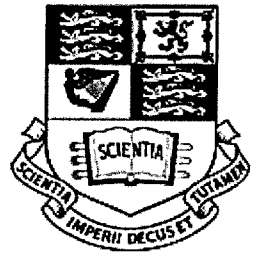
Imperial College
London

IN SITU BIOLOGICAL DENITRIFICATION OF GROUNDWATER ~~FOR POTABLE SUPPLY~~: AN ~~LABORATORY MICROCOSM STUDY OF WATER QUALITY AND BIOFOULING~~

IN THE UK

EXPERIMENTAL STUDY OF WATER QUALITY AND BIOFOULING WITHIN THE DENITRIFICATION TREATMENT ZONE'

A thesis submitted to the University of London
for the degree of Doctor of Philosophy and for the Diploma of the
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ABSTRACT

Increasing concentrations of nitrate (NO_3^-) in groundwater, approaching or exceeding the drinking water standard of 50 mg l^{-1}, have caused the Water Utilities to become progressively more reliant on NO_3^- removal methods. *In situ* biological denitrification (ISBD) is a prospective NO_3^- removal technology harnessing the denitrification potential of indigenous microbial communities within aquifers to biochemically reduce NO_3^- to nitrogen (N_2) gas. This approach has not been adopted in the UK for the treatment of groundwater intended for drinking, due to a reluctance to inject extraneous substances into potable supply aquifers (Dahab, 1993), and a paucity of information on the effects of ISBD on other regulated water quality parameters and on the aquifer environment. In the UK, a lack of confidence with regards to ISBD as a NO_3^- removal treatment is apparent.

A series of laboratory experiments were undertaken to examine the effects of stimulated heterotrophic denitrification in aquifer rock on the chemical and biological quality of water travelling through aquifer material, utilising a flow through microcosm apparatus to simulate the sub-surface environment. Furthermore, the impacts of the organic carbon amendment on the microbial community within the microcosms were assessed with regards to biofouling and community structure.

Experimental results showed that under controlled C/N ratio, with an appropriate carbon substrate, complete denitrification could be achieved in UK aquifer material, without additional nutrient or bacterial inoculation requirements. Selection of carbon substrate was critical in optimising the water quality of the denitrified water, with regards to residual chemical species, bacterial concentrations or turbidity, for example. Acetic acid emerged as a superior substrate in this respect, compared to glucose and acetate, and additionally, neutral pH conditions were maintained when acetic acid was used as the substrate. Therefore, water treated by ISBD should not require additional post-abstraction treatment beyond the scope of the usual groundwater treatments, prior to supply.

Biofouling within the porous sandstone matrix was not detected and the hydraulic conductivity of the denitrifying microcosms, though slightly reduced, was stable over the duration of the experimental incubations. Molecular microbiological examination techniques (PCR-DGGE) provided insights into the microbial ecology of the enhanced aquifer community, revealing that the dominant species in both acetate- and glucose– supplied microcosms, were facultative anaerobes, associated with denitrifying environments

The results demonstrate that ISBD is potentially an environmentally sustainable and economic alternative to conventional NO_3^- removal methods. The treatment technique is most likely to be suited to aquifers with a homogenous granular matrix, such as the Triassic sandstone, since uniform dispersion and mixing of injected carbon is paramount to achieving the desired C/N ratio that ultimately controls the biochemical reaction and hence, water quality.

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CHAPTER 1

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1.10	**SUMMARY**

1.1 INTRODUCTION

Nitrate (NO_3^-) is commonly regarded as the most prevalent contaminant of global groundwater resources. Over the last 30 years, growing trends in groundwater NO_3^- concentrations have raised concerns for health, environmental and economic reasons (e.g. Korom, 1992; Spalding and Exner, 1993; Smith, 2001; EC DGENV, 2002). Environmentally, groundwater NO_3^- contributes to the eutrophication of surface waters but health issues are of more immediate concern in the short term and have associated economic impacts. For example, potable supply within the European Union, regulated under the EU Drinking Water Directive (98/83/EC) (CEC, 1998), stipulates a maximum admissible concentration (MAC) of 50 mg NO_3^- l^{-1}. As NO_3^- concentrations in many groundwater resources have begun to exceed this limit, the affected abstraction facilities have had to secure low NO_3^- resources to blend with the high NO_3^- water, invest in additional NO_3^- removal process or alternatively, be abandoned. Since groundwater is ordinarily regarded as a pristine resource with minimal treatment requirements compared to surface water, additional NO_3^- removal treatments have had considerable economic repercussions, particularly in regions which rely heavily on groundwater resources.

In the UK, 30% of potable supply comes from groundwater, with regional variations; for example up to 80% in the south east of England (Burt and Trudgill, 1993; Gooddy *et al.*, 2002). The concentration of NO_3^- in the UK's three major aquifers is either approaching or has exceeded the MAC during the last 20 years, as illustrated in Figure 1.1.

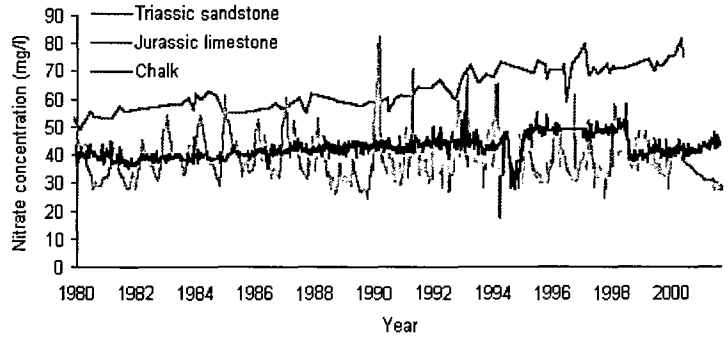


Figure 1.1: NO_3^- concentration in major UK aquifers, (Environment Agency, 2004)

Similar trends throughout Europe prompted adoption of legislation tackling NO_3^- pollution within European Union Member States including the Nitrates Directive (CEC, 1991), for example, and more recently, the Water Framework Directive (CEC, 2002).

However, these preventative strategies do little to ameliorate existing contamination problems owing to the time scales involved with implementing the policies and moreover, the long residence times of groundwater in aquifers (CEC, 1997). In the interim, increasing numbers of groundwater abstraction wells have been abandoned or equipped with NO_3^- removal technologies. The comparative expense and inefficiency of traditional physicochemical NO_3^- removal methods have driven the recent interest in biological denitrification. The United States and European countries such as France and Germany in particular have pioneered development of engineered biological denitrification for drinking water purposes. In the UK, water utilities and regulators have approached such novel methods with caution, but the increasing pressure on groundwater resources necessitates the evolution of biological denitrification as a more sustainable and economically feasible treatment alternative to ensure the future use of groundwater for potable resource. This Chapter reviews contemporary literature on biological denitrification for drinking water remediation and gives an account of the causes and effects of NO_3^- pollution. The issues of uncertainty concerning the application of this technology in the UK are summarised and developed into the motivational issues and research themes for this PhD thesis.

1.2 NITRATE AS A GROUNDWATER CONTAMINANT

1.2.1 The Nitrogen Cycle and Groundwater Nitrate Contamination

The occurrence of N in the biosphere is described by the nitrogen cycle, as depicted in Figure 1.2. Although N may exist as an element in molecular form (N_2); in soil and water environs, it typically combines with H or O to form molecules or ions with varying oxidation states, as described in Table 1.1.

Table 1.1: Common N-species in soil and water Environs (Canter, 1997)

N-species	Chemical Formula	Oxidation State
Ammonia	NH_3	- 3
Ammonium ion	NH_4^+	- 3
Molecular Nitrogen (gas)	N_2	0
Nitrite ion	NO_2^-	+ 3
Nitrate ion	NO_3^-	+ 5

Nitrate is naturally present in soils and sediments and is regarded as an integral component in the microbial cycling of inorganic N. It is formed from NH_3 in the soil by a two phase aerobic oxidation process; 'nitrification,' as described by Hawker and Linton (1971):

(i) $2NH_4^+ + 3O_2 \rightarrow 2NO_2^- + 4H^+ + 2H_2O$

(ii) $2NO_2^- + O_2 \rightarrow 2NO_3^-$

Nitrification is carried out by specialised bacterial autotrophs: *Nitrosomonas* and *Nitrobacter* respectively.

