soil cores from the Centre for Alternative Technology wetland, and inflow (○) to cores. (mean, $n = 5$, \pm s.e.).

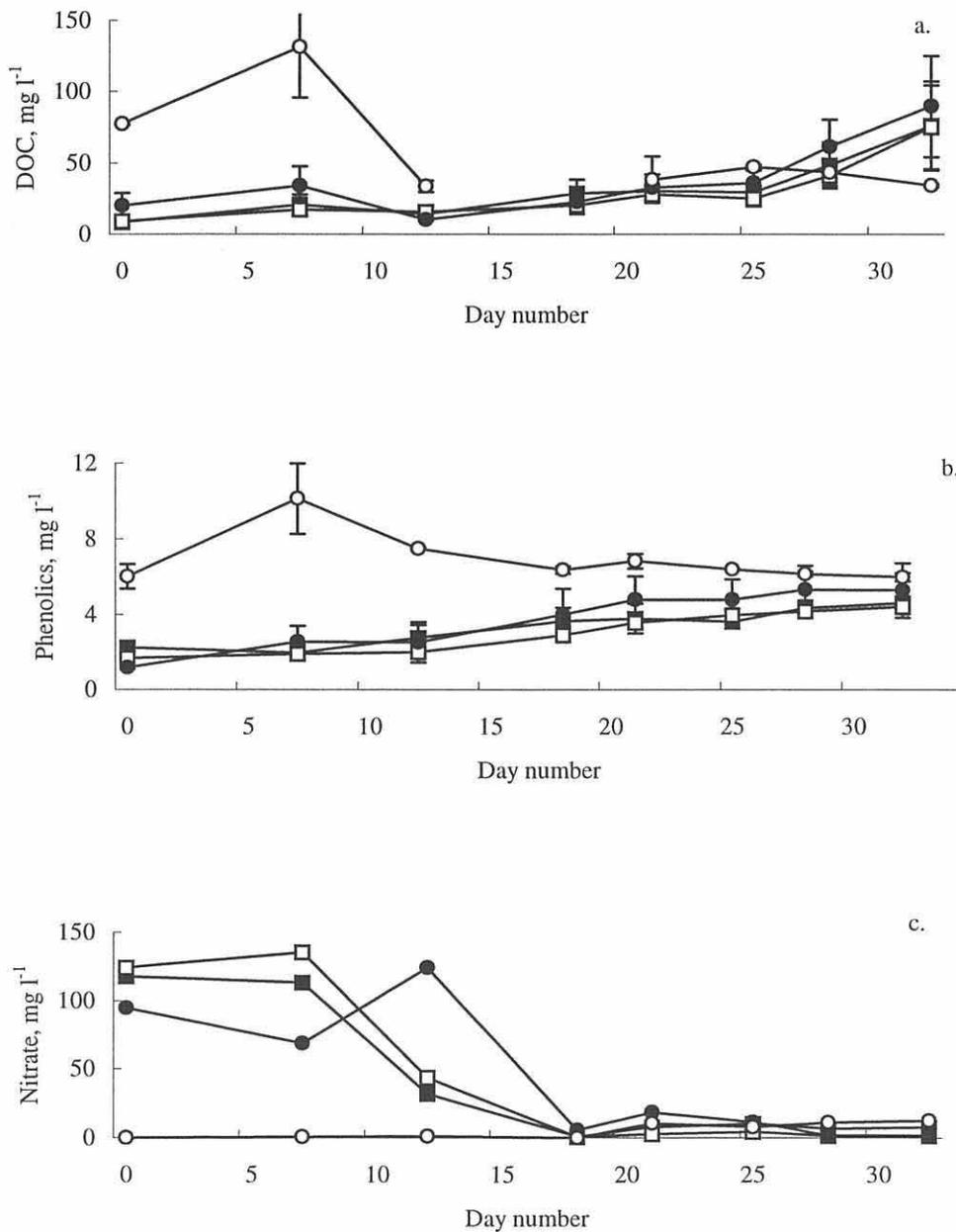
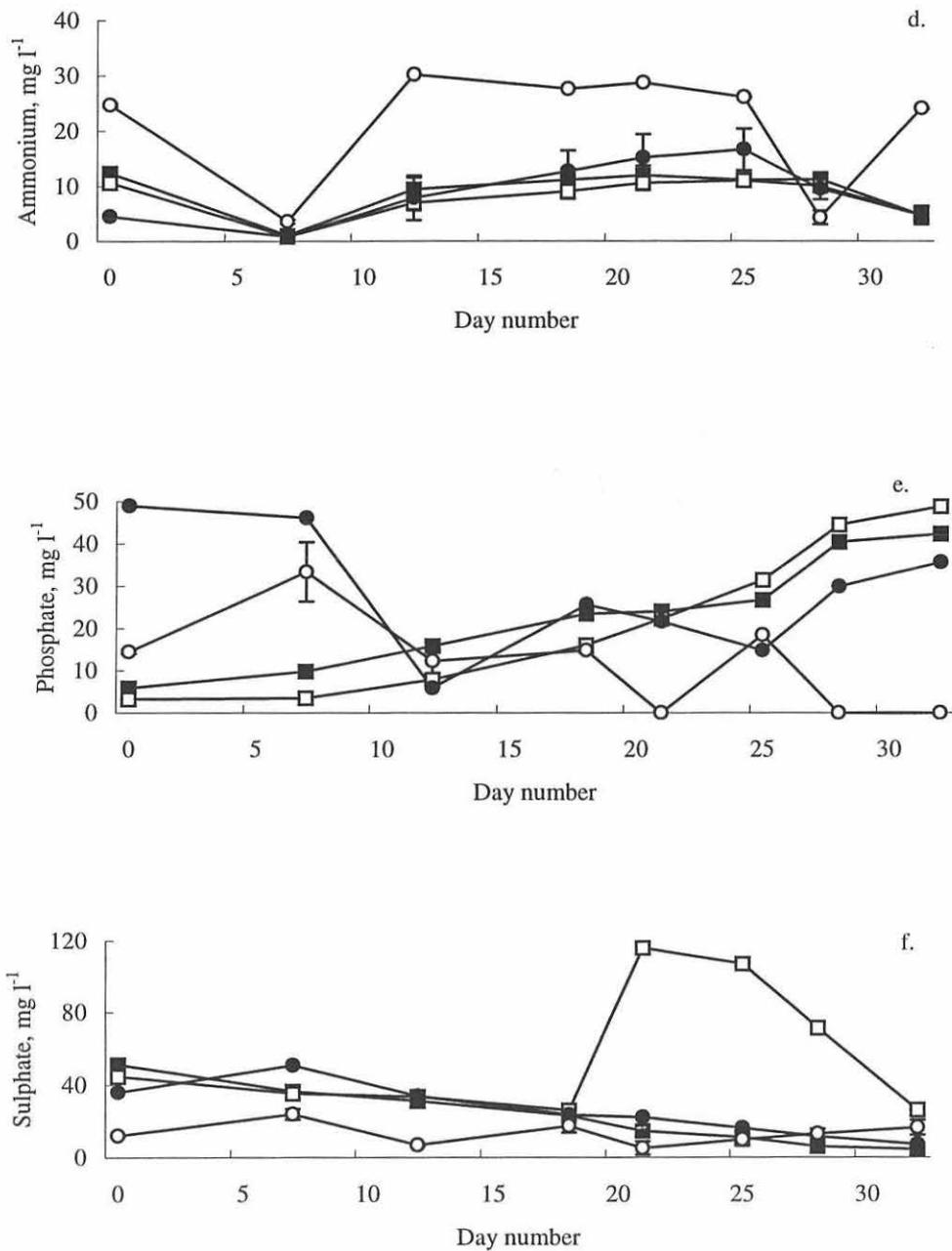


Figure 8.6 contd.

Ammonia (d), phosphate (e) and sulphate (f) in outflow from control (■), cellulase-amended (□) and β -glucosidase-amended (●) soil cores from the Centre for Alternative Technology wetland, and inflow (○) to cores. (mean, $n = 5$, \pm s.e.).



c. Discussion

The longevity of enzymes in soils depends on

- their resistance to degradation by micro-organisms and proteases; enzymes are prime sources of protein in soils but require hydrolysis before they can be taken up as amino acids or nitrogen compounds
- how quickly and how effectively they become immobilized on soil colloids and with humic acids; this depends on the availability of soil colloids and humic acids, and on the amount of enzyme already immobilized
- the stability of the organic matter on which the enzymes are immobilized.

In treatment wetlands an additional cause of reduced enzyme activity is the continuous flow of wastewater through the soil. Once enzymes are added to soil they will be vulnerable to degradation by protease enzymes and may become inactive, unless they quickly become immobilized on some part of the soil matrix; immobilization can alter the kinetic properties of enzymes, both advantageously and deleteriously. Urease immobilized on a calcium polygalacturonate matrix (Ciurli *et al* 1996) showed a slight change in V_{max} , but a considerable increase in K_m , compared to free urease. Therefore although immobilization did not greatly change the rate of the reaction, it did decrease the substrate affinity of urease in this case, as lower K_m values equate to higher substrate affinities (Nannipieri *et al* 1978). This suggests that immobilization did not cause conformational changes to the protein, but did produce substrate partitioning (Ciurli *et al* 1996). Changes in the activity of an enzyme on binding may depend on the proximity of the binding and active sites (Boyd & Mortland 1985). The optimum pH was not changed, but did become broader, and activity was increased at low pH in the immobilized form; both of these effects could be advantageous for water treatment.

Zantua & Bremner (1977) reported greater stability of naturally-occurring soil urease than of urease added to soils. Sterilized soil rapidly lost urease activity which was derived from supplemented enzymes, and Zantua & Bremner speculate

that different soils have different stable levels of urease, determined by their ability to protect the enzyme. This may be applicable to other enzymes, although it would be unwise to generalize without specific evidence.

In both of the CAT, and in the Pelenna β -glucosidase core experiments, adding extracellular enzymes to the soil resulted in a significant increase in enzyme activity which was sustained beyond the initial supplementation.

In these experiments, with a continuous flow of effluent, the activity of added enzymes declined much more rapidly than in the batch experiments. This indicates that the enzymes were secured in some way to or in the soil, but incompletely. Measurements of enzyme activity in outflowing water would have been interesting. Dissolved organic carbon measurements of CAT core outflows did show an increase over the course of the experiment, but it is impossible to attribute this to either enzymes themselves, or their products, being present in the outflow without fractionation or enzyme assay. Pelenna cores did not show the same increase in DOC output. The rapid decline in enzyme activity ties in with the hypothesis of Sarkar & Burns (1984) that adsorption and entrapment (the mechanisms probably at work in these experiments) are comparatively insignificant in stabilizing soil enzymes. If enzymes are to be added to wetlands, then it may have to be in a previously immobilized form to achieve extended benefit. The maintenance of high levels of activity after enzyme addition in the lab experiments is probably due to the retention of all water and soil components within the experimental units; the only loss of enzyme activity was presumably through protease activity or through incorporation into humic material. Contrastingly in the continuous flow experiments enzyme activity may additionally have been lost as humic material left the cores in the outflow. In experiments which involved adding (among others) almond-derived β -glucosidase to soil, Sarkar and Burns (1984) found that soluble enzyme lost 38 % of its activity after 3 days, and 80 % after 21 days; however immobilized enzyme

showed no loss in 9 days, and only 25-44 % loss in 21 days. The loss of activity in both cases may be due to adsorption or chemical denaturation or proteolysis (Sarkar & Burns 1984).

