Carbon supply and the regulation of enzyme activity in constructed wetlands

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Abstract
Regulation of extracellular enzyme activity in constructed wetlands (i.e. artificial wetlands for wastewater treatment) has not been elucidated; the objective of this work was to investigate the contribution of one possible regulator (carbon supply) to rates of activity of three enzymes involved in organic matter decomposition. Soil samples from a constructed wetland were supplied with carbon (cellulose or glucose) at different concentrations (10 and 100 mg l⁻¹) and with carbon-free water (control) or sewage effluent. The activities of β-D-glucosidase, phosphatase and arylsulphatase in the soil were monitored over a 54-day period using methylumbelliferyl substrates, which are cleaved only extracellularly (Jones 1990). When compared to controls receiving no carbon, phosphatase activity under all treatments showed no significant differences over the course of the experiment. Sulphatase activities over the course of the experiment were significantly reduced by glucose (10 and 100 mg l⁻¹), cellulose (10 and 100 mg l⁻¹) and sewage effluent addition, to between 53 and 79% of the activity in controls. β-glucosidase activity gradually increased by 127–161% over the 54 d in response to cellulose addition (10 and 100 mg l⁻¹), and decreased after sewage effluent addition to 84% of the activity in controls. These findings suggest that by manipulating the quantity and quality of carbon supply in constructed wetlands, it may be possible to modify extracellular enzyme activities in order to maximize the efficiency of water treatment.

Keywords: Constructed wetlands; Carbon supply; Enzyme activity; Regulation

1. Introduction
During treatment of wastewaters (such as sewage) by constructed wetlands, high molecular weight organic pollutants are degraded to low molecular weight nutrients, utilisable by microorganisms. Current knowledge of soil biochemistry suggests that this mineralization is achieved via the metabolism of microorganisms (Brix and Schierup 1989) and the enzymic activity of the soil (Martens et al., 1992; Kang et al., 1998). Soil enzyme activity includes enzymes excreted by microorganisms as part of extracellular metabolism, and enzymes immobilized on soil colloids and humic materials (Burns 1982), and is sensitive to changes in soil (micro-environmental) conditions such as temperature, pH, plant exudates and soil water chemistry.

Soil enzyme activity has been proposed to be an important determinant of soil quality (Alef and Sparling, 1995) and, in the context of this paper, water quality improvement in wetland systems (Freeman et al., 1997; Kang et al., 1998). Therefore, manipulating enzyme activity may enable increases in wetland treatment efficiency. Enzyme activity in soil may increase because: suitable microbial substrates are at a premium (e.g. de-repression allows microorganisms to produce enzymes to liberate more low molecular weight compounds); growth of the microbial population as a whole is stimulated (i.e. more microbes = more enzymes); or extracellular enzymes that are already immobilized 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, higher or lower levels of nutrient input could be obtained by altering the water chemistry of the inflowing wastewater.

Methods to manipulate soil enzyme activities have been proposed in earlier studies of natural soil systems (Martens et al., 1992; Sparling et al., 1981). 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. Breakdown of organic components in wastewater, resulting in mineralization, is essential.

The hypothesis investigated in this study is based on the essential role of carbon in microbial processes as: (i) substrates for microbial enzyme synthesis; (ii) substrates for enzyme activity; and (iii) 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. Our objectives were to determine the effect on β-glucosidase, phosphatase and arylsulphatase activities of adding different amounts of high and low molecular weight carbon.

2. Materials and methods

2.1. Source of wetland soil cores

Soil samples were taken from a constructed wetland at the Centre for Alternative Technology (CAT), mid-Wales. This reed bed treats the domestic sewage of staff and visitors at the Centre, receiving on average 210 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 (±3°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 1.

2.2. Wetland soil core preparation and treatment

Thirty soil cores, 100 mm high × 110 mm diameter were placed in a controlled temperature room at 12°C (±3°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 1.

2.3. Source of carbon

Carboxymethylcellulose (Sigma–Aldrich, Poole, England) was chosen as a soluble form of cellulose. Control and organic carbon solutions were supplied from reseroirs 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 (1 min⁻¹ 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. (1995). On each assay day, four 0.2 cm³ 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. Substrate (MUF-β-D-glucoside 200 μM L⁻¹, MUF-phosphate 200 μM L⁻¹ or MUF-sulphate 400 μM L⁻¹; Sigma–Aldrich, Poole, England), 1.4 ml, or methylumbelliferone free acid (50 μM L⁻¹) was added to soil and thoroughly mixed. After incubation at 12°C for 60 min, the reaction was terminated by centrifuging at 10,000 × g for 5 min, and 0.5 ml of the supernatant fraction 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 fluorimeter (Perkin–Elmer LS50). 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 cm³ was calculated by drying at 105°C for 24 h.

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.

3. Results

3.1. 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 (Fig. 1). Adding 10 mg L⁻¹ (C 10) or 100 mg L⁻¹ (C 100) 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 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 (r² = 97.7%), dropping to 0.119 after day 33 (r² = 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 \( \beta \)-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, and 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 2 summarizes the effects of different treatments on \( \beta \)-glucosidase activity.