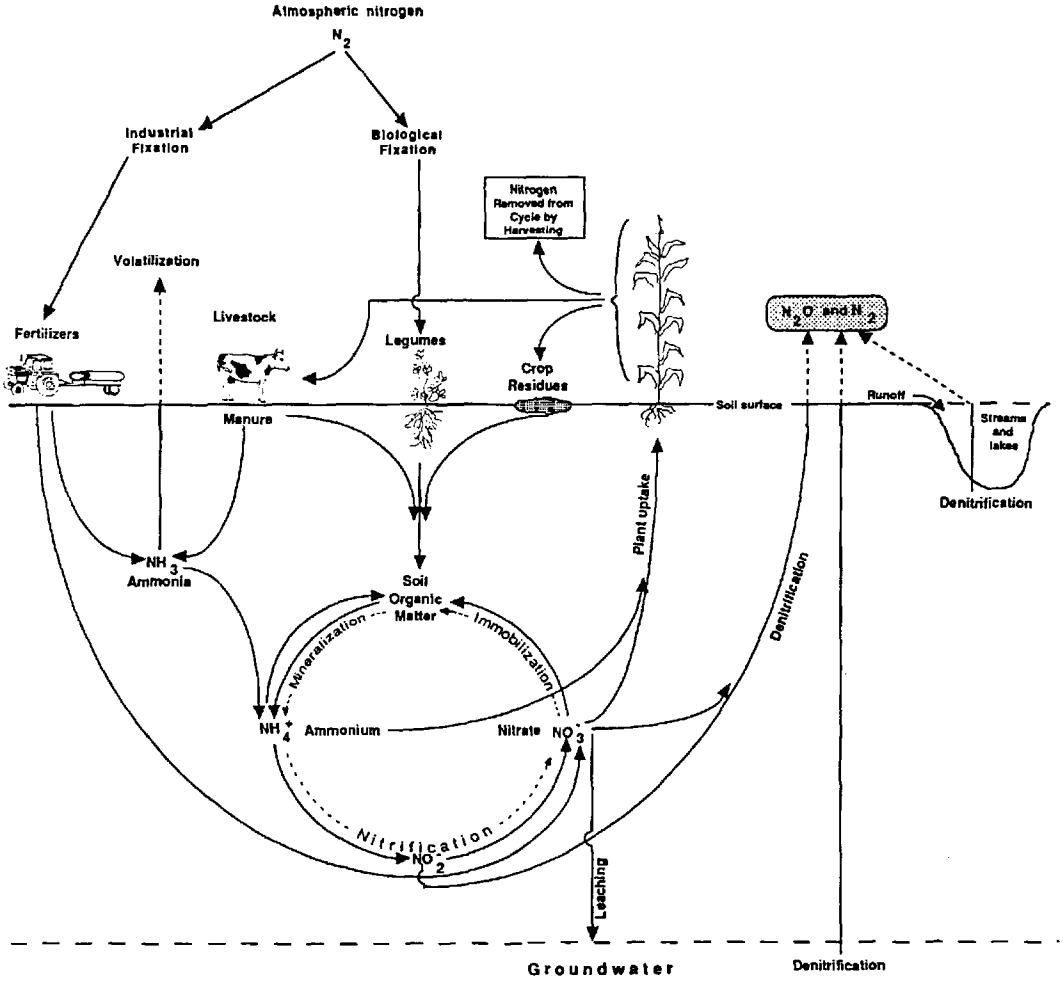


Figure 1.2: Nitrogen Cycle (N fixation from lightenting not shown) (Stevenson, 1991).

Conversely, NO_3^- is lost from the soil via denitrification reactions, which typically convert NO_3^- to N_2 gas under soil conditions, where O_2 supply is limited, described by the equation:

$5\,C + 4\,NO_3^- \rightarrow 2\,N_2 + 2\,H_2O + 4\,HCO_3^- + CO_2$ (Korom, 1992)

Nitrate is also subject to removal from soil via plant assimilation and physical processes such as leaching to the saturated zone. Consequently, although NO_3^- natually occurs in soils, its concentration is theoretically low owing to these removal processes. However, with additional NO_3^- loading to soils from anthropogenic sources, the natural cycling processes; denitrification and assimilation, are unable to remove the superflous NO_3^- and so the quantity leached to groundwater courses increases (Korom, 1992; Canter, 1997).

Anthropogenic sources of NO_3^- loading to soils are of industrial, urban or agricultural nature. Treated nitrogenous wastewater or effluents discharged directly to the environment from industrial processes constitute industrial sources whilst treated sewage effluents, leachates from landfill sites, seepage from storage facilities and run off from hazardous sites have all been identified as significant urban sources of anthropogenic N (Canter, 1997). The most widespread and significant source of anthropogenic loading to soils, however, is from the agricultural sector (Korom, 1992; Van der Voet *et al.*, 1996; Canter, 1997; CEC 1997). The UK Department of Food and Rural Affairs (DEFRA) estimates that 70 % of the NO_3^- occurring in 'natural waters' is of agricultural origin (Lake *et al.*, 2003).

1.2.2 Agricultural Nitrate Pollution

Within the European Union, the introduction of the Common Agricultural Policy (CAP) in 1957 intensified operations in fisheries, livestock and arable industries, with the aim of maximizing productivity. As a result, increases in chemical fertilizer use in addition to manure, and concentrated livestock husbandry, have been observed during the last 50 years, contributing significantly to the anthropogenic N load to soils (CEC, 1997; EC DGENV, 2002). Similarly, in the US, the EPA identify chemical pesticides and fertilizers together with the high volumes of wastes associated with Animal Feeding Operations (AFO's), where animals are kept and raised in concentrated areas, as major sources of anthropogenic N.

In 2002, the EC Directorate-General for the Environment (DGENV) reported annual animal husbandry contributions of approximately 8 million t of N to EU agricultural land (EC DGENV, 2002). In some areas of the EU, intensive livestock farming has lead to the production of more manure than can be disposed of without creating NO_3^- excess in the soil (CEC, 1997). The greater density of livestock operations has reportedly resulted in strong NH_3 volatilization and subsequent atmospheric deposition of N in neighbouring soils and waters (EC DGENV, 2002). In spite of this, many authors regard fertilizer as the ultimate cause of groundwater NO_3^- contamination (Van der Voet, 1996). In Western Europe, for example, the use of

chemical fertilizers increased exponentially between the 1950's and the 1980's. In the UK, this increase was by an order of magnitude, from approximately 100,000 t in 1950 to 1.6×10^6 t in 1980 (Novotny, 1999). The EU as a whole, observed a peak in inorganic N fertilizer consumption during 1985-87; at approximately 11 million t annually (EC DGENV, 2002). In conjunction with increased fertilizer use, changes to the farmed landscape since the introduction of the CAP, such as reduction in permanent grassland areas and buffer zones (ditches and hedgerows), encourage quicker drainage and run-off of fertilizer to adjacent watercourses. Moreover, timing of fertilizer application, irrigation and tillage practices and the type of crop cultivated, all influence NO_3^- leaching from soils. In areas of high precipitation, excessive irrigation, or in soils that are well drained and permeable, NO_3^- pollution from fertilizer is compounded (US EPA, 2000).

The retention of pollutants in soil depends, to some extent, on the soil composition (organic content, pH, clay and moisture contents, cation exchange capacity) and its redox status. Once a soil has reached saturation with NO_3^-, excess is leached to groundwater. In such cases, there is a marked positive correlation between 'excess' NO_3^- entering the soil and the groundwater concentration (Novotny, 1999). Agricultural land use is frequently associated with high NO_3^- in the local groundwater (Burt and Trudgill, 1993); alluvial and shallow aquifers, which experience considerable agricultural leaching inputs during episodes of high rainfall, for example, are particularly vulnerable (Kelly, 1997; Karaczun, 2003).

1.2.3 Influence of Aquifer Characteristics on Nitrate Contamination

Groundwater contamination is further influenced by the characteristics of the aquifer itself. Unconfined aquifers, for example, are highly variable in their topography, but are generally recharged regularly by water percolating from soils above (Burt and Trudgill, 1993). Since the amount of recharge tends to show seasonal variation, so does the groundwater NO_3^- content. Conversely, confined aquifers are much less vulnerable to contamination from agricultural sources. Owing to their nature, they are only recharged in small regions, usually receiving recharge from an adjacent unconfined aquifer rather than from percolating water. Confined aquifers therefore provide an important source of low NO_3^- water which is exploited to provide potable water by blending with groundwater of high NO_3^- concentration (Canter, 1997). However, groundwater from confined aquifers is a finite resource; moreover, there is concern that NO_3^- contamination from unconfined aquifers may eventually migrate to confined aquifers. This could be induced by pumping, for example, or by gravitational flow as the quantity of groundwater in the confined aquifer is reduced (Burt and Trudgill, 1993).

Within unconfined aquifers, distribution of NO_3^- in the saturated zone depends largely on (Burt and Trudgill, 1993):

- depth of the overlying unsaturated zone
- the saturated hydraulic conductivity of the aquifer
- the influence of pumping and lithological variation within the aquifer

Dissolved NO_3^- is transported throughout the aquifer by means of dispersion, which is not uniform but affected by factors such as porosity and tortuosity of the rock matrix. Flow through a porous medium is complex; it is determined by hydraulic conductivity and hydraulic gradient; the ability of the medium to transmit liquid, and the driving force acting on the liquid respectively. Such physical characteristics vary spatially as the rock composition of the aquifer changes.