Cellulase activity in Pelenna cores did not show any significant increase compared to control cores after the addition of enzymes. There are two possible explanations for this; firstly, the enzyme may have been unable to bind with soil components to remain in the soil, and were immediately lost from it. However the lack of increase in outflow DOC does not agree with this. Secondly, the enzyme may have been successfully bound and immobilized within the soil, but not in such a way as to retain its activity. Why this would occur in one soil, yet the enzyme appears able to bind and remain functional in the CAT soil, is unclear, but the type of organic matter available may have a strong influence. The CAT wetland probably has more plant-derived organic matter than the Pelenna wetland, due to its greater age; since plant-derived matter forms the basis of humic material, it follows that there may be a greater humic reservoir at the CAT for enzyme binding.

The slower loss of β -glucosidase activity from Pelenna wetland soil, compared to that in CAT wetland soil, agrees with Zantua & Bremner's (1977) hypothesis that soils low in organic matter show a faster loss of activity. The Pelenna soil is high in organic carbon (47 % by weight) compared to the CAT soil (5 % by weight), and may therefore have a greater potential for immobilizing supplemented enzymes.

If the enzymes added to wetland soil are retained (for however short a period) in an active form (which they appear to have been in the case of cellulase at CAT and of β -glucosidase at CAT and Pelenna), then the products of their activity might be expected to be detectable. The release of glucose and other low molecular weight molecules by β -glucosidase should stimulate the soil microbial

population and result in an increase in carbon dioxide emission. No such increase was noticeable in either the CAT or Pelenna experiments. Whilst there were not significant differences in flux between treatments, the trend of declining carbon dioxide emissions and rising methane emissions can be seen in soil cores from both wetlands; it seems possible that the soil has become more anaerobic (through increased saturation) during the course of the experiment.

Glucose availability could also potentially increase nitrous oxide production by providing energy for denitrification if nitrate was available. However there were no significant differences in nitrous oxide emission between cores treated with enzymes and controls, suggesting that the denitrifying microorganisms were not stimulated by the increased amounts of carbon available. Carbon dioxide emissions do not show convincing evidence of metabolic stimulation; all of the cores appear to have maintained similar levels of carbon dioxide production after enzyme addition.

Nutrient ion concentrations in the CAT experiment outflow showed some changes after enzyme addition. With the exception of sulphate, cellulase-treated cores followed the same pattern as control cores. Sulphate showed a large increase approximately ten days after the cellulase addition, possibly a delayed reaction to increased cellulase activity, or an artefact of heterogeneous inflow.

8.4 General discussion

Adding enzymes to soil may only be effective, that is produce a lasting increase in activity, if the soil has sites available for protection of enzymes. Zantua & Bremner (1977) found that soils with lower organic matter contents showed a faster decline in activity of added enzymes, and this seems likely to agree with the general theory that enzyme immobilization depends on humic matter adsorption (Burns 1983).

Approximately 33 % of the dissolved organic carbon in landfill leachate is material with a high molecular weight (> 1000 Da), which is broken down slowly or hardly at all (Harmsen 1983). Since this DOC represents a potential water quality problem, wetlands constructed for landfill leachate treatment may benefit from additions of enzymes which specifically attack humic and fulvic acids, or from conditions which favour the activity of such enzymes.

If soils do not contain sufficient, or suitable, sites for adsorption, supplemented enzymes are rapidly degraded by soil proteases. Newly formed soils, and artificial soils, may therefore take time to build up a pool of humic material, so that at first extracellular proteins excreted by micro-organisms immediately become substrates for proteases. Later, any accumulated pool of humic material is increasingly able to immobilize, and so retain the activity of, extracellular enzymes. In addition, if humic matter turnover rates change, then soil extracellular enzyme pools could change similarly; phenol oxidase may be implicated in this as it is involved in humus formation.

With a continuous flow of effluent, the activity of added enzymes declined much more rapidly than in the laboratory experiments, indicating that the enzymes were secured in some way to or in the soil, but incompletely. Removal of enzymes by physical (soil washed out of the cores) or chemical (leaching of enzymes after immobilization) processes are possible explanations for this, and suggest that constructed wetlands operating on a through-flow basis are less suitable for enzyme supplementation than those operating on low-flow or soak-away routines. By placing enzymes in a wetland in an immobilized form which cannot physically be removed by water flow, e.g. on membranes buried in the soil, it may be possible to retain their activity for much greater periods of time whilst obtaining the benefits of increased organic matter turnover to wastewater improvement.

However there are still potential applications for enzyme supplementation of soil,

particularly in the treatment of land contaminated with toxic organic chemicals (Bollag 1992, Dick & Tabatabai 1993). A single enzyme application may potentially retain its activity for long enough to break down the contaminant to substances more accessible to microfauna which can survive the contamination.

Conclusions

- Adding the enzymes cellobiohydrolase and β -glucosidase (from commercial sources) to fresh wetland soil produced significant increases in activity. Autoclaved soil from the same wetland showed a small increase in activity which declined quickly.
- Increased activity lasted for over six weeks in batch experiments, but only a few weeks in continuous flow experiments.
- Soil with a low organic matter content may lead to a faster decline in enzyme activity than soil with more organic matter.
- Adding enzymes to wetland soil did not appear to stimulate microbial respiration or denitrification.

Chapter IX

Simulating enzyme activity

Simulating enzyme activity and gas emissions

9.1 Introduction

Temperature is one of the most important factors affecting many decomposition processes (Linkins *et al* 1984) because it influences both extracellular and intracellular rates of organic matter breakdown. The link between temperature and extracellular enzyme activity (involved in nutrient cycling) was seen in Chapter III, although over half of the enzyme activity data did not correlate with soil temperature. In cases where such correlations were not significant, it appeared that other edaphic or environmental factors may be more strongly influencing the soil enzyme activity.

However, temperature must inevitably have some influence, and by simulating enzyme activity according to soil temperature it should be possible to establish the potential response of an enzyme to changes in temperature throughout the year. By subsequently comparing the simulated enzyme activity to the actual activity recorded from the field study, patterns of good and poor fit between the data sets can be identified. Once the periods when temperature does not accurately predict enzyme activity are identified, investigations could be focussed on these times to establish which other factors are the most important in enzyme regulation.

If such regulatory factors can be found for these periods of poor fit with the temperature simulation, it may be desirable to modify enzyme activity only at certain times of year to obtain better treatment performance from a constructed wetland. The type and method of enzyme manipulation would depend on the factor or factors which were found to regulate enzyme activity.

Simulation of enzyme activity may provide a useful tool for predicting the potential rate of decomposition of specific substrates. Soil factors such as pH (Spier & Ross 1978), available carbon (Falih & Wainwright 1996) redox potential (Pulford & Tabatabai 1988) and electron acceptors (McLatchey & Reddy 1998),

as well as temperature, are known to influence soil enzyme activity; if more detailed models incorporating these factors could eventually be constructed for key enzymes, the manipulation of soil extracellular enzyme activity in constructed wetlands would not need to be by trial and error.

To achieve the simulation of enzyme activity and gas flux, mathematical relationships were derived from the temperature responses of enzymes and soil gas fluxes (from Chapter V a), and were used to simulate field enzyme activity and gas fluxes. Only relationships which were statistically significant were used to derive equations. The results were compared to the actual field data using descriptive statistics. The aim of this work was to investigate the proportion of the year in which temperature was the over-riding regulator of enzyme activity, and therefore possibly also of nutrient cycling, and to identify periods when other factors were more influential than temperature.

The soil used in the temperature bar experiments from which the mathematical relationships were derived was collected from the Centre for Alternative Technology (CAT) wetland, which treats domestic sewage, and from the Pelenna wetland, which treats mine drainage water.

9.2 Methods

Correlations between soil temperature and enzyme activity or gas flux, determined from the experiments described in Chapter V a, are given in Table 9.1.

For each enzyme temperature response which showed a significant relationship, (β -glucosidase, N-acetylglucosaminidase and phosphatase, at the CAT and Pelenna wetlands), the spreadsheet package MS Excel was used to find the equation of the (linear or polynomial) best fit line for the data. This gave equations of the form:

ACTIVITY = FUNCTION (TEMPERATURE)

Table 9.1

Pearson correlations determined in the laboratory between soil temperature and enzyme activity or gas flux in soil from the CAT and Pelenna wetlands

	Enzyme	r	p
CAT	β -glucosidase	0.947	0.053*
	NAG	0.960	0.040**
	Phosphatase	0.951	0.049**
Pelenna	β -glucosidase	0.920	0.080*
	NAG	0.919	0.081*
	Phosphatase	0.940	0.060*
CAT	N ₂ O	0.849	0.151
	CH ₄	-0.226	0.774
	CO ₂	0.993	0.067*
Pelenna	N ₂ O	0.273	0.727
	CH ₄	0.052	0.948
	CO ₂	0.822	0.178

* indicates significant at $p < 0.1$, ** at $p < 0.05$

Some relationships which were significant only at the 10 % level were used to produce equations for enzyme simulations because they supported the central idea that temperature is a significant regulatory factor on soil enzyme activity and gas fluxes.

The field temperature for each month during 1997-1998 for CAT, and during 1997 for Pelenna (data from Chapter III) was then used in each of these equations to simulate the activity of each enzyme throughout the year. The statistical parameters mean difference, standard error (of the mean difference) and mean absolute deviation of the simulated activities were then compared to those of the actual activities. Whereas *mean difference* (MD) gives the average distance of the simulated values from the actual values, the *mean absolute deviation* (MAD)

gives the average distance regardless of direction (positive or negative). By comparing the two values it is possible to evaluate the accuracy with which the enzyme activity has been simulated; a perfect simulation would result in identical MD and MAD.

The same technique was applied to data regarding the variation of gas emissions with temperature, and the results compared to field data from Chapter III.

Although the laboratory results gave only one significant relationship between gas flux and temperature (carbon dioxide in soil from the CAT wetland), the field work (Chapter III) showed significant relationships with temperature for nitrous oxide ($r = 0.651$, $p = 0.001$) and carbon dioxide ($r = 0.844$, $p < 0.001$) at the CAT wetland, and between carbon dioxide and temperature ($r = 0.542$, $p = 0.069$) at the Pelenna wetland.