### 3.2. Phosphatase

Cumulative phosphatase activity showed linear accumulation (Fig. 2), 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 to 0.609). However, there were significant differences \( (p < 0.05) \) between C 10 and G 10, G 100 and C 100 cores. Table 3 summarizes the effects of different treatments on phosphatase activity.

### 3.3. Sulphatase

Sulphatase activity in all cores was approx. 10% of either \( \beta \)-glucosidase or phosphatase activity throughout the experimental period. In all cores, cumulative sulphatase activity was linear throughout the experiment (Fig. 3). Compared to Milli-Q control cores \( (mMQ = 0.0929) \), 10 mg \( l^{-1} \) glucose \( (mG10 = 0.0537) \), 100 mg \( l^{-1} \) glucose \( (mG100 = 0.0731) \), 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 \( (p < 0.05) \). Table 4 summarizes the effects of different treatments on sulphatase activity.

### 4. Discussion

Adding carbon to soil (as cellulose or glucose in this experiment) changes the ratio of carbon/nitrogen/phosphorus. Nitrogen and phosphorus 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 and Sparling, 1995), while Anderson and Domsch (1975) used \(\alpha\)-glucose at 800 mg l\(^{-1}\) and higher to obtain maximum stimulation of respiration. In this experiment, carbon was added at two levels (10 and 100 mg l\(^{-1}\)) which were considered to be low and high (respectively) compared to the inflow to the wetland (average approx. 50 mg l\(^{-1}\) DOC in 1998).

In water samples supplemented with \(\alpha\)-glucose and amino acids, Chrost (1991) recorded drastically reduced \(\beta\)-glucosidase activity, which was presumed to be the result of repression of enzyme synthesis by the added enzyme products. Chrost (1991) also reported greatly decreased affinity of \(\beta\)-glucosidase for \(\alpha\)-glucose in the presence of excess \(\alpha\)-glucose, by substrate inhibition. In the results of this experiment there is support for the hypothesis that \(\beta\)-glucosidase activity was inhibited by excess substrate because cumulative activity in G 100 cores initially proceeded at a similar rate to \(\beta\)-glucosidase activity in G 10, C 100 and MQ cores, but after day 26 declined to less than 20% of the initial rate.

Studies have suggested that increased levels of \(\beta\)-glucosidase activity may be explained in terms of enzyme activation or induction. The synthesis of cellulase enzymes is induced indirectly by the presence of cellulose in soil, and the activity of these induced enzymes hydrolyzes subunits such as cellobiose, cellotriose, cellotetraose and cellopentaose from large polymers (Killham 1996). Chrost (1991) reported that cellobiose, in particular, strongly induces the synthesis of \(\beta\)-glucosidase; which suggests an explanation for the increased activity of this enzyme in cores treated with 10 and 100 mg l\(^{-1}\) cellulose. The proposed sequence of events is summarized in Fig. 4.

Sewage effluent-treated cores showed significantly lower \((p < 0.05)\) \(\beta\)-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 soluble carbon is freely available, it has been suggested that there is no need for microorganisms to acquire it enzymically (Chrost and 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 and 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 C 10 cores. There are many reports of an inverse relationship between phosphatase activity and inorganic phosphate availability (Siuda and Chrost, 1987; Cotner and Wetzel, 1991) and of a correlation between organic phosphorus

![Fig. 3. Cumulative sulphatase 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) (▲).

![Fig. 4. Effect on soil enzyme activity of adding cellulose to soil.](image)
mineralization and phosphatase activity. On this basis, Sinsabaugh and Moorhead (1994), suggested that increased activity is a response to low environmental availability of phosphorus, and Chróst (1991) suggested that 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 phosphorus was not limiting, or phosphatase activity is regulated by factors other than carbon availability.

Tabatabai and 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 and 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 and 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 was sugarbeet, which is likely to have contained organic phosphorus 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 studies by Sinsabaugh and Moorhead (1997).

The microbial biomass and enzyme activities of a soil are a reflection of the capacity of that soil to maintain these parameters (Nannipieri et al., 1983); biomass and enzymes in excess of this capacity are rapidly destroyed. Soil has a carrying capacity in which microbial communities are able to maintain approximately steady state conditions. The increases in β-glucosidase activity in this experiment after cellulose addition (which could be viewed 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 non-limiting not all microbial process will accelerate, 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 — carbon acquisition, nitrogen acquisition and phosphorus 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, phosphorus and sulphur cycling in the wetland soil. Sinsabaugh and Linkins (1988) described β-glucosidase as a mediator of cellulose degradation, phosphatase as involved in the acquisition of phosphorus from organic sources 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 phosphorus acquisition enzymes is puzzling, especially given the essential role that phosphorus 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 and Ross 1978). Sulphatase on the other hand may be under a much greater degree of microbial regulation.

The soil of this constructed wetland appears to behave similarly to other non-saturated soils with respect to microbial stimulation and inhibition. Different enzymes are differently affected by the addition of labile and complex carbon sources, which has implications for the optimization of wetland efficiency. 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 systems, 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 release of organically bound sulphate. However, stimulating carbon-cycling could compromise metal removal, as organic matter degradation would potentially release heavy metals immobilized on 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.

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References