Once NO_3^- has reached the saturated zone, it may remain in the groundwater for decades, accumulating and being transported over considerable distances. Consequently, groundwater NO_3^- pollution is both long term and trans-boundary (Van der Voet *et al.*, 1996), necessitating integrated management approaches to preserve groundwater as a drinking water resource and prevent further environmental impacts such as eutrophication.

1.2.4 Effects of Nitrate Contamination in Groundwater: Health and Environmental Considerations

Nitrate content of drinking water is regulated in the EU, under the Drinking Water Directive (1998), which stipulates a MAC of 50 mg NO_3^- l^{-1} (11.25 mg l^{-1} as N)(DWI, 2000). This legislation aims to safeguard human health against illness caused by NO_3^- ingested from drinking water. The adverse health effects of NO_3^- are controversial, perhaps because they are indirect; the NO_3^- ion itself is usually regarded as non-toxic at groundwater concentrations (Gulis *et al.*, 2002). Conversely, once ingested it may be reduced to NO_2^-, which is acutely toxic and essentially responsible for the illnesses associated with NO_3^-. The most familiar NO_3^-/NO_2^- related condition is methaemoglobinaemia, which occurs when NO_2^- in the body oxidises the haemoglobin found in red blood cells to methaemoglobin, a form which cannot carry O_2. As a result, O_2 supply to body tissues is restricted, leading to cellular anoxia and clinical cyanosis (Canter, 1997). This illness may affect humans of all ages as the reduction of ingested NO_3^- occurs in human saliva, although infants less than 3 months old are especially vulnerable, hence the term 'blue baby syndrome.' Further transformation of NO_3^-/NO_2^- in the gastrointestinal tract and bladder can produce N-

nitroso compounds; a potent group of chemical carcinogens. Whilst the toxicity of these compounds is well documented, the epidemiological evidence linking cancer with NO_3^- ingestion is somewhat more conflicting because of the different research approaches, such as the use of mortality data compared to incidence data (Gulis, 2002). However, elevated NO_3^- in drinking water has been previously associated with non-Hodgkin Lymphoma, and cancers of the oesophagus, nasopharynx, bladder and gastrointestinal tract (Canter, 1997; Gulis, 2002). More tenuously, NO_3^-/NO_2^- toxicity has also been linked to defects of the central nervous system and high blood pressure (Spalding and Exner, 1993).

Aside from the potential health effects, NO_3^- contaminated groundwater is considered hazardous to the environment as it is a plant nutrient and may cause eutrophication of surface waters when in excess (Spalding and Exner, 1993). In addition to the more documented relationship between P and eutrophication, NO_3^- specifically has been identified as the main contributor to eutrophication in coastal and inland water bodies throughout the EU, in particular the North and Baltic Seas (Van der Voet *et al.*, 1996). Increasingly sophisticated monitoring technology, such as G.I.S and improved sampling programmes has facilitated better documentation of the environmental impacts of anthropogenic NO_3^-.

The rising costs of treating drinking water resources compromised by NO_3^-, and the cost of repairs to dams and coastal waters damaged in NO_3^- related eutrophication incidents has raised the profile of NO_3^- pollution in Europe. An economic assessment of groundwater protection commissioned by the EC DGENV observed that protection measures are always cheaper than clean-up processes (BRGM ECOLOGIC, 2003), hence EU Member States, though initially slow to act, are now actively engaged in NO_3^- management programmes as required by the EU Nitrates Directive (EC DGENV, 2002).

1.3 MANAGEMENT AND TREATMENT OF NITRATE IN GROUNDWATER

1.3.1 Management Strategies: European Legislation

The amelioration of environmental NO_3^- pollution in the long term requires management strategies targeted towards minimising and controlling NO_3^- pollution at source. Within the EU, this is accomplished through environmental policies or Directives that Member States are legally bound to adhere to, for example: the Urban Wastewater Treatment Directive (CEC, 1991b), regarding the regulation of NO_3^- inputs to surface waters via discharges/effluents; the Nitrates Directive (CEC, 1991a), regarding the control and prevention of NO_3^- pollution in water bodies from

agricultural sources; and the recent Water Framework Directive (WFD) (CEC, 2000), which is concerned with achieving 'good status' in European waters on a river catchment basis. The various directives require EU Member States to achieve specific goals within specified time frames and to provide evidence of continual monitoring. European Union legislation is translated into law and implemented by individual member states, which are subsequently accountable to the European Commission. In cases where a Member State fails to comply with the EU directives to a 'satisfactory' level, prosecution and subsequent penalties may follow. The Nitrates Directive is the most relevant to groundwater as it focuses on pollution from the agricultural sector. Conversely, the Urban Wastewater Treatment Directive legislates for N- emissions from urban wastewater, a major source of N pollution in surface waters (Baumann *et al.*, 1997). The more stringent controls on N-emissions under the Urban Wastewater Treatment Directive led to the modernisation of wastewater treatment plants (WWTPs) to include biological (secondary) treatments for nitrogen removal, including denitrification. Without reduction in nutrient (NO_3^-) losses from agriculture, however, advances in wastewater treatment processes would be ineffective, regarding the overall status of nutrient pollution in the environment (EC DGENV, 2002). Indeed the ongoing importance of the Nitrates Directive was recognised in the extensive overhaul of European water policy of the 1990s, which resulted in the adoption of the WFD (2000/60/EC). In the review processes which streamlined existing EU water policy, with the loss of seven previous water-directives, a resolution was passed to uphold the full 'topicality' of the Nitrates Directive without revision (resolution: A5-0386/2000), in conjunction with the new WFD. Furthermore, whilst the modernisation and gradual reform of the European CAP encourages better agri-environmental practices, the regulation and control of NO_3^- emissions from agriculture remains the principal concern of the Nitrates Directive (EC DGENV, 2002).

1.3.2 The EU Nitrates Directive and UK Implementation

The Nitrates Directive (91/676/EEC), passed into law European law in 1991, aims to reduce existing water pollution "caused or induced by nitrates from agricultural sources" and prevent further such pollution (CEC, 1997). The Directive has facilitated a harmonised approach to ameliorating agricultural NO_3^- pollution through improved farming practices and more comprehensive monitoring of water quality. Under the Directive, Member States are required to achieve the following principal objectives:

i. Identify waters which could be affected by NO_3^- pollution and designate these area as Vulnerable Zones,
ii. Draw up Action Programmes for these Vulnerable Zones, containing mandatory agricultural practices, including the stipulation of maximum amounts of manure that may be applied to land per year,

iii. Establish at least one code of "Good Agricultural Practice" to be mandatory within the Vulnerable Zones, but implemented voluntarily outside of them.

In addition, the Member States are obliged to monitor the concentrations of NO_3^- in water and thereby assess the impacts of measures put in place (CEC, 1997).

In the UK, implementation of the Nitrates Directive is currently managed by the government Department of Environment, Food and Rural Affairs (DEFRA), with monitoring work carried out by the Environment Agency. Initially, 66 Nitrate Vulnerable Zones (NVZs) were identified throughout England in 1996, covering approximately 8 % of England's land area, as depicted in Figure 1.3. The designation of original NVZs was based primarily on catchments that provide drinking water resources, however, in 2000, the European Court of Justice ruled that the UK had failed to designate sufficient NVZs to protect 'all' waters. Consequently, after an open period of consultation and a more comprehensive process of identifying surface and groundwaters which 'are' or 'could' become polluted, the government designated additional NVZs throughout England in 2002, as shown in Figure 1.3 *(the responsibility of designation in remaining areas of the UK falling to the devolved parliamentary administrations)*(DEFRA, 2002). An alternative approach to the identification of individual NVZs, adopted by many Member States including Germany, Austria, Denmark and the Netherlands, is to designate the whole country as an NVZ (CEC, 1997). This approach was not deemed appropriate in the UK as extensive monitoring and modelling etc demonstrated that NO_3^- pollution varied regionally (MAFF, 1995a).

Within the NVZs, farmers are required to keep formal records of cropping, livestock, fertiliser and manure use for each field. Furthermore, the timing and rate of application of nitrogen fertiliser and organic manure must also be controlled. In 1999, the UK Environment Agency also enforced a "Closed Period," where certain practices such as application of slurry and liquid digested sewage sludge for example, are prohibited during the months of August – October (with some exceptions) (Environment Agency, 1999). Throughout the implementation of this programme, the Environment Agency, the Ministry of Agriculture, Fisheries and Food (MAFF) and now DEFRA have provided technical and financial support to farmers affected by the NVZ scheme.