9.3 Results

Enzymes

Data from the CAT soil produced the following equations relating soil temperature to enzyme activity (where A = activity and T = soil temperature):

β -glucosidase

Linear: $A = -0.24 + (T \times 0.0784)$ $r = 0.947$

Polynomial: $A = 0.495 - (T \times 0.0682) + (0.00586 \times T^2)$ $r = 0.998$

N-acetylglucosaminidase

Linear: $A = -0.128 + (T \times 0.067)$ $r = 0.960$

Polynomial: $A = 0.0054 - (T \times 0.041) + (0.0011 \times T^2)$ $r = 0.963$

Phosphatase

Linear: $A = 0.312 + (T \times 0.072)$ $r = 0.965$

Polynomial: $A = 0.94 - (0.053 \times T) + (0.005 \times T^2)$ $r = 0.996$

Data from the Pelenna soil produced the following equations relating soil temperature to enzyme activity (where A = activity and T = soil temperature):

β -glucosidase

Linear: $A = 0.195 + (T \times 0.034)$ $r = 0.920$

Polynomial: $A = 0.206 + (0.032 \times T) + (8 \times 10^{-5} \times T^2)$ $r = 0.920$

N-acetylglucosaminidase

Linear: $A = 0.036 + (T \times 0.034)$ $r = 0.919$

Polynomial: $A = 0.444 - (0.048 \times T) + (0.0033 \times T^2)$ $r = 0.999$

Phosphatase

Linear: $A = 0.72 + (T \times 0.033)$ $r = 0.940$

Polynomial: $A = 0.9699 - (0.017 \times T) + (0.002 \times T^2)$ $r = 0.974$

Gases

Methane flux did not show a sufficient response to temperature in the laboratory experiments to obtain a mathematical relationship. Data from the CAT soil produced the following equations relating soil temperature to gas flux (where G = gas flux and T = soil temperature):

Nitrous oxide

Linear: $G = -7 \times 10^{-5} \times (T \times 10^{-5})$ $r = 0.849$

Polynomial: $G = 7 \times 10^{-5} - (2 \times 10^{-5} \times T) + (1 \times 10^{-6} \times T^2)$ $r = 0.959$

Carbon dioxide

Linear: $G = -0.18 (T \times 0.0234)$ $r = 0.933$

Polynomial: $G = 0.069 - (0.026 \times T) + (0.002 \times T^2)$ $r = 0.996$

Data from the Pelenna soil produced the following equations relating soil temperature to gas flux (where G = gas flux and T = soil temperature):

Carbon dioxide

Linear: $G = -0.072 (T \times 0.009)$ $r = 0.822$

Polynomial: $G = 0.0852 - (0.023 \times T) + (0.0013 \times T^2)$ $r = 0.975$

The values given by these equations were compared with the actual values from the field study, and the mean difference, standard error of the mean difference and mean absolute deviation are given in Table 9.2. The data are plotted graphically in Figures 9.1 – 9.3; where two equations were derived from the temperature relationships, the one with the least difference between mean difference and mean absolute deviation is used to plot the differences. Actual and simulated values of gas fluxes were plotted on separate axes for comparison, as the simulated values were underestimates. For enzymes in soil from the Centre for Alternative Technology wetland, the equations used were therefore: β -glucosidase, linear; N-acetylglucosaminidase, polynomial; phosphatase, polynomial. For gases the equations were both linear. For enzymes in soil from the Peledda wetland the equations used were all linear, and for carbon dioxide polynomial.

The differences between actual and simulated values of enzyme activity and gas fluxes were also plotted against month for each of the simulations (Figure 9.4). Negative values of the difference result from an underestimation, by the mathematical equation, of enzyme activity or gas flux. Conversely, positive values of the difference are overestimates.

Table 9.2

Mean difference (MD), standard error of the mean difference (SE), and mean absolute deviation (MAD), between actual and simulated values of enzyme activity and gas data.

	Equation type	CAT			Pelenna		
		MD	SE	MAD	MD	SE	MAD
β-glu	Linear	-0.097	0.063	0.235	-0.0057	0.087	0.234
	Polynomial	-0.176	0.057	0.26	-0.0058	0.087	0.233
NAG	Linear	0.078	0.061	0.25	0.0726	0.053	0.14
	Polynomial	0.072	0.059	0.24	0.0318	0.048	0.12
Pho	Linear	-0.19	0.138	0.43	0.092	0.15	0.411
	Polynomial	-0.255	0.128	0.433	0.066	0.147	0.398
N₂O	Linear	-0.0028	0.0018	0.0028			
	Polynomial	-0.0029	0.0018	0.0029			
CO₂	Linear	-1.185	0.912	1.262	-0.845	0.281	0.867
	Polynomial	-1.081	0.833	1.163	-0.813	0.271	0.842

Figure 9.1

β -glucosidase (a), N-acetylglucosaminidase (b) and phosphatase (c) activity simulated using laboratory-derived temperature coefficients (\diamond linear, \square polynomial) and compared to field data (\bullet) from the Centre for Alternative Technology wetland.

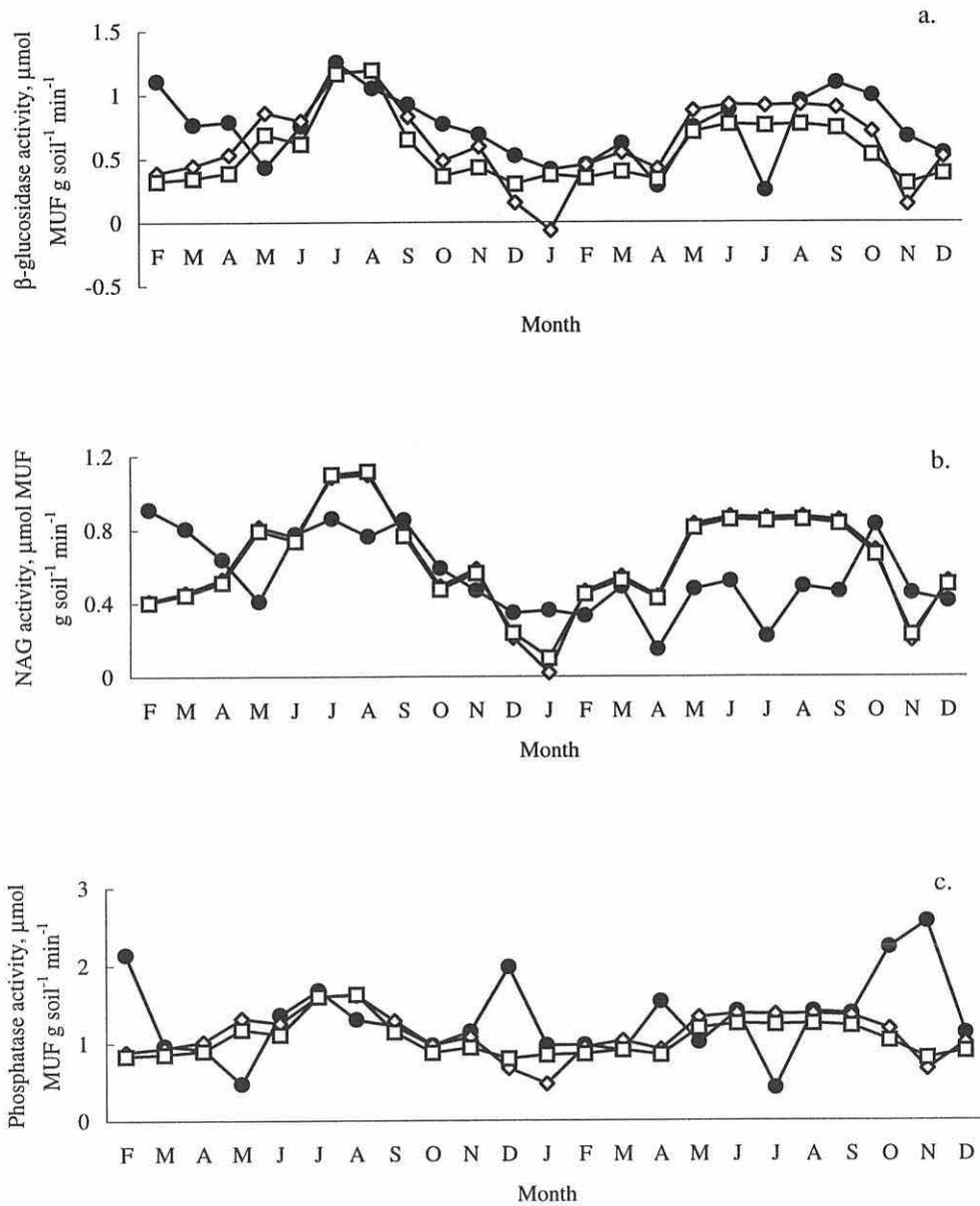


Figure 9.2

β -glucosidase (a) and N-acetylglucosaminidase (b) activity simulated using laboratory-derived temperature coefficients (\diamond linear, \square polynomial) and compared to field data (\bullet) from the Peledda wetland.

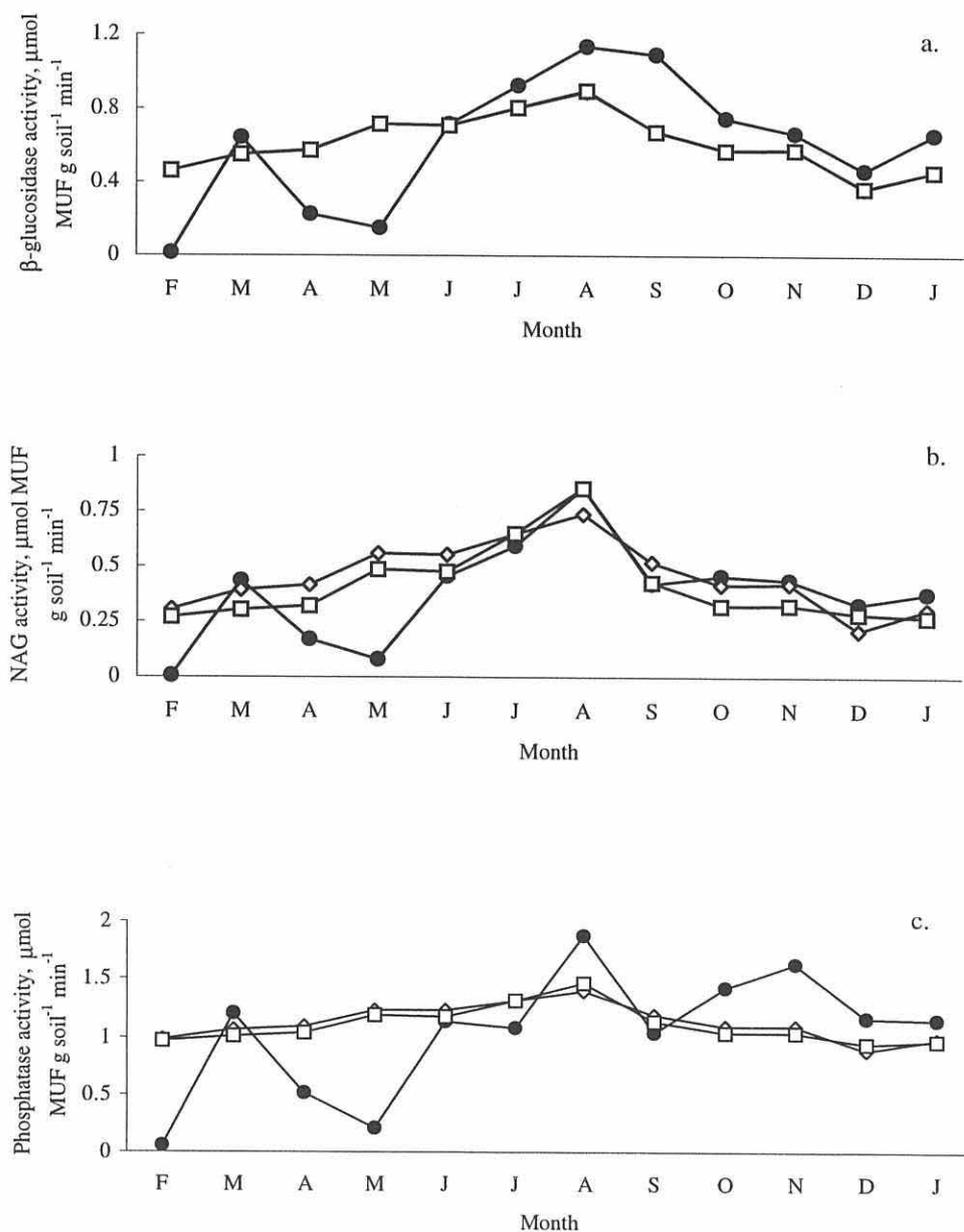


Figure 9.3

Nitrous oxide (a) and carbon dioxide (b) at Centre for Alternative Technology and carbon dioxide at Pelenna (c) simulated using laboratory-derived temperature coefficients (\square) and compared to field data (\bullet).

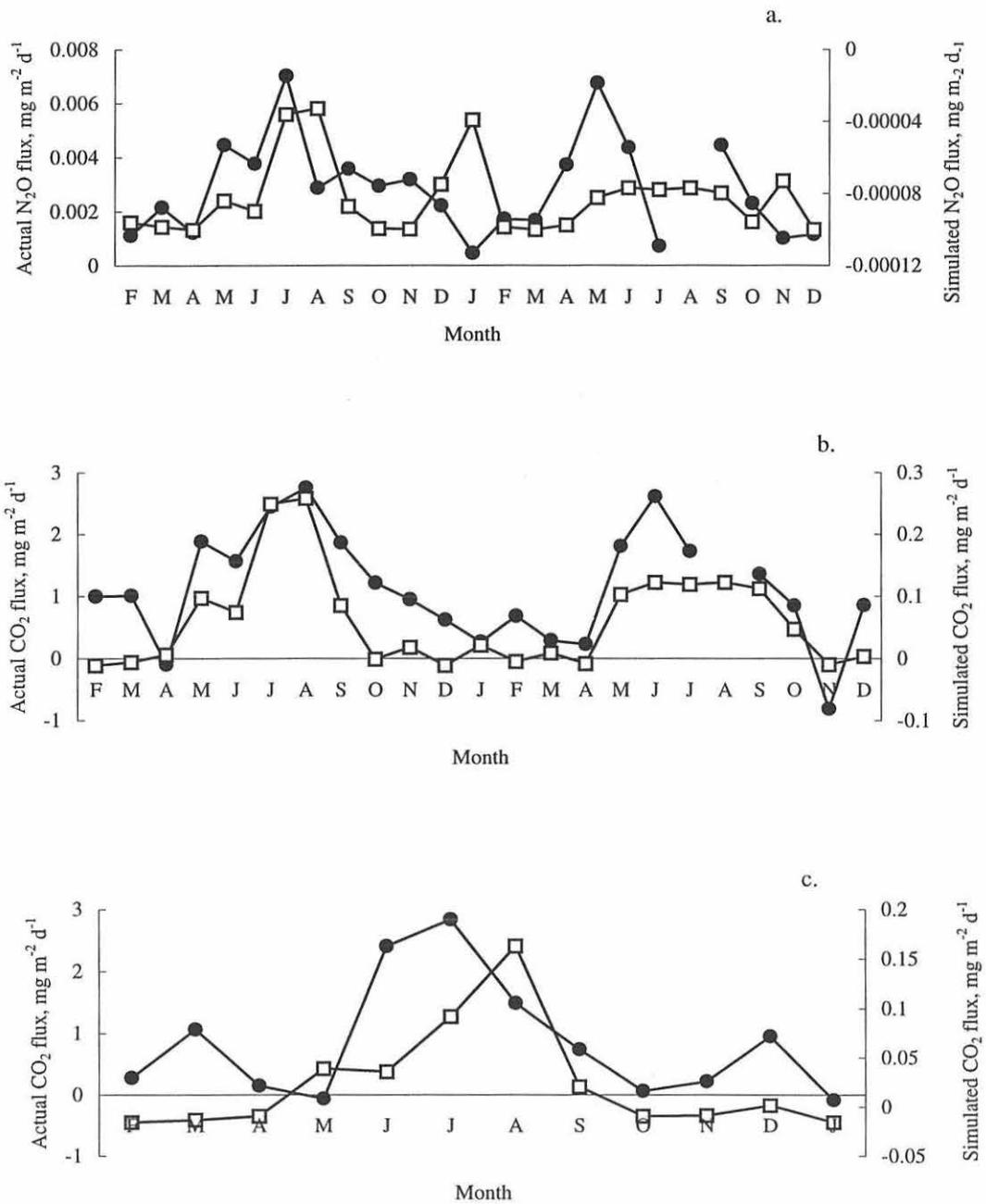


Figure 9.4

Differences between actual and simulated enzyme activity values by month for β -glucosidase (\blacklozenge), N-acetylglucosaminidase (\square) and phosphatase (\bullet) at the CAT (a) and Pelenna (b) wetlands.

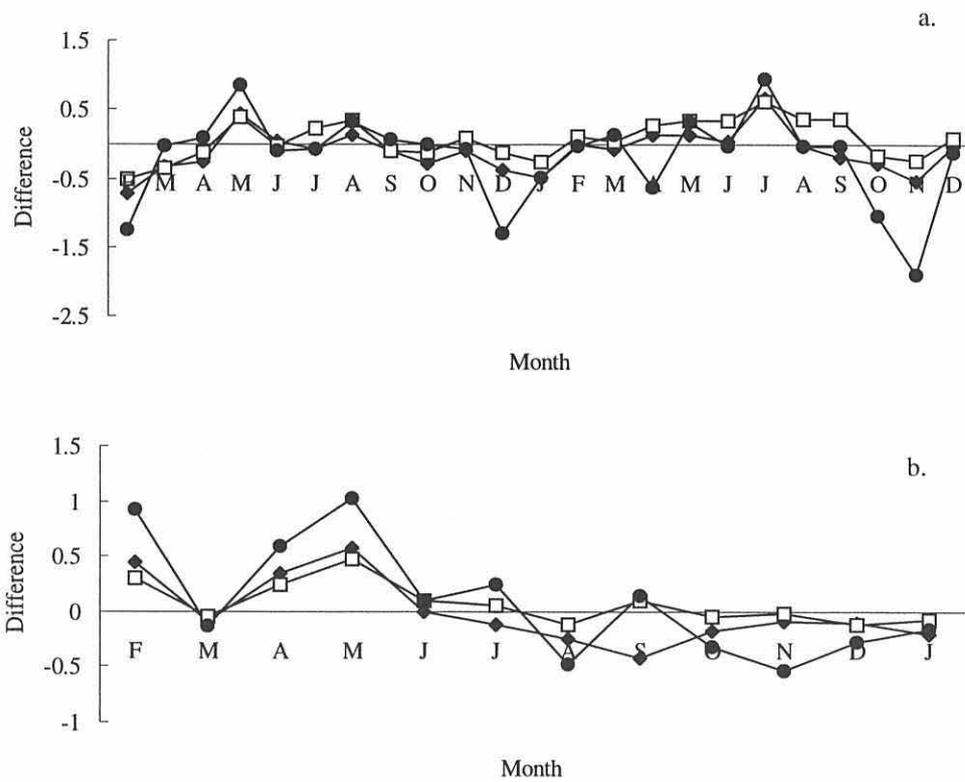
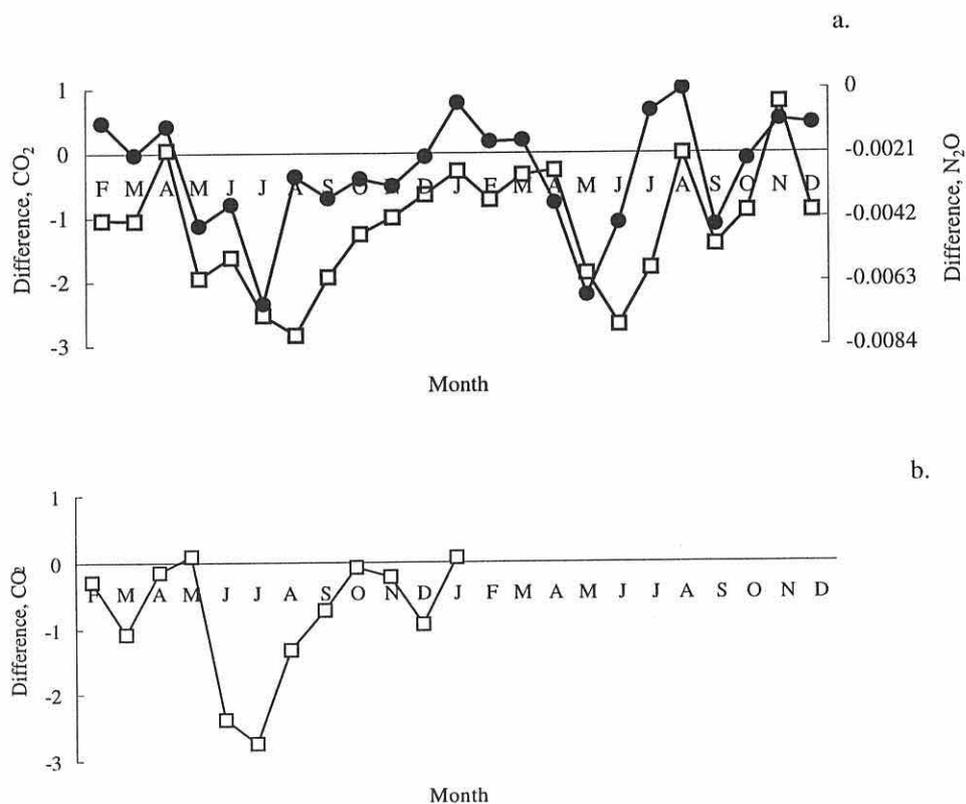


Figure 9.5

Differences between actual and simulated gas flux values by month for nitrous oxide (●) and carbon dioxide (□) at the CAT (a) and Peledda (b) wetlands. (Scale in (b) extended for comparison to (a)).



9.4 Discussion

Enzymes

The relationships between actual and simulated values for β -glucosidase, N-acetylglucosaminidase and phosphatase activity at the CAT and Pelenna wetlands were not significant, although in some cases the mean difference and mean absolute deviation were not greatly different. Plotting the difference between the two values over time (Figure 9.3) showed that the biggest deviations at the CAT wetland occurred in the middle of winter (November to February; large under-estimation) and the middle of summer (May, July; large over-estimation) each year. At the CAT wetland it was also clear that all three enzymes varied in the same way, with under- and over-estimations at the same points during the year. The correlations between these differences for each enzyme were significant (r values = 0.868, 0.785, 0.655, p all < 0.001).