In addition to the fundamental changes to farming practices, the agrichemical industry has also been under pressure to improve fertilizers and new formulations of fertilizer have emerged designed to minimise NO_3^- leaching losses. For example,

nitrification inhibitors, which delay the nitrification process, have been incorporated into fertilizers, thus maintaining more ammonia than NO_3^- in soils (Canter, 1997).

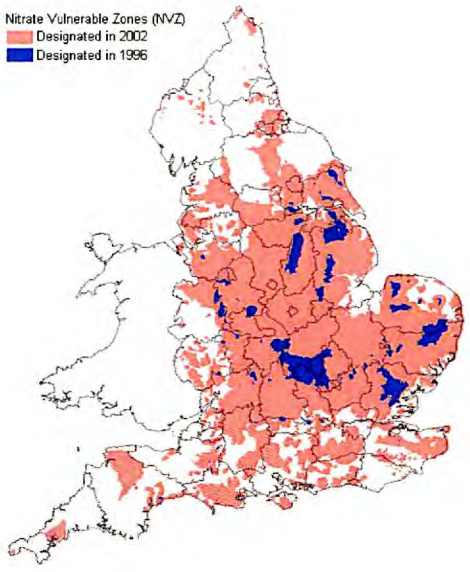


Figure 1.3: Designation of NVZs in the UK (DEFRA, 2002)

Alternatively, slow-release nitrogen (SRN) fertilisers have been designed to minimise losses from soils prone to leaching by providing nitrogen to crops in a time-release fashion. As the rate of conversion of the fertiliser components to NO_3^- is low, less NO_3^- will be in the soil when leaching events occur (Canter, 1997).

In 2002, the EU Directorate-General of the Environment reported that all Member States had embarked on implementation of the Nitrates Directive; designating NVZs, establishing comprehensive monitoring networks and good codes of agricultural practice. However, it is also acknowledged that in spite of the early successes in some regions (e.g. Denmark, East of France, Algarve), in general, it will take many years for the effects of the Nitrates Directive Action Programmes to have a significant impact on groundwater NO_3^- concentrations, owing to the time scales involved in soil and groundwater activity (EC DGENV, 2002). Indeed, some authors estimate that it will be up to 3 decades before groundwater protection efforts are realised leading to a decline in NO_3^- concentrations. Therefore, advanced NO_3^- removal technologies are necessary to treat contaminated groundwater in the short-mid term (Mercado *et al.*, 1988) so that it complies relevant drinking water legislation.

1.3.3 Nitrate Removal Treatments for Drinking Water Sources

Historically, remedial solutions for water resources that fail to comply with the NO_3^- Drinking Water MAC have involved the blending of 'high' NO_3^- water with local 'low' NO_3^- supplies or NO_3^- removal by physicochemical processes. Blending was in widespread use in the UK during the 1990s as it offered an 'easy-to-operate' solution to NO_3^- control (Hall and Croll, 1993). However, this treatment is clearly unsustainable as uncontaminated groundwater is a finite resource. Other disadvantages include: potential introduction of other water quality impairments via the 'low NO_3^-' water; and changes to ionic composition of the water which may result in corrosion problems in the water distribution network. Such factors may contribute to render the blending option unsuitable and in some cases, the expense of equipment necessary to deliver low NO_3^- water to the contaminated source may be prohibitive and thus, NO_3^- removal treatments must be used.

Nitrate removal from water is typically difficult and expensive because the NO_3^- ion is stable and highly soluble and therefore not easily precipitated out of solution or adsorbed. Common chemical treatments used in water purification processes such as coagulation and filtration are not successful in NO_3^- removal and specialised treatments are required (Luk and Au-Yeung, 2002). Established NO_3^- removal processes are either of a physico-chemical nature; ion exchange (IE), reverse osmosis (RO), electrodialysis (ED); or involve some form of biological denitrification.

Physico-chemical processes essentially involve the separation of a concentrated stream of NO_3^- away from the main body of water undergoing treatment. In IE, this is achieved by passing the contaminated water through a bed of ion-exchange resin beads. The resin absorbs NO_3^- ions in exchange for another anion – typically chloride, and is functional until the exchange capacity of the resin is exhausted. The resin may then be regenerated by backwashing with sodium chloride to return the resin to the chloride form, producing a final waste 'brine' of concentrated NO_3^- (Hall and Croll, 1993). Conventional ion exchange processes experiences two major disadvantages; the preferential uptake of sulphate (SO_4^{2-}) ions over NO_3^- ions and the intrinsic production of concentrated waste brines on regeneration of the resin, which required further disposal. However, the advent of 'nitrate-to-sulphate-selective' (NSS) resins has improved the efficiency of the process so that > 90% NO_3^- removal is achieved, even in 'high sulphate' groundwaters (Bae *et al.*, 2002; Hall and Croll, 1993). Regarding the issue of brine disposal, modern ion exchange plants have evolved to incorporate further brine 'polishing' so that recycling may be an option. Biological denitrification reactors may be coupled to the main body of the ion exchange plant, enabling brine denitrification to give better quality waste water. Furthermore, the

considerable quantities of sulphate that accumulate when NSS resins are used can also be treated in sulphate reducing reactors added to the brine processing phase. Finally, granular activated carbon (GAC) treatment is typically employed to remove suspended solids such as bio-polymers and organics that are produced in the biological brine-treatment (Bae *et al.*, 2002).

Similarly, reverse osmosis (RO) and electrodialysis (ED) are physico-chemical processes in which NO_3^- is separated from the treated water via the filtering action of a membrane. During RO, hydraulic pressure is used to force the water undergoing treatment across a semi-permeable membrane, leaving salts (including NO_3^-) deposited on one side. The resulting proportions of treated water to concentrated waste are determined by the pressure differential across the membrane (Hall and Croll, 1993). Alternatively ED uses stacked membranes, alternating in anion and cation permeability, which allow ions to pass through but not water. An electric current carries ions through the membranes, producing streams of treated water and concentrated waste. Nitrate selective membranes are available for RO and ED which improve cost efficiency by reducing the overall power requirement per unit of NO_3^- removed (Hall and Croll, 1993). Inherently, as with IE, both membrane separation processes generate concentrated waste streams which require further disposal. Since the costs of RO and ED are already relatively high, and the power demand to drive the processes is high; the additive cost of waste disposal prohibits application and examples of use in drinking water treatment are scarce. Reverse osmosis has specifically been advocated for small agricultural communities in developing countries based on experimental trials in South Africa. Here, the feasibility of applying RO in rural villages where the local community could be trained to operate the plants and wastes could be used for stock-watering was demonstrated (Schoeman and Steyn, 2003). However, of all three processes, only IE has been employed on any considerable scale (Dahab, 1993). In spite of the relatively high cost of using NO_3^- selective resins and the additional treatment/disposal requirements of the brine, the IE process is considered fast, efficient, easily automated, unaffected by seasonal temperature changes (in the ordinary operating range) and has conventionally been recommended for small-medium sized plants (Luk and Au-Yeung, 2002).

Currently, the most efficient NO_3^- removal process for treating large volumes of water is biological denitrification, which is a relatively stable process that can achieve 100 % NO_3^- removal (described in section 1.4). However, although biological denitrification is widely acknowledged as a superior treatment method compared to existing physico-chemical processes; pioneers of novel electro-chemical technologies do not

view biological denitrification as the future of NO_3^- removal for drinking water treatment. Criticisms of biological denitrification include: the comparatively long start up and process times; the production of microbial waste products and partially degraded N- or organic carbon compounds in the treated water; limited operational temperature; extensive process-maintenance, including precise monitoring of the carbon source in heterotrophic examples and limited opportunity for automation (Luk and Au-Yeung, 2002; Berndt *et al.*, 2001; Horold *et al.*, 1993; Peel *et al.*, 2003). As an alternative, electro/chemical processes such as chemical-, catalytic- and electro-catalytic NO_3^- reduction, are in development. Briefly:

- Chemical NO_3^- reduction involves the abiotic reduction of NO_3^- in solution by a zero-valent metal, which may reduce anions owing to their electron-donating tendency (Shrimali and Singh, 2001). Chemical reduction of NO_3^- has been successfully achieved using zero-valent iron (Fe^0) (Huang *et al.*, 1998; Hu *et al.*, 2001; Devlin *et al.*, 2000) and also aluminium powder (Luk and Au-Yeung, 2002).
- Catalytic NO_3^- reduction involves the use of a bimetallic catalyst such as palladium-copper or palladium-tin which can be used to catalyse the hydrogenation of NO_3^- to form N_2 (Horold *et al.*, 1993; Berndt *et al.*, 2001) or potentially, the reduction of NO_3^- by formic acid (Prusse *et al.*, 2000).
- Electro-catalysis, a more efficient catalytic process, uses a small, direct electric current delivered via an electrode such as rhodium-coated carbon cloth to catalyse NO_3^- reduction (Reddy and Lin, 2000; Peel *et al.*, 2003).