At the Pelenna wetland the differences between simulated and actual enzyme activity values were smaller and much more uniform (Figure 9.2), apart from phosphatase which showed a large under-estimation during the summer. This difference is opposite to that during the summer at the CAT wetland. For all enzyme activities simulated at the Pelenna wetland, the differences between actual and simulated values were positive early in the year (1997) and declined significantly during the year, resulting in simulated values being much closer to the actual (field) values in the second half of the year than in the first half. The correlations between these differences for each enzyme were significant (r = 0.847, 0.82, 0.942, p all < 0.001). This would seem to indicate that a factor which is exerting a large influence from February to June weakens after the summer to allow temperature to become the dominant influence on enzyme activity. Where temperature alone is not an accurate predictor of enzyme activity, one or more other factors are substantially influencing activity.

Factors which might cause under-estimation of the enzyme activity would be those which resulted in an increase in the actual level of enzyme activity measured

in the wetland soil. Under-estimation at the CAT wetland occurred in the winter. For example, a rise in the microbial production of extracellular enzymes would increase soil enzyme activity and would not necessarily be due to increasing soil temperature. Similarly, an increase in the quantity of enzymes immobilized within the soil would raise the soil enzyme activity, but would probably depend on an increase in the supply of organic matter. Thirdly, a decline in the rate at which extracellular enzymes were broken down or inactivated would effectively increase soil enzyme activity. Induction of enzymes, due to the presence of excess substrate, might also be responsible. Changes in the soil enzyme pool due to greater immobilization (slower turnover of organic matter during the winter may supply more sites for immobilization) or slower removal of enzymes seem the most likely explanation for the underestimation of enzyme activity during the winter at the Centre for Alternative Technology wetland.

Over-estimation of enzyme activity in the simulation would be related to factors which caused a decline in enzyme activity. At the CAT wetland, over-estimation occurred in the summer. Increased rates of enzyme breakdown, or breakdown of the material on which the enzymes are immobilized, leading to their release and ultimate degradation, might cause such a decline. A decrease in microbial synthesis of extracellular enzymes would have the same effect, but seems less likely to occur during the summer. End-product inhibition may also decrease enzyme activity, and in turn might be due to a change in the uptake of the enzyme end-product(s). Plant uptake of nutrients may slow in summer when growth has finished, prior to senescence in the autumn.

There may be different relationships between temperature and soil enzyme activity in the field and in the temperature bar. Temperature relationships were determined using soil collected on only one sampling occasion, therefore it is possible that if the experiment had been repeated using soil collected at a different time of year, then different factors may have been more influential, leading to different mathematical relationships.

Like simulated enzyme activity, simulated gas fluxes appeared to vary with similar patterns, with different gases showing a poor fit at the same time of year (Figure 9.5). The differences between simulated and actual values of nitrous oxide and carbon dioxide flux at the CAT wetland were significantly correlated ($r = 0.624$, $p = 0.002$). At this wetland, under-estimation of gas fluxes occurred in summer, which was at the opposite time of year to under-estimations of enzyme activity. There were no considerable over-estimations of gas fluxes.

Under-estimation of nitrous oxide and carbon dioxide fluxes by the mathematical equation may be due to higher microbial activity at the times of year when the under-estimates occur. A greater supply of microbial substrates, rather than increased soil temperature, at these times could provoke the higher microbial activity.

Interestingly the differences between simulated and actual values of carbon dioxide flux at the Pelenna wetland also correlated (at the 10 % level) with those of carbon dioxide at the CAT wetland ($r = 0.556$, $p = 0.060$). Gas fluxes tended to be most under-estimated in June and July, gradually improving towards October/November. At the Pelenna wetland, as at the CAT wetland, under-estimations of gas fluxes occurred during the summer; there were no real over-estimates of gas fluxes.

End-product inhibition might be one reason why enzyme activity sometimes failed to fit the model well. The non-phenolic Dissolved Organic Carbon (npDOC) pool is likely to contain compounds which are inhibitory to β -glucosidase. Using npDOC in the linear equation simulating β -glucosidase activity improved the relationship by shifting the simulated values vertically down, so that they coincided better with the actual data, but this did not improve the mean difference between simulated and actual values of enzyme activity.

Deviations from the simulated values did not correlate significantly with chemical or physical factors also measured at the wetlands such as dissolved organic carbon, phenolic materials, soil pH or nutrient ions. Underestimation of carbon dioxide fluxes at the CAT and Pelenna wetlands occurred during the summer, and may therefore be due to increased photosynthesis at this time of year.

Conclusions

- Simulations of enzyme activity and gas flux can reveal the potential activity/flux which is due to temperature changes during the year, when field temperature data is available. Simulations can also therefore identify periods when temperature is not the primary regulator of enzyme activity.
- Deviations from the simulated values occur at the same time of year and in the same direction for different enzymes at one wetland.
- Over-estimation of enzyme activity occurred at the same time as under-estimations of gas fluxes at the Centre for Alternative Technology wetland.

Chapter X

Concluding discussion

Concluding discussion

10.1 Application of model substrates

In this thesis, fluorescent (methylumbelliferyl) model substrates were used to study the activity of six extracellular hydrolase enzymes, and a colorimetric model substrate (L-dihydroxyphenylalanine) was used to investigate extracellular phenol oxidase activity, in the soils of four constructed wetlands. Field surveys and laboratory experiments using these methods were combined to determine the kinetics of the extracellular enzymes acting on these substrates, to investigate the range of their activities within constructed wetlands and their potential for manipulation, and to assess the longevity of enzymes added to wetland soil.

The use of fluorescent substrates for this study was highly appropriate given the low levels of enzyme activity in constructed wetlands' soil, which require a sensitive method. In order to gauge the levels of activity under field conditions, rather than under the more usual optimal (with respect to temperature and pH) conditions of laboratory biochemical work, a relatively simple method which did not need buffer or long incubation was necessary; the fluorescent substrate method fulfilled this requirement. The relative enzyme activity of each sample could therefore be determined using the natural soil pH, and incubating at field temperature. Methylumbelliferyl substrates were found to provide a rapid and sensitive method for determining wetland enzyme activities, with good reproducibility. L-dihydroxyphenylalanine provided a rapid, although rather less sensitive, method for determining phenol oxidase activity; the generation of negative values sometimes resulted in large standard errors.

Kinetic analysis of extracellular enzymes in soil from each of four constructed wetlands (Chapter II) revealed differences in substrate affinity and maximum velocity, which probably resulted in part from different forms of enzyme binding in each of the four wetland soils. The highest activities were always found in soil from the Pelenna wetland, which also had the highest organic matter content,

suggesting that organic matter is of fundamental importance in retaining and immobilizing extracellular enzymes. Substrates in this wetland also showed the fastest turnover time, which is a result of the high substrate affinity and low maximal velocity of the relevant enzymes. A number of enzymes in different wetlands showed substrate inhibition at high substrate concentrations.

Laboratory experiments performed on soil collected from constructed wetlands were a valuable means of investigating relationships discovered during the field studies. Although laboratory studies of ecological processes have been criticised in the past as being too small or short-term (e.g. Verhoef 1996), in this thesis they were chosen as a first step to investigating constructed wetlands, and the methods used often built upon previous work which found laboratory experiments to be satisfactory (e.g. Nannipieri *et al.*, 1978, 1983, McLatchey & Reddy 1988). However, disruption of soil hydrology and structure is an inherent problem of moving soil into the laboratory, as processes such as nutrient release may be altered. The use of intact cores (*sensu* Freeman *et al.* 1993) would be preferable wherever possible, although even then it can be difficult to replicate field conditions such as the hydrologic regime. Comparisons between laboratory and field studies should be made cautiously.

10.2 Nutrient balances at the Centre for Alternative Technology wetland

Estimated mass balances for nitrogen and carbon were calculated based on the water chemistry data obtained for $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$ and DOC entering and leaving the wetland, and for $\text{N}_2\text{O-N}$, $\text{CH}_4\text{-C}$ and $\text{CO}_2\text{-C}$ emissions from the wetland, throughout 1998. No water chemistry analysis for organic forms of nitrogen, phosphorus and sulphur was performed on the inflow, soil water or outflow from the CAT wetland, so the mass balances cannot be completed, but serve as a rough guide.

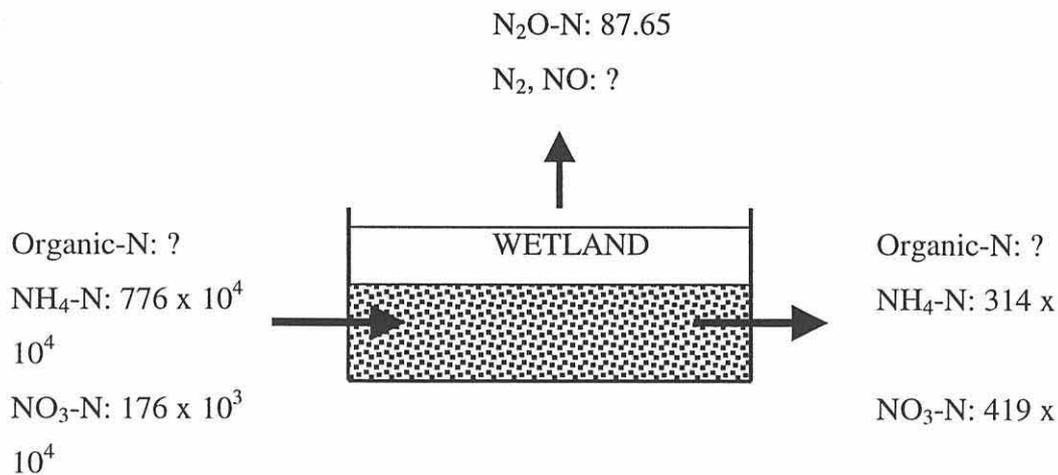
The calculations were based on a known inflow volume of $290,000 \text{ l yr}^{-1}$. As no hydrological data were available regarding outflow volume, estimates of plant

transpiration and precipitation were made. In the area of Wales surrounding the Centre for Alternative Technology, precipitation averages 1400 mm per year, which equates to an input of 224,000 l yr⁻¹ over the 160 m² area of the treatment wetland. Evaporative losses through plant transpiration were estimated using an average transpiration rate of 1-5 mmol m⁻² s⁻¹ (Grace 1997), which gives approximately 270,000 litres of evaporation per year over the area of the treatment wetland. Thus the water loss from the bed which is not included in the outflow may be around 46,000 l yr⁻¹. The data for nitrogen and carbon are set out in Figures 10.1 and 10.2 respectively.

Figure 10.1

Nitrogen balance in the CAT wetland throughout 1998. All figures are in mg yr⁻¹.

Total nitrogen in: 794 x 10⁴; total nitrogen out: 734 x 10⁴.