Abiotic processes such as these provide a relatively pure water-product in time scales of minutes to hours and do not have the post-denitrification treatment requirements associated with biologically denitrified water. Furthermore, they do not generate the concentrated waste brines inherent to physico-chemical processes. Reported NO_3^- removal efficiencies vary between 60-99.9 % and therefore such novel technologies appear promising. However, these processes are all currently at laboratory scale and thus far, observed disadvantages include: difficulties in operational control, fouling of catalyst, sensitivity to environmental conditions, and preferential production of NH_4^+ rather than N_2 in the case of catalytic NO_3^- reduction (Horold *et al.*, 1993; Berndt *et al.*, 2001; Luk and Au-Yeung, 2002). In chemical reduction processes with zero-valent metals, observed disadvantages include a very strong pH-dependence, which necessitates the addition of either a strong base, or strong acid, in order to maintain the narrow operational pH ranges in zero-valent aluminium and iron processes, respectively. Moreover, significant amounts of NH_3 may be produced, and residues of soluble ferrous iron (Fe^{2+}) and suspended aluminium can accumulate in the

denitrified product, although these could be easily removed with filtration and settling phases (Huang *et al.*, 1998; Luk and Au-Yeung, 2002). Extensive process engineering is required to optimise these abiotic NO_3^- removal technologies, with regards to reactor designs and raw materials. It is uncertain how long it would take to fully engineer these processes for field scale application, indeed, in the case of NO_3^- reduction with zero-valent metals, the mechanism for the reaction has not yet been fully defined. Therefore, these processes cannot be considered as NO_3^- removal treatments at present and biological denitrification continues to be the most efficient alternative method to IE for NO_3^- removal from drinking water resources.

1.4 BIOLOGICAL DENITRIFICATION

Biological denitrification refers to the microbially mediated process of dissimilatory reduction of NO_3^- to N_2 gas via a series of intermediate N-oxides. It is a well documented process that occurs naturally in soils and water, as depicted by the N cycle (Figure 1.2). The reaction is of economic and historical importance, harnessed as a treatment for the removal of N from wastewater as early as the 1800s (Van Loosdrecht and Jetten, 1998). Recently, biological denitrification has also gained attention for groundwater NO_3^- removal treatment (Bhatti *et al.*, 2001).

Dissimilatory NO_3^- reduction is a method of bacterial respiration, the process by which non-photosynthetic bacteria generate energy. Cellular respiration involves the transfer of electrons from a donor to an acceptor via an electron transport chain, generating cellular energy compounds such as adenosine tri-phopshapte (ATP), (Mateju *et al.*, 1992). Bacterial respiration may utilise a variety of electron acceptors including oxygen, N-oxides, and inorganic ions such as Mn^{4+}, Fe^{3+} and SO_4^{2-}. Because these molecules/ions are final in the respiratory electron transport chain, they are usually referred to as 'Terminal Electron Acceptors,' (TEA) (e.g. Chapelle, 2001). In biological denitrification, NO_3^- acts as the TEA and must receive 5 electrons to be fully reduced to N_2, in conjunction with the oxidation of an electron donor, which loses 5 electrons. Organic carbon or various inorganic substances may act as the electron donor in the case of heterotrophic and autotrophic denitrification, respectively. Since the electron donor becomes oxidised in the reaction, and the electron acceptor is reduced, biological denitrification is an oxidation-reduction (redox) reaction, which can be mediated biochemically.

Denitrification is generally taken to be an anaerobic reaction since facultative anaerobic bacteria will preferentially use O_2 as the terminal electron acceptor in respiration rather than NO_3^- because of the higher amount of energy released

(discussed in section 1.5.1). When denitrification conditions occur, the necessary denitrification enzymes are synthesised in a highly regulated process (Mateju *et al.*, 1992), where individual enzymes are not induced simultaneously, but sequentially (Baumann *et al.*, 1997). The step-wise denitrification reaction proceeds via a series of N-oxides; each reaction step representing the further reduction of the original NO_3^- ion, as described, for example, by Bates *et al.* (1998):

$$\underset{\text{Nitrate reductase}}{NO_3^{-(+5)} \rightarrow} \quad \underset{\text{Nitrite reductase}}{NO_2^{-(+3)} \rightarrow} \quad \underset{\text{Nitric Oxide reductase}}{NO^{(+2)} \rightarrow} \quad \underset{\text{Nitrous Oxide reductase}}{N_2O^{(+1)} \rightarrow} \quad N_2^{(0)}$$

(Nitrogen oxidation state indicated in superscript)

Whilst both heterotrophic and autotrophic denitrification may occur in the natural environment, engineered biological denitrification processes have previously focussed on heterotrophic denitrification rather than autotrophic denitrification, as autotrophic bacteria are typically slower-growing and denitrification rates are lower (Mateju *et al.*, 1992). However, the potential presence of bacterial wastes and partially degraded organics in the denitrified effluent/water during heterotrophic denitrification has lead to increased interest in the autotrophic process.

1.4.1 Autotrophic Denitrification

Autotrophic denitrification refers to dissimilatory NO_3^- reduction by an inorganic ion which serves as an electron donor, whilst an inorganic carbon such as CO_2 or HCO_3^- is assimilated for growth (Hall and Croll, 1993). In general, bacteria of the genera *Paracoccus, Thiobacillus and Thiosphaera*, for instance, can denitrify using hydrogen or reduced sulphur compounds as the electron donor; alternately, *Ferrobacillus, Gallionella, Leptothrix* and *Sphaerotillus* species can utilise ferrous iron (Mateju, 1992). Natural denitrification in aquifers is frequently driven by autotrophic bacteria, since the organic carbon content of un-amended/uncontaminated aquifers is typically very low. Case studies have reported examples of natural aquifer denitrification with ferrous iron (Fe^{2+}) and ferrous disulphide (FeS_2 - pyrite), by indigenous bacterial species *Gallionella ferruginea* and *Thiobacillus denitrificans* respectively, (Korom, 1992). The most commonly employed reaction for engineered autotrophic denitrification is the oxidation of elemental sulphur by *Thiobacillus denitrificans*, as defined by Soares (2002), for example:

$$5S^0 + 6NO_3^- + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$$

The use of elemental S is popular for denitrification of contaminated groundwaters owing to its widespread availability, comparatively low cost, non-toxicity and stability

under ambient conditions (Soares, 2002). Inorganic carbon for biomass assimilation, in the form of HCO_3^- can be added or a solid calcareous material such as limestone, which additionally provides the support matrix, can be used. A pH buffer is typically required as the overall reaction consumes alkalinity, resulting in decreased pH. If not buffered, the pH falls below the optimal range for biomass growth; *Thiobacillus denitrificans* has an optimal growth range of pH 6.8 - 8.2 (Liu and Koeing, 2002). If limestone is used as the inorganic C source or biofilm support, no additional buffer is required as the limestone dissolves in proportion to pH decrease, with the effect of raising pH and thus maintaining pH equilibrium (Liu and Koeing, 2002). However, Soares (2002) reported that during treatment of NO_3^- contaminated groundwater using reactors containing only granular elemental sulphur with *Thiobacillus denitrificans*, the reaction had negligible affect on pH, and no buffer was required.

Elemental S and limestone reactors using *Thiobacillus denitrificans* have reported denitrification efficiencies of 75 – 100 % and have demonstrated good stability of operation, including oxygen tolerance, low temperature capacity (10 °C), and extended operational periods before washing is required (24 days compared to 2 days in typical heterotrophic denitrification reactors) (Kuai and Verstrate, 1999; Trouve and Chazal, 1999). However, a potential disadvantage of elemental sulphur and other reduced sulphur compounds for autotrophic denitrification is the formation of sulphate as a reaction product, since sulphate is a regulated water quality parameter (DWI, 2000). This could prohibit elemental sulphur denitrification, for instance in groundwater sources where there is already a high sulphate content (Soares, 2002).

Alternatively, hydrogenotrophic bacteria, which can use H_2 as an electron donor, have also been studied with regard to engineered autotrophic denitrification. Indeed, hydrogen is one of the most thermodynamically favourable electron donors for autotrophic denitrification, as described by Till *et al.*, (1998):

$$2NO_3^- + 5H_2 \rightarrow N_2 + 4H_2O + 2OH^-$$

In spite of this, engineered H_2 denitrification has been constrained by its relatively high cost and difficulties associated with the storage and handling of H_2 as a consumable resource, notably its low solubility, and its hazardous (explosive) properties (Till *et al.*, 1998l; Devlin at al., 2000). Surrogate methods of supplying hydrogen are currently under experimentation, for example the evolution of cathodic hydrogen from iron corrosion, according to Till *et al.* (1998):

$$Fe^0 + 2H_2O \rightarrow H_2 + Fe^{2+} + 2OH^-$$

Although it has been demonstrated that cathodic hydrogen generated by iron corrosion could support autotrophic denitrification, research is currently at laboratory scale and it remains unclear whether this technique could be developed into an engineered process.

The most advanced autotrophic denitrification currently involves elemental sulphur/limestone reactors, which have been advocated for use particularly in small scale wastewater and groundwater applications (Kuai and Verstraete, 1999; Trouve and Chazal, 1999; Soares, 2002). This technology is also being further developed in conjunction with electrochemical treatments, to provide two-phase denitrification (Wang and Qu, 2003).

In the long term, autotrophic denitrification offers an attractive alternative to the use of heterotrophic denitrification in treatment plants because of its comparative simplicity, and its ability to remove NO_3^- more cleanly, with less organic residual in the denitrified product. However, the low reaction rates compared to heterotrophic denitrification, would lead to increased process times, or perhaps larger reactor size, to achieve the equivalent amount of treatment as heterotrophic systems. Such factors could result in higher capital costs, ultimately prohibiting the use of autotrophic methods on any considerable scale and limit application to small/rural treatment plants only (Hall and Croll, 1993). In the interim, heterotrophic denitrification has become an established treatment for potable water (Hamon and Fustec, 1991).

1.4.2 Heterotrophic Denitrification and Dissimilatory Reduction of Nitrate to Ammonium

Heterotrophic denitrification refers to the dissimilatory reduction of NO_3^- to N_2 gas, where an organic carbon substrate is used both as electron donor and for biomass assimilation. It is thought that most denitrifying bacteria are heterotrophic, commonly of the *Pseudomonas*, *Alcaligenes* and *Flavobacterium* genera (Kesseru *et al.*, 2002; Green and Shelef, 1994). Some bacterial species may perform complete denitrification, whilst others may only perform one stage of the step-wise reaction, thus, heterotrophic denitrification is commonly described as a community function (Green and Schelef, 1994). Similarly to autotrophic denitrification, the heterotrophic reaction occurs naturally in aquifers and is the principal method of NO_3^- removal in engineered biological denitrification, described by the basic reaction:

$$5C + 4NO_3^- + 2H_2O \rightarrow 2N_2 + 4HCO_3^- + CO_2$$ (e.g. Korom, 1992)

The electron donor is generically denoted as 'C,' and the equation simply describes the oxidation of 5 moles of organic carbon, which would supply the 20 electrons needed to fully reduce 4 moles of NO_3^-.

Many different sources of organic carbon (OC) can be 'degraded' by denitrifying microbial communities, ranging from complex organics typically occurring in natural environments to more simple molecules such as sugars and alcohols, commonly used in treatment processes. The OC substrate, together with dissolved oxygen (DO) concentration and the speciation of the denitrifying population exert a critical influence over the dynamics of the heterotrophic reaction; hence optimal conditions are necessary to achieve maximum denitrification yield.

Under suboptimal conditions, denitrification may proceed at a lower rate. Moreover, the sequential NO_3^- reduction may be arrested at one of the intermediate products in the reaction chain, resulting in the evolution of nitrite (NO_2^-), a toxic ion, or nitrous oxide (N_2O), a major greenhouse gas and catalyst of stratospheric Ozone depletion (Otte *et al.*, 1996; Baumann *et al.*, 1997; Cavigelli and Robertson, 2001). These intermediates are inherently produced in the natural environment although NO_2^- would be quickly re-oxidised back to NO_3^- by ubiquitous nitrifying bacteria. The formation of NO_2^- in engineered denitrification plants however, represents a serious water/effluent quality concern, as its toxicity means it is more stringently regulated than NO_3^- (e.g. MAC for drinking water = 0.5 mg NO_2^- l^{-1} compared to 50 mg NO_3^- l^{-1}). Emission of N_2O from denitrification plants, on top of natural N_2O evolution would have serious implications for global air quality, particularly with regard to ozone depletion and climate change.

In addition to the intermediate N-oxides, ammonium (NH_4^+) may also be formed during NO_3^- reduction as an alternative final product of the dissimilatory reaction. Dissimilatory reduction of nitrate to ammonium (DNRA) occurs in similar conditions to those required for dissimilatory NO_3^- reduction to N_2 (denitrification), and the two processes may occur simultaneously, although bacteria capable of DNRA are thought to be fermentative rather than respiratory (Yin, *et al.*, 2002; Fazzolari *et al.*, 1998). Whilst denitrification is a well documented reaction, DNRA is currently less defined particularly with regards to factors affecting the partition between denitrification and DNRA as pathways of NO_3^- reduction. It is proposed that OC supply and relative abundance of ammonifying bacteria are the determining factors of DNRA activity (Fazzolari *et al.*, 1998; Bengtsson and Bergwall, 1999; Yin *et al.*, 2002). In particular, DNRA appears to be favoured by OC rich environments, reportedly occurring under C/N regimes in excess of 5.0 (Fazzolari *et al.*, 1998).

Dissimilatory reduction of nitrate to ammonium could therefore be a potential NO_3^- reduction pathway in C-amended biological denitrification processes. This has implications for *in situ* denitrification schemes, where high quality potable water is desired as an end product, although there has been little attention devoted to this in previous *in situ* trials. Therefore, DNRA cannot be dismissed as a potential outcome of NO_3^- reduction, even though it is generally considered that denitrification is the dominant dissimilatory process (Yin *et al.*, 2002). An understanding of the parameters influential to denitrification dynamics, including the formation of intermediate N-oxides and NH_4^+ would facilitate greater process control and increase process-stability.

1.5 CRITICAL ENVIRONMENTAL PARAMETERS IN HETEROTROPHIC DENITRIFICATION

1.5.1 Oxygen Concentration

Denitrification is generally described as an anaerobic activity, inhibited in the presence of oxygen (O_2). Tiedje (1988) proposed that O_2 inhibition of denitrification might follow a threshold model, where denitrification would be completely inhibited once the O_2 concentration had reached a critical threshold (Oh and Silverstein, 1999). Since the majority of denitrifying bacteria are facultative anaerobes, there must be a point at which O_2 becomes inhibitory to denitrification activity. However, this is not universal and the point at which O_2 concentration affects denitrification activity in cultures varies between organisms depending on: species, strain, culture medium, physical structure of the culture and culture history (Korom, 1992; Otte *et al.*, 1996; Oh and Silverstein, 1999; Gomez, 2002). Denitrification activity has been reported at O_2 concentrations as high as 3.5 mg l^{-1} in WWTPs, and several researchers have noted pure strains growing on O_2 and NO_3^- simultaneously in culture (Oh and Silverstein, 1999). It is important to recognise the differential O_2-sensitivity of the facultative denitrifiers, but fundamentally O_2 has a negative affect on denitrification. There are no all-encompassing correlations between the O_2 concentration and denitrification rate, but optimal concentrations are generally < 2.0 mg O_2 l^{-1} (Hamon and Fustec, 1991; Bates and Spalding, 1998; Oh and Silverstein, 1999; Gomez, 2002). The inhibitory mechanism of O_2 is apparent on two levels; from a holistic perspective, O_2 prevents denitrification by competitive inhibition, whereas at a cellular level, O_2 interferes with the synthesis and activity of denitrification enzymes (Hernandez and Rowe, 1987; Gomez, 2002).

Competitive inhibition of denitrification by O_2 occurs because O_2 yields a higher amount of energy in bacterial respiration compared to NO_3^-. For example, the

oxidation of glucose using O_2 yields 686 Kcal mol^{-1} compared to 570 Kcal mol^{-1} from glucose oxidation with NO_3^-, as described by Green and Shelef (1994):

i) oxygen respiration:

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$

$\Delta G = -686$ kcal / mol glucose

ii) nitrate respiration:

$5C_6H_{12}O_6 + 24KNO_3^- \rightarrow 30CO_2 + 18H_2O + 24KOH + 12N_2$

$\Delta G = -570$ kcal / mol glucose

Facultative anaerobes will therefore preferentially utilise O_2 rather than NO_3^- as a terminal electron acceptor because it will provide more energy, per mole of OC consumed. The enzymes involved in O_2 respiration are thought to remain functional within bacterial cells and therefore, in fluctuating aerobic/anaerobic conditions, it is assumed that O_2 respiration will immediately take precedence where conditions permit.