(If precipitation greater > evaporation, these figures may be higher)

Oxygen Demand and dissolved organic carbon across the wetland both suggest that organic matter is largely mineralized within the wetland. Concentrations of nitrate-N increase across the system, further suggesting mineralization. Inflow to the CAT treatment wetland is the liquid component of domestic sewage from the site, and has a high organic content; such liquid typically contains 8-35 mg l⁻¹ organic nitrogen (Kadlec & Knight 1996), and the ratio of BOD: N: P in sewage is of the order 100: 18: 2.7 (Borne 1979). Analysis of dissolved organic nitrogen can be made relatively simply using the Kjeldahl method; after removing naturally occurring ammonium, organic nitrogen is converted to ammonium for analysis. Organic phosphorus is similarly analysed using acid digestion followed by orthophosphate measurement; the inorganic phosphorus must be measured independently and organic phosphorus is then calculated by subtraction.

10.3 Conclusions

Biogeochemical cycles in constructed wetlands

The twelve month field study established the range of soil enzyme activities in constructed wetlands of different ages, and built for different purposes (Chapter III). The relative activities of enzymes indicate the rates of cycling of different nutrients (Sinsabaugh *et al* 1991). Despite the differences in age, design and substrate type, these showed surprisingly similar levels of activity (within one order of magnitude) to each other, although they were somewhat lower than activities measured in natural wetlands and sediments (Ueno *et al* 1991, Chappell & Goulder 1994a, Kang 1999). When the microbial population was inhibited, there was very little effect on extracellular enzyme activity (Chapter V a).

As far as is known, these data comprise the first temporal study of enzyme activity in the artificial soils of constructed wetlands. However it is by no means an exhaustive survey, and has raised many questions about the exact roles of different extracellular enzymes in wetlands constructed for different purposes; the range of enzymes investigated was necessarily narrow, and represented only a fraction of the total which exist in these soils. It is also probable that different

wastewaters need different balances of nutrient cycling (for example sewage may need more nitrogen-cycling activity than sulphur-cycling activity), and therefore further work which examined enzymes specifically relevant to a particular constructed wetland would be of value. Wetlands constructed for different treatment purposes will have different dominant nutrient cycles, and the roles of extracellular enzymes in such systems are therefore likely to vary.

Nitrate and phenolics had the biggest influence on soil enzyme activity in the field study (Chapter III), correlating significantly with cellobiohydrolase and β -glucosidase (CAT), N-acetylglucosaminidase and phosphatase (Waun-y-Cwrt), cellobiohydrolase and sulphatase (Waun-y-Cwrt and CAT) and sulphatase (Tollesbury) activities. It was difficult to draw conclusions about the relationship between particular enzymes and their products (for example between phosphatase and phosphate) from the data set, which in turn made conclusions about the relationships between enzyme activity and treatment efficiency elusive. Poor removal of nitrate was attributed to a high ammonia concentration in the wetland inflow at the CAT wetland. Analysis of total soil nutrient or soil element content, as well as soil water chemistry, may have helped to clarify these relationships.

Dissolved organic carbon present in the soil water was found to be a good indicator of enzyme activity from the field study (Chapter III), and in Chapter V the importance of carbon in determining enzyme activities was confirmed. Changes in β -glucosidase and sulphatase activities during this experiment supported correlations which were found in the field data. However different enzymes, in both field and laboratory studies, showed different responses to carbon concentration, highlighting the importance of a holistic approach to enzyme manipulation; whilst increased carbon may stimulate decomposition of organic pollutants or prevent sulphate release, in doing so it may compromise metal immobilization. In all constructed wetlands a supply of microbially available carbon is essential for electron donation, and this experiment

demonstrated its importance in non-carbon nutrient cycles. The nature of the carbon content of wastewater entering a wetland may therefore considerably influence the very nutrient cycles which are involved in its remediation.

The order of relative activities of some enzymes were found to be identical to those in other systems such as freshwater epilithon (Chappell & Goulder 1994a), which was attributed to the relative importance of their products to the microbial communities. This revealed an unexpected similarity in nutrient cycling between the artificial soils of constructed wetlands, and a range of natural soil and sediment systems.

Emissions of nitrous oxide, methane and carbon dioxide were used as indicators of nitrogen cycling and anaerobic and aerobic microbial metabolism respectively. Measurement of nitrous oxide flux at the soil surface has been reported to underestimate actual production in more saturated soil (McCarty *et al* 1999); however the fluxes measured in these field work studies and laboratory experiments were used as indicators, rather than quantitative measures, of nitrogen cycling. Emissions of nitrous oxide, methane and carbon dioxide were lower from the constructed wetlands studied than those reported from natural wetlands (Kang *et al* 1998b, Freeman *et al* 1993, Whiting & Chanton 1993, Williams & Crawford 1984) (Chapter III), suggesting altogether lower levels of microbial activity in these artificial soils. This was in agreement with the slower nutrient cycling suggested by lower levels of enzyme activity. Soil temperature was the strongest determinant of nitrous oxide and carbon dioxide fluxes, while methane did not show the seasonal pattern of flux which is usually seen in natural wetlands (Yavitt *et al* 1987, Press *et al* 1995). At the Pelenna wetland an interesting aerobic/anaerobic switching was evident, with methane emission inversely correlated with soil water nitrate concentration (nitrate inhibiting methanogenesis via competition for substrates), and with carbon dioxide emission. Depending on the depth to which this alternation penetrated, the efficiency of mine-drainage remediation may be impaired by inhibiting

(anaerobic) sulphate-reducing bacteria. The reliance of methanogens on organic substance availability (Bollag & Czlonkowski 1973, Crill *et al* 1991) was apparent at the Waun-y-Cwrt wetland, where dissolved organic carbon correlated significantly with methane release (Chapter III). However the pH of the wetland substrate did not seem to influence methanogenesis at any of the four sites.

Good correlations between nitrous oxide emission and nitrogenous substrate concentrations in soil water at two sites (CAT, and Pelenna in the latter half of 1997) (Chapter III), suggest that substrate availability exerts more control over nitrous oxide flux than soil temperature does. This conclusion with regard to substrate availability supported the work of Freeman *et al* (1997) on a flushed fen natural wetland. Good nutrient supplies from wastewater may preclude nitrous oxide flux being substrate-limited.

Effectiveness of water treatment

The Centre for Alternative Technology wetland showed good removal of ammonia, dissolved organic carbon, Biological Oxygen Demand and total *Coliforms*, but poor or zero removal of nitrate, phosphate and sulphate. Whilst the concentrations of these nutrients discharged from the wetland are unlikely to cause serious water quality problems, the poor removal shows room for improvement in the wetland's performance. Generally the percentage removal efficiency of the wetland was not predictable from the activity of related extracellular enzymes.

Factors regulating nutrient cycling

In the past there have been many reports of the importance of temperature in determining soil decomposition processes (Linkins *et al* 1984), which suggests that constructed wetlands function more efficiently in the warmer climates of tropical, rather than temperate, latitudes. Temperature was significantly correlated with the activities of several enzymes, and there was extensive intercorrelation in activity between enzymes at each site. This suggested that temperature operates an

important control on enzyme activity in these systems, and that at higher temperatures faster extracellular decomposition may, indeed, contribute to faster nutrient cycling. However correlations between enzyme activities and soil temperature seem to have been disrupted by the marked decline in activity which occurred in late spring at the CAT, Waun-y-Cwrt and Pelenna wetlands. This springtime decline in enzyme activities suggested that at particular times of year temperature was not the primary regulator of enzyme activities.

Laboratory experiments showed that pH markedly influenced the activities of all seven of the enzymes studied (Chapter V a). Fieldwork data from constructed wetlands (Chapter III and IV a) however did not indicate such relationships. The effects of redox potential and oxygenation on enzyme activities were not detectable in field studies, where interactions of effects may have masked them. Field study results using *in situ* redox probes partly agreed with the laboratory work, in which only some enzymes showed definite responses to changes in redox potential.

Phenolic concentration had a mixed influence on enzyme activity in the laboratory experiments (Chapter V a), yet in the fieldwork data several positive correlations (Chapter III) between soil water phenolics and enzyme activity were seen. These results contradict previous work (Freeman *et al* 1990) which found an inhibitory effect of phenolics.

Simulation of soil enzyme activity using field temperature data and equations derived from the laboratory temperature bar work (Chapter V a) gave good predictions of some actual soil enzyme activities measured in the field (Chapter IX). This enabled the influence of temperature on field enzyme activity and gas flux to be clarified throughout the year. Where temperature alone was not an accurate predictor other factors must have been more influential in determining enzyme activity.

Investigation of gas fluxes after microbial inhibition demonstrated the importance of a live microbial biomass for nitrogen removal (Chapter VII); wetlands treating mixed organic pollutants which may harm the microbial population may have to be carefully adapted to achieve reliable treatment, or risk releasing pollution to water courses. Although the extracellular enzyme pool may cope with much of the pollution without being harmed by it, if intracellular processes such as nitrogen mineralization are also important for treatment of a specific waste then two- or three- stage wetlands might be needed to separate the different treatment processes.

Supplementing wetland soil with enzymes

Adding the enzymes β -glucosidase and cellulase in unimmobilized forms successfully supplemented the natural enzyme activity of wetland soil; this increase was sustained over a period of months in fresh soil, but disappeared quickly in heat-sterilized soil. Supplementation using other enzymes was less successful, as different enzymes appeared to behave differently when added to soil in this way. These differences may reflect the different abilities of various enzymes to bind to soil constituents in a way which preserves their activity. The humic and colloidal reservoir of a constructed wetland substrate could markedly influence its ability to sustain the activity of added enzymes; as the wetlands age, organic matter will accumulate (particularly if they are planted) and provide immobilization capacity for enzymes. Adding humic or colloidal material may therefore be a better way of increasing extracellular enzyme activity, particularly in new treatment wetlands, than adding enzymes directly. This suggested that the soil used in the experiment had a good capacity for immobilizing enzymes in addition to those which microorganisms added to it. Other constructed wetlands may have different capacities to immobilize additional enzymes.

Adding extra enzymes to the wetland soil did not appear to stimulate microbial respiration (through increased availability of labile substrates) or denitrification. As soil which has a lower organic matter content showed a faster decline in the

activity of supplemented enzymes, it may be necessary to increase the organic matter content of the wetland substrate before adding enzymes. Ironically, wetlands with an inherently low organic matter content are quite likely to be those requiring supplementation; increasing the organic matter content of such wetlands by mulching in plant biomass etc, may lead to a natural increase in enzyme activity over a period of months or years, and eliminate the need for enzyme additions.

Research objectives

The specific aims of this research were to investigate:

- 1 What levels of enzyme activity are found in treatment wetlands, and how these compare with those of natural wetlands
- 2 How long extracellular enzymes survive in constructed wetlands
- 3 Whether extracellular enzyme activity can be modified or influenced to improve wastewater treatment

These aims have been fulfilled, and in the process more questions have arisen. (1)

The levels of extracellular enzyme activity found in constructed wetlands generally vary within one order of magnitude, and tend to be lower than those seen in a variety of natural wetlands; this may be a consequence of the shorter time over which activity has been able to build up in constructed wetlands.

Nutrient cycling indicated by gaseous emissions also tended to be lower than that found in natural wetlands. (2) Whilst some free extracellular enzymes appeared to persist for periods of months in the soil of constructed wetlands, others were rapidly inactivated or denatured, presumably by microbial activity. Studies of long-term changes in enzyme activity, over years or decades in constructed wetlands, may prove surprising. (3) The activity of extracellular enzymes was susceptible to manipulation by changes in soil temperature, pH, redox potential, oxygen availability and carbon supply; not all of these factors would be easy to manipulate at a field scale, but some offer definite possibilities for the improvement of water treatment by constructed wetlands.

Practicalities of manipulating enzyme activity in wetlands

Whilst the temperature of a constructed wetland is determined by latitude, climate and geographical location, the pH, redox potential and level of oxygenation could be relatively easily manipulated in order to adjust enzyme activities for optimal water treatment. It would be essential to first characterize the wastewater and determine the type of enzyme activity which will best accomplish the desired remediation. Some enzyme activities may need to be increased or decreased, either by changing soil conditions such as pH or water table, or by supplementing the soil with commercial enzymes (possibly immobilized on inert carrier materials). The effect on other nutrient cycles of such an alteration must be considered.

Constructed wetlands compared to natural soils

Overall, the biogeochemical processes in constructed wetlands are remarkably similar to those in some natural wetlands, and to those in wet soils. Microbial stimulation and inhibition also compare well (Chapter VII). Given that the levels of enzyme activity and gas flux at the studied wetlands are only slightly lower than those published for natural systems, the substrate of treatment wetlands must rapidly develop microbial populations with characteristics which resemble those of natural soils. The substrate of a treatment wetland must therefore become colonized by bacteria whose community evolves to a certain level or state which mimics, at least functionally, that found in natural soils. If it is thus clear that constructed wetlands become inoculated with a bacterial population (which doubtless evolves as the wetland ages), where do those bacteria come from? They may be carried on soil particles distributed by wind or precipitation, or perhaps survive intact through human intestines or in domestic refuse to colonize sewage treatment and landfill leachate wetlands. Mine drainage wetlands, in which conditions are presumably unfavourable for the majority of bacteria which will be carried to the surface in mine water from deep, anaerobic strata, may be inoculated with bacteria introduced when the wetlands are planted. If this is so, unplanted wetlands may have an entirely different community. The similarity

between artificial and natural soils is somewhat surprising given the differences in organic matter content.

Either artificial substrate communities eventually come to resemble those in natural soils, or soils/substrates simply promote a defined function in their communities. It may be that the exact nature (species types etc.) of the microbial community is unimportant, as the environment defines the function. If the microbial communities are similar, or function in similar ways, to those in natural soils, the time taken for an artificial substrate community to reach an ecological climax which functionally resembles natural soils could be determined. If extracellular enzyme activities accumulate gradually as cells turn over and new species arrive, then wastewater treatment may not be fully effective right from the start. Even if extracellular enzymes are present, there may not be adequate humic and colloidal material for enzyme immobilization at the outset; this may take time to accumulate, during which nutrient cycling is not optimal because extracellular enzymes and electron sources are scarce. For example sand-based wetland treatment beds rely on an accumulation of plant organic matter after a few growing seasons for organic carbon supply.

10.4 Scope for application

Constructed wetlands are now employed for diverse purposes, and manipulations of decomposition and nutrient cycling will not be universally applicable. Broadly, treatment wetlands may be divided into (1) those whose purpose is to decompose and break down organic pollutants into substances which either form enzymic or microbial substrates rather than recalcitrant materials, or are labile enough to be taken up directly by microbes, plants or soil animals, or which can be incorporated into the complex humic fraction of the soil; and (2) those whose purpose is to sequester and retain inorganic pollutants in order to minimize the impact of these on the environment.

For wetlands in category (1), it may initially appear that any changes which increase organic matter turnover and nutrient cycling will improve treatment efficiency. However, consideration of long-term effects would be necessary to avoid creating imbalance in a treatment wetland through favouring some processes which exhaust important resources such as plant-derived organic matter input. However fast soil nutrient cycling occurs, plant growth will still be limited by latitude, which determines climate (therefore sunlight hours) and the extent of seasons. Although some improvements in treatment efficiency via enzyme activity manipulation may well be possible, there must be a minimum wetland area/retention time which will be required for effective remediation of a mixed waste such as sewage, which has a very variable strength and composition. Wastewaters with steady concentrations of only one or two pollutants (for example some industrial process effluents) may be more effectively treated in bioreactors containing specific bacterial cultures.

Wetlands in category (2) operate by precipitating and adsorbing metals onto organic matter, and by plant uptake of metals (Wood & McAtamney 1996). Although such binding limits remobilization and/or bioavailability of heavy metals (Mungur *et al* 1997), the capacity of any wetland for such retention will be finite (Wieder *et al* 1988). Any change in soil conditions which enhances the decomposition of soil organic matter will almost certainly be deleterious to treatment efficiency. It is possible that the heavy metal content of the inflow to such a wetland helps to minimize enzyme activity (by inhibition).

Minewater treatment

Minewater discharges must be carefully assessed before the method of treatment is decided upon. In particular the pH and iron content are important factors which direct the choice of treatment. Inappropriate use of wetlands for the treatment of acidic minewaters may be ineffective, or even exacerbate the problems involved (DB Johnson, pers. comm.). For acidic discharges (pH < 3), treatment with wetlands containing plants may result in no net decrease in Fe^{2+} , because carbon

exudates from plant roots allow micro-organisms to reduce the Fe^{3+} produced directly back to Fe^{2+} . Carbon starvation is necessary for these autotrophic iron bacteria (King & Garey 1999), and so unplanted wetland stages may be appropriate for treatment of some mine water discharges.

Contrastingly, in treatment of organic wastes, wetlands can be extremely useful. In these cases there is no possibility of “reverse” treatment occurring as the enzymes involved in organic matter decomposition are catabolic. For wastewaters containing a mixture of organic and trace element pollution (for example some landfill leachates), wetland treatment may be inappropriate due to the conflicting requirements for high and low (respectively) extracellular enzyme activity; alternatively, sequential treatment with primary trace element removal in a “low activity” bed, followed by organic amelioration in a “high activity” bed may provide a solution.

Summary

The biogeochemistry of constructed wetlands appears to be very similar to that of natural wetlands, despite the comparatively low organic matter content of the substrates in constructed wetlands. Enzymic and gaseous flux evidence seemed to show that most nutrient cycling proceeded rather more slowly in constructed wetlands than in natural wetlands. Increasing the turnover of nutrients in artificial wetlands could improve water treatment efficiency for organic pollution; one way to achieve this might be to increase the organic matter content of the wetland substrate. Soil enzymes in constructed wetlands showed some similarities in susceptibility to factors which regulate enzyme activity in natural wetlands and soils. These may provide useful targets for more specific manipulation of enzyme activity. Alternatively, the activity of some enzymes may be effectively increased by supplementing constructed wetlands with commercially produced enzymes. Constructed wetlands provide simple and effective treatment for organic pollution, and further research will improve their suitability for the treatment of inorganic pollution.

10.5 Further work

Springtime decline in enzyme activity

The declines in hydrolase activity in late spring each year were attributed to plant growth. As the decrease was apparently unconnected with climatic factors such as air or soil temperature, further investigations would most usefully be focussed on the interactions between plant growth, microbial population changes and existing soil enzymes. Changing day length, which stimulates plant growth in spring and consequently alters photosynthesis rates, photosynthate translocation in the plant, root exudation and extension, and ultimately therefore rhizosphere microbial populations, could be the key to the timing of the decline. A late start to spring could potentially delay the spring growth spurt of plants and thus move the springtime decline in enzyme activity by a few weeks.

Kinetic investigation of inhibition

Despite the widespread assertion that phenolic substances inhibit enzyme activity, experiments using a source of phenolics added to constructed wetland soil did not produce any evidence of a change in enzyme activity (Chapter V a). Field data showed positive relationships between phenolic materials in the soil water and enzyme activity. Even if there had been evidence for phenolic inhibition, simply investigating the change in rate of product formation (fluorescence) would not provide information about the nature of the inhibition. A more enlightening technique would be to study inhibition using kinetics; partial (competitive, non-competitive or uncompetitive) and complete inhibition give distinctly different responses when fractional velocity (ordinate) is plotted against the reciprocal of the inhibitor concentration (abscissa). Partial inhibition results in straight lines converging on the abscissa at a point which is not the origin, while complete inhibition gives lines intercepting at the origin (Whiteley 1997, 1999). Changes in the kinetic parameters K_m and V_{max} in the presence of suspected inhibitors may also provide useful information about the type of inhibition occurring (if any). Once the nature of enzyme inhibition is determined, the factors which cause it

may be manipulated as desired (increased or decreased) to adjust enzyme activity according to the wastewater to be treated in a wetland.

Enzyme supplementation

During experiments in which unimmobilized enzymes were added to wetland soil, the mechanisms of immobilization were not investigated. Before adding unimmobilized enzymes to wetlands in order to improve treatment efficiency, it would be prudent to determine the capacity of the wetland substrate for immobilization, and also the likely half-life of unimmobilized enzymes. The mechanisms of immobilization could affect both of these factors. To date, wastewaters which are toxic to the majority of soil bacteria have been treated in wetlands which have been allowed to gradually adapt to using the chemicals in question as carbon and nitrogen sources. This usually requires a greater wetland area than would be required for, e.g., sewage treatment. However by supplementing soil with extracellular enzymes which specifically targeted the waste products, their decomposition may be achieved within a smaller area. Heavily contaminated soil or water, which could not support a microbial population, could still be remediated using enzymes alone until toxicity was sufficiently reduced to allow treatment in a “live” wetland.

Using wetlands to treat specific pollutants

Constructed wetlands are capable of treating complex organic compounds by facilitating their extracellular decomposition to molecules which form suitable substrates for microorganisms. These systems can develop greater efficiency for treatment of specific pollutants after a short acclimatization period during which the pollutant is applied, but recirculated (McKinlay & Kasperek 1996).

Monitoring the changes in activity of different soil enzymes, which occur as the soil microbial population adapts to a novel pollutant, may provide information about the most useful types of enzymes which could be added to constructed wetlands to speed up the treatment of recalcitrant compounds. Laboratory studies

of the impact of soil pH and redox potential on degradation of specific organic pollutants would also be useful.

Nitrogen cycling

Combining nitrogen-rich wastewater (e.g. sewage) with carbon-rich wastewater (e.g. paper mill effluent) may restore the C:N balance which these wastewaters tend to lack, and thereby promote better denitrification. An investigation into the partitioning of nitrous oxide production between nitrification and denitrification may help to minimize the release of this atmospheric pollutant from constructed wetlands. The contribution of urease, and other enzymes involved in extracellular parts of the nitrogen cycle, to nitrogen supply in constructed wetlands, remains to be investigated, as do the relative contributions of urea nitrification and chitin decomposition to nitrogen availability in soil.

Redox and enzyme activities in field conditions

More detailed investigation, at a field scale, of the influence of redox potential on extracellular enzyme activities is still needed. This could be achieved by using the *in situ* redox probes (Chapter IV b) to obtain detailed information about redox potential, and collecting soil samples from between the probes on a weekly or daily basis. In between sampling, the probes have time to re-stabilize ready for the next reading and soil sampling. A further study would be needed to establish the potential for field-scale changes and their effect on extracellular enzyme activity, using water table manipulation or the addition of different electron acceptors. Lastly, the effect at a field scale of nutrient enrichment, for example to determine the effect on enzymes and/or redox potential of adding phosphate, sulphate, nitrate or ammonium (Reddy & D'Angelo 1998), would be worthy of investigation.