Conversely, the various enzymes involved in denitrification are not all constitutively present in bacterial cells and may have to be enduced. Denitrification involves a series of enzymes, as described in Section 1.4: nitrate reductase (NaR); nitrite reductase (NiR); nitric oxide reductase (NOR); and nitrous oxide reductase (N_2OR). The synthesis of these enzymes generally occurs under anaerobic conditions (Baumann *et al.*, 1997) and initiation of enzyme synthesis in response to anaerobic conditions may be further intensified by the presence of an N-oxide (Hernandez and Rowe, 1987). In the presence of O_2, synthesis may be repressed, although the mechanism of this repression remains uncertain, particularly since sensitivity to O_2 varies between the different enzymes and the response of the same type of enzyme may vary between species (Otte *et al.*, 1996). Once synthesised, the activity of the enzymes may also be repressed by O_2. In spite of the ambiguities, it is generally agreed that synthesis of the various enzymes is sequential and that imbalances in the concentration/activity of the enzymes leads to incomplete denitrification with the production of NO_2^- or N_2O as the result (Otte *et al.*, 1996; Baumann, 1997).

Mixed microbial communities may become increasingly acclimatised to transient oxygen concentrations, and small quantities of denitrification enzymes may remain present, allowing an increasingly efficient response to the onset of denitrification conditions (Otte *et al.*, 1996; Baumann, 1997). In engineered biological denitrification, the removal of DO, for example by supplying a reducing reagent such

as sodium sulphite (Na_2SO_3), facilitates more efficient denitrification. Alternately, this could be achieved by increasing the OC dose so that there is enough electron donor, i.e. OC, to reduce the O_2 and subsequently NO_3^- (Gomez, 2002). Therefore, the availability of OC is also critical in the control of denitrification activity, including the regulation of O_2 respiration.

1.5.2 Organic Carbon Substrate

Fundamentally, the OC substrate drives the denitrification reaction by supplying the electrons required to reduce NO_3^- (and associated N-oxides). The dosing regime of OC relative to N, the 'C/N ratio,' is of therefore primary importance in managing biological denitrification systems (e.g. Hamon and Fustec, 1991). The reaction stoichiometry (described in section 1.4.2) states that 5 moles of carbon reacts with 4 moles of NO_3^-. In terms of mass, this gives a stoichiometric C/N ratio of 1.07. Stoichiometric C/N ratios such as this are typically used to define the dosing regime for the specific OC substrate in a biological denitrification reaction. If O_2 is present, an additional quantity of OC must be supplied to consume the O_2, as previously described, and further allowances must be made for some of the OC to be consumed in biomass assimilation (e.g. Dodd and Bone, 1975; Gomez, *et al.*, 2000, 2002). Empirically, the OC requirement is therefore higher than the theoretical stoichiometric quantity, with the actual OC amendment dependant on initial NO_x^- concentration, DO and biomass assimilation factor. The amount of OC that is likely to be assimilated is difficult to predict at the outset of an experiment and reported values range from 15-30 % (Dodd and Bone, 1975; Mateju *et al.*, 1992; ITRC, 2002). The biomass assimilation factor is typically estimated at the outset (ITRC, 2002), inherently adding an element of ambiguity to the determination of a suitable OC dose to achieve complete denitrification.

Denitrification yield increases linearly with increased C/N dose, until the optimum dose to sustain complete denitrification is reached (Mateju *et al.*, 1992; Her and Huang, 1995). It has also been observed that the number of denitrifying bacteria increases with increased C/N ratio; the affect being more pronounced with certain types of substrate (Gomez *et al.*, 2000). Once complete denitrification is achieved, further OC addition may prove toxic to the microbes or render the process unstable, although this appears to depend on type of substrate (Martienssen and Schops, 1997). Moreover, an excessive C/N ratio is likely to result in the enhancement of biomass to the point of biofouling and continued decline in the redox status, so that alternative electron accepting processes may become a nuisance (further discussion in Section 1.7.3).

Conversely, it is imperative that enough OC is provided to achieve complete denitrification, as deficiencies in this respect result in the formation and accumulation of intermediate products: NO_2^- and N_2O. In drinking water applications, NO_2^- accumulation is a particular concern and authors have noted that 100 % denitrification efficiency must be realised to avoid sustained NO_2^- production (e.g. Hamon and Fustec, 1991). Electron limiting conditions leads to competitive inhibition of NO_2^- reduction, as the various N-oxides must effectively compete for electrons and NO_2^- accumulates because NO_2^- reductase is the least competitive of the denitrification enzymes (e.g. Almeida *et al.*, 1995; Burns *et al.*, 1996).

An efficiently managed denitrification system therefore has an optimised OC dose to attain 100% denitrification, thus minimising intermediate production; whilst avoiding OC-excess, which could lead to the breakthrough of influent OC into the effluent or undesirable alternative electron accepting processes and biofouling. The type of OC substrate is also important in this regard because the ease with which a substrate is metabolised or assimilated, inherently affects the availability of electrons from that substrate.

An important example of this is the use of fermentable substrates for biological denitrification, which allow fermentative organisms to compete with denitrifying organisms for the electron resource. Whilst fermentation may produce an organic metabolite which could be used, secondarily in denitrification, it is generally described as a process by which electrons are lost, and hence fermentable substrates are not recommended for engineered denitrification (Mercado *et al.*, 1988; Devlin *et al.*, 2000). The selection of the OC substrate should therefore by critically assessed (e.g. Wilderer *et al.*, 1987).

An assortment of OC compounds have indeed been applied to biological denitrification, in accordance with the diversity of biological denitrification applications, including: remediation of point-source pollution, wastewater treatment, *in situ* reactive barriers for aquifer remediation, and drinking water treatment. The type of substrate may be a simple compound, such as an alcohol or an organic acid; a waste product, particularly from the food processing industry, for example distillery water; or a complex carbonaceous material such as cellulose. Methanol, ethanol, acetate, acetic acid, benzoic acid, glucose, sucrose, formate, molasses, whey, distillery stillage, natural gas, cotton, sawdust and leaf compost have all been employed as OC substrates (Blaszczyk *et al.*, 1980; Mateju *et al.*, 1992; Robertson *et al.*, 2000; Her and Huang, 1995; Rajapakse, 1999; Gomez *et al.*, 2000; Soares, 2000; Smith *et al.*, 2001). A specific substrate is not necessarily suitable for all denitrification

processes, and various selection criteria must be taken into account. Practical criteria such as: operational suitability (handling, storage etc.), costs, resource availability, and legality, are the foremost concern from a commercial perspective and will render many substrates unsuitable. From a reduced list of practicable substrates, further selections can be made on the grounds of denitrification efficiency and treated water quality. For instance, drinking water treatments benefit from the use of pure compounds such as alcohols or organic acids, because there are likely to be a smaller variety of OC metabolites in the treated water and the denitrification process is considered 'cleaner.' Comparatively, WWTPs can operate denitrification reactors using wastewater containing an array of different organics, because the potential metabolites are of less importance in the treated effluent, compared to drinking water. In drinking water applications, the use of simple organic substrates facilitates control over the reaction stoichiometry. Examples of substrates previously examined for drinking water denitrification are: methanol, ethanol, glucose, sucrose, acetate and acetic acid (Mateju *et al*, 1992; Her and Huang, 1995; Gomez *et al.*, 2000). In some EU countries, choice of substrate may be limited by legislation, for example in France, only ethanol and acetic acid are authorised for use in biological denitrification for drinking water.

The type of substrate used affects the overall efficiency of the denitrification reaction, with some substrates linked to sustained NO_2^- production in place of complete denitrification to N_2. Stief (2001) observed that NO_2^- accumulation was associated with the differential biodegradability of the substrate, and found that glucose experienced significant NO_2^- accumulation compared to methanol. Simple sugars such as glucose and sucrose have commonly been associated with NO_2^- accumulation compared to the alternative substrates for drinking water denitrification; alcohols and organic acids (Blaszczyk *et al.*, 1980; Mercado, 1988; Mosheni-Bandpi *et al.*, 1999; Gomez, *et al.*, 2000). This has been attributed to the relative ease with which alcohols and organic acids may be metabolised, compared to glucose and sucrose and additionally the availability of the substrate for fermentation reactions. In this regard, fermentable substrates such as glucose and sucrose may be utilised in fermentative processes, creating competition for electrons between respiring bacteria (denitrifiers) and fermentative bacteria. Furthermore, the presence of fermentable substrates such as glucose and glycerol has been linked to the establishment of DNRA as the predominant pathway in a denitrification system (Akunna *et al.*, 1993).

The type of substrate used may also influence the potential for biofouling to occur in the denitrification environment. Inherently, all denitrification systems may become

fouled as the necessary enhancement of the biomass to achieve a sufficient level of metabolism will also result in biomass proliferation. Biofouling has been reported in engineered denitrification applications using ethanol (Hamon and Fustec, 1991; Abdelouas *et al.*, 1999), formate (Smith *et al.*, 2001) and sucrose (Gomez *et al.*, 2000); whilst applications using acetate and acetic acid have fewer incidences reported in the literature (although this may be due to a shortage of reported trials with acetate and acetic acid overall).