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Biogeochemistry of water quality amelioration using wetlands – a role for enzymes

V. Shackle, C. Freeman and B. Reynolds

Introduction

Constructed wetlands are used to treat a diverse range of waste waters – domestic sewage (BRIX & SCHIERUP 1989), landfill leachate (KOWALIK et al. 1996), acid mine drainage (WEBB et al. 1998), road drainage (WIEDER et al. 1988), unused pesticides (MCKINLAY & KASPEREK, 1996), chemical process effluents (COBBAN et al. 1998) and aquaculture outflow (REDDING et al. 1997). The use of aquatic plants to remove nutrients and contaminants from water is documented since the 1950s (PRIDE et al. 1990), but the more detailed biochemical processes which achieve this are poorly understood (FARAHBAKSHAD & MORRISON 1997, WEBB et al. 1998).

Constructed wetlands can treat waste waters without the costly material constructions, large fossil fuel energy inputs and chemical processes which conventional purification requires. Wetland energy sources are renewable (solar radiation, microbial metabolic energy and gravity), their products are more easily disposed of, and maintenance and operating costs are a fraction of those of conventional treatments (BOYT et al. 1976, PRIDE et al. 1990).

Organic matter entering a wetland is mineralized and incorporated into nutrient cycles via the metabolism of micro-organisms, plants and soil animals, mediated by intra- or extra-cellular enzymes (BURNS 1978). There has been some research into the biogeochemistry of bogs and fens (FREEMAN et al. 1996), but no parallel work describing constructed wetlands. Soil enzyme activities in two constructed wetlands were investigated over 12 months using a fluorescent substrate method (FREEMAN et al. 1995), and related to seasonal changes in soil chemistry. The aim of the study was to determine the relative importance of temperature, pH and “inhibitory” phenolics in the regulation of enzyme activities in two constructed wetlands.

The two constructed wetlands are in Wales, UK; the first is at the Centre for Alternative Technology (CAT), Machynlleth, Powys, and receives domestic sewage and waste water from 1000 people/day. The

wetland, constructed in 1991, is approximately 600 m² and consists of four vertical flow cells planted with *Typha latifolia*, with a single large horizontal flow bed below. Solid material is screened out of the raw sewage and composted, and outflowing water discharges to the Afon Dulas.

The second wetland is at Tonmawr in the Gwenffrwd valley near Neath, Carmarthenshire. It was constructed to improve the water quality and appearance of a river receiving water discharging from an abandoned coal mine. Such discharges are common in South Wales where workings at most coal mines were abandoned in the early 1960s; the water contains iron pyrites, which stains river gravels orange when it is oxidized to insoluble iron compounds, and then precipitated. As well as the vivid discolouration, the acidity and metal content of mine discharges is toxic to aquatic life, restricting its reproduction and diversity and so impacting on entire food chains. The studied wetland, Whitworth No. 1, was constructed in 1995 and consists of four cells (total area 900 m²); the cell sampled has a bark substrate and is planted with *Juncus effusus*. Treated water discharges to the Nant Gwenffrwd river.

Methods

At both sites five replicate 10-cm³ samples of wetland surface soil were collected each month, and soil temperature (at 10 cm depth) adjacent to each sampling point was measured. Soil pH was measured in the laboratory, and soil water obtained by the centrifugation method of REYNOLDS (1984). The activity of six groups of hydrolytic enzymes was determined in each of the five soil samples from each site each month, using the fluorogenic methylumbelliferyl (MUF) substrates β -D-cellobioside, β -D-glucoside, *N*-acetyl- β -D-glucosaminide, phosphate, sulphate and β -D-xyloside (FREEMAN et al. 1995).

For each enzyme assay, a 1-cm³ portion of soil from each sample from each site was prepared and equilibrated to the average field temperature. Seven millilitres of 400 μ M (200 μ M for MUF phosphate)

MUF substrate solution at field temperature were added to each soil portion and thoroughly mixed; the resulting slurries were incubated at field temperature for 60 min (45 min for phosphatase). After incubation the reaction was terminated by centrifuging 1.5 ml of each slurry at 10,000 rev. min⁻¹ for 5 min. Supernatant (0.5 ml) from each vial was mixed with 2.5 ml of deionized water in a cuvette, and the fluorescence measured at 330 nm excitation, 450 nm emission, (slit width 2.5 nm), with a Perkin Elmer LS50 fluorimeter. For each sample each month, a MUF-free acid calibration was assayed to correct for quench due to phenolics (FREEMAN et al. 1995). The dry mass of 1 cm³ of soil from each sample from each site was measured each month to calculate the rate of enzyme activity as $\mu\text{M MUF released min}^{-1} \text{g}^{-1}$.

The phenolic content of the soil waters was determined using soil water samples, sodium carbonate (200 mg L⁻¹) and Folin-Ciocalteu phenol reagent in the ratios of 10:1.5:0.5 as recommended by BOX (1983). The absorbance at 750 nm was measured after 90 min, and a standard curve prepared using phenol standards, from which the phenolic content of the samples was determined.

Total and inorganic carbon were quantified in soil water samples using a Shimadzu TOC-500 Total Organic Carbon Analyser; ions in soil water samples were analysed with a Skalar Auto Analyser.

Results and discussion

At both sites, all soil enzyme activities showed seasonal variations throughout the sampling period (Figs. 1a, 1b). At the CAT (sewage treatment) wetland the spring peak for all enzymes averaged 40% above the average for the year, with a range between enzymes of 20–69% above average. At the Pelenna (acid mine drainage treatment) wetland the spring peak for all enzymes averaged 62% above the average for the year, with a range between enzymes of 23–95% above average. Enzyme activities showed a distinctive drop in May. At the CAT wetland this drop averaged 53% below the average for all enzymes (range 36–79% between enzymes) and at the Pelenna wetland averaged 81% below the average for all enzymes (range 78–82% between enzymes). In summer enzyme activities rose again, at the CAT wetland to 47% above the average for all enzymes (range 32–65% between enzymes), while at the Pelenna wetland activities rose to 56% above the average for all enzymes (range 22–86%

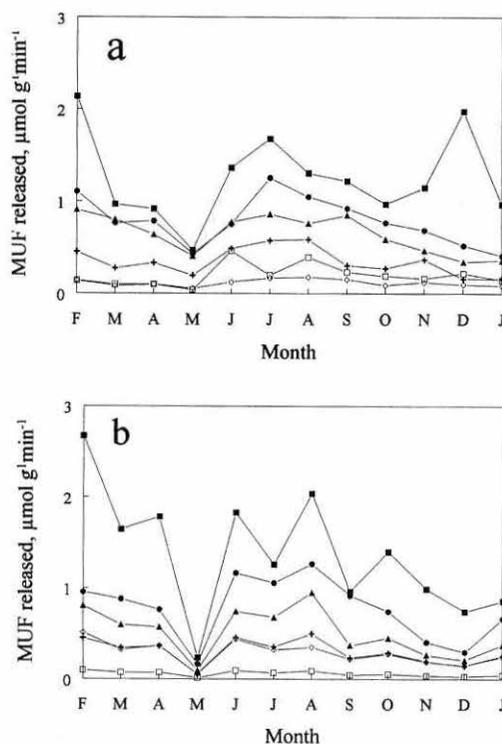


Fig. 1. The activity of six hydrolytic enzymes (cellobiosidase (+), glucosidase (●), chitinase (▲), phosphatase (■), sulphatase (□) and xylosidase (◇) from February 1997 to January 1998 at the Centre for Alternative Technology sewage treatment wetland (a), and Pelenna acid mine drainage treatment wetland (b).

between enzymes) at the highest point in August. Enzyme activities tailed off in the autumn and remained low during the winter. Phosphatase showed rather different behaviour at the CAT wetland in winter, as its activity rose in December to 56% above the average for the year.

Soil enzyme activity had been expected to follow field temperature patterns, but as these showed typical seasonal responses (Figs. 1a, 1b), there were clearly other factors involved in regulating enzyme activities. The pH at both sites remained stable throughout the sampling period (Figs. 2a, 2b), and thus showed no regulatory effect on enzyme activities. We also anticipated finding an inverse relationship between

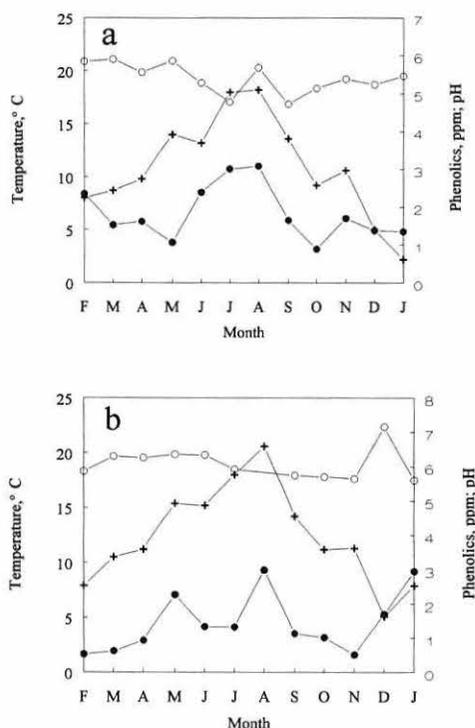


Fig. 2. Mean soil temperature (+), phenolic content of soil water (●) and soil pH (○) from February 1997 to January 1998 at Centre for Alternative Technology sewage treatment wetland (a), and Pelenna acid mine drainage treatment wetland (b). ($n = 5$).

the phenolic content of the soil water and soil enzyme activity, as phenolics are regarded as enzyme inhibitors (FREEMAN et al. 1990, WETZEL 1992, APPEL 1993). However, the results conflicted with this hypothesis; at the CAT wetland the relationship between mean enzyme activity and phenolic content (Fig. 2a) of the soil water showed a strong positive correlation ($r = 0.830$, $P < 0.001$), while at the Pelenna wetland there was apparently no correlation ($r = -0.189$, $P > 0.05$) (Fig. 2b). In neither case was there evidence that high levels of phenolics reduce enzyme activity, despite the suggestion that phenolic and humic substances can contribute up to 80% of the total dissolved organic matter in a system (WETZEL 1993).

A weak relationship between dissolved organic carbon (DOC) and soil enzyme activity

at both sites suggested an association between these factors. However, DOC includes a broad range of substances (carbohydrates, amino acids and sugars, cellulose, lignin, proteins, etc; PAUL & CLARK 1989), which can represent either products of, or substrates for, enzymic hydrolysis.

Clearly the enzyme activity in constructed wetlands is regulated by more than the three variables that we have considered in this study. Future studies should consider a more diverse range of variables including redox, oxygen availability, inorganic nutrient abundance and enzyme activities located within the rhizosphere. If such controlling variables can be identified it may prove possible to modify biodegradative processes in constructed wetland systems.

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