Finally, the microbial community structure of the OC-enhanced biomass has also been related to substrate. Gomez *et al.* (2000) reported higher ratios of denitrifying bacteria to non-denitrifying bacteria in denitrification reactors fed with sucrose, compared to methanol and ethanol. This supported earlier work by Blaszczyk *et al.* (1980), who found that the proportion of denitrifiers in batch cultures with NO_3^- was 100 % with ethanol, compared to 42 % with acetic acid and only 5 % with glucose. Interestingly, cultures fed with methanol, often cited as the most superior substrate for denitrification, only yielded a denitrifier population of 36 %. Isolates from these cultures (inoculated from a single batch of wastewater) revealed that the denitrifying community in the ethanol culture was composed of two denitrifying species, compared to four species in the acetic acid culture whilst the glucose culture contained only a single denitrifying species. The influence of substrate on bacterial species selection and consequently denitrification activity has been frequently noted (Dodd and Bone, 1975; Wilderer *et al.*, 1987; Fourmentraux-Chevron *et al.*, 1998; Gomez *et al.*, 2000). Several authors specifically relate the predominance of fermentative conditions with the selection of bacterial species capable of NO_3^- respiration (NO_3^- reduction to NO_2^-) at the expense of true denitrifiers, i.e. those capable of NO_3^- reduction to N_2 (Wilderer *et al.*, 1987; Devlin *et al.*, 2000).

1.5.3 Microbial Community

Engineered biological denitrification processes may utilise naturally occurring, environmental strains, or may be inoculated with specific cultured strains of bacteria, for example. Once initiated, denitrification is a relatively stable process; however, this depends on the development of a balanced denitrifying microbial community (Baumann *et al.*, 1997). The environmental conditions during 'start up' of denitrification are crucial to the development of stable denitrification conditions, but nonetheless, shifts in the bacterial population may occur over extended periods of time. Although community structure appears to stabilise under steady-state conditions, it remains continually responsive to aberrations in the environment; for example, seemingly established communities were found to alter in response to changes in C/N ratio after 40 days (Martienssen and Schops, 1997). When an

'unbalanced' community develops, it typically results in NO_2^- accumulation and diminishing denitrifying capacity. Microbial community structure thus exerts a significant influence over the metabolic function, where population selection mechanisms operate in response to the microbial environment. Species best adapted to the specific conditions of the environment will effectively out-compete less efficient species, although the biocommunity as a whole remains dynamic. In addition to competitive inhibition processes, the microbial community structure will be influenced by other environmental conditions such as oxygen concentration and pH (e.g. Cavigelli and Robertson, 2000).

1.5.4 pH

The pH of a bacterial environment affects most biological processes, particularly in sub-surface environs (Ellis *et al.*, 1998; Simek *et al.*, 2002). The preferred pH of the majority of heterotrophic soil microbes ranges from pH 5.5 to pH 8.0; pH outside of this range may affect the microbe's ability to metabolise C compounds and contaminants (Rust *et al.*, 2000). Indeed, neutral to slightly alkaline conditions are reportedly optimal for denitrification; between pH 7.0 and 8.0 for most denitrifying strains (Daum and Schenk, 1998; Glass and Silverstein, 1998). However, the relationship between pH and denitrification activity remains uncertain, particularly with regards to the mechanism by which pH influences denitrification (e.g. Almeida, *et al.*, 1995; Simek, 2002).

Speculatively, acidic-tending pH appears to slow denitrification activity overall, and diminish the size of the denitrifying population. In particular, N_2O-reductase activity appears to be repressed in more acidic environments, as exhibited by an increased N_2O/N_2 ratio in gaseous emissions from denitrifying communities (Daum and Schenk, 1998; Ellis *et al.*, 1998; Simek *et al.*, 2002). Alternatively, increasingly alkaline conditions are associated with greater NO_2^- accumulation, although it appears that this is mutually dependent on the presence of NO_3^-, where increased pH coincides with increased competitive inhibition of NO_2^- reductase by NO_3^- (Almeida *et al.*, 1995; Glass and Silverstein, 1998; Jianping *et al.*, 2003). However, it has recently been suggested that the response of denitrifying cultures to pH depends largely on the previous culture/exposure history of the community. Simek *et al.* (2002) indicated that a denitrifying soil community exhibited maximum denitrification capacity at the native pH, and sudden pH increase or decrease retarded denitrification activity. The authors concluded that there is no simple relationship between pH and denitrification, and that longer incubation/experimental duration would result in the acclimation of the biocommunity to pH conditions (Simek *et al.*, 2002). This supported the earlier suggestions of Ellis *et al.* (1998), who proposed that in soil communities,

antecedent soil regimes could influence the pH affect on denitrification. The mechanism by which pH influences denitrification activity is also ambiguous, although it is acknowledged that the effect is likely to be on denitrifying enzyme activity (Almeida *et al.*, 1995; Ellis *et al.*, 1998; Jianping *et al*,. 2003).

Therefore, it is imperative that pH in engineered denitrification systems is monitored, thus allowing the use of buffers to maintain neutral pH where necessary, avoiding production of reaction intermediates: NO_2^- and N_2O. *In situ* bioremediation schemes often require an extraneous buffer, as microbial degradation activity results either in increased acidity, where CO_2 is evolved or increased alkalinity, where hydroxyl ions are evolved. However, in some instances, the aquifer matrix itself may act as a buffer, depending on the dissolution equilibrium of the rock minerals; for example calcareous rocks dissolve under increasingly acidic conditions, thereby releasing calcite and buffering the pH increase (Dahab, 1993). Alterations to aquifer pH are considered further in Section 1.7.3.

1.5.5 Nutrient Supply

In addition to the use of extraneous buffers, it may be necessary to supplement the water undergoing treatment with nutrients to support optimum microbial growth and denitrification activity. D'Angelo and Reddy (1999) demonstrated that nutrient availability is usually correlated with microbial respiration rate. In this respect, groundwater treatments may require additional nutrient supplies whilst surface and wastewaters are more likely to contain sufficient nutrients. For instance, denitrification experiments using chalk groundwaters found that micronutrients were lacking in the aquifer and amendment of the natural groundwater significantly improved heterotrophic denitrification in reactors using chalk from the aquifer (Fourmentraux-Chevron *et al.*, 1998. In this example, P, NH_3 and various metals: Cu, Co, Mo and Fe, were required to support bacterial growth. Some of these metals are essential co-factors in denitrifying enzymes. Phosphate or phosphoric acid is commonly added in conjunction with the OC amendment to supply the necessary P requirement for bacterial growth (Mercado *et al.*, 1988; Abdelouas, 1999). Cartmell (1997) defined a general list of elements necessary to support bacterial growth:

- H, P, N and S as major elements
- K, Na, Mg, Ca and Fe as minor elements
- Mn, Zn, Cu, Co, and Mo as trace elements

However, it may not be necessary to amend the raw groundwater with synthetic nutrients for denitrification treatments *in situ*. For example, there are many examples of natural denitrification in aquifers (e.g. Korom, 1992) where only OC is a limiting

factor, and therefore, it is assumed that the trace elements and nutrients required for bacterial growth must be available from the groundwater or aquifer minerals.

1.5.6 Temperature

Temperature can be an important parameter in managing engineered heterotrophic denitrification. The issue of 'low temperature' denitrification is of considerable economical impact in northern climates, where low temperatures (in the 0-10 °C range) prevail in surface and wastewaters for up to nine months of the year and groundwater is perpetually at a low temperature. Temperature influences the rate of bacterial growth and metabolism, therefore can affect the volume and rate of denitrification. Dawson and Murphy (1972) demonstrated that raising temperature exponentially increased the denitrification rate over their experimental range of 5-27 °C. This exponential relationship is quantified by the *Arrhenius equation*:

$$d(\ln k)/dT = E/RT \qquad \text{(e.g. Metcalf and Eddy, 2003)}$$

Where: k = reaction rate constant

T = temperature (K)

E = a constant characteristic of the reaction (e.g. activation energy)

R = ideal gas constant (8.314 J/mol·K)

When biological reactions in water-treatment processes are modelled, the effect of temperature is commonly expressed as a ratio between reaction rate constants, for example, as defined by Chapra (1997):

$$k(T_2)/k(T_1) = \theta^{(T2-T1)}$$

Where θ represents the temperature activity coefficient and the centigrade scale is used for temperature. The temperature activity coefficient essentially describes the sensitivity of a reaction to temperature change; temperature coefficients for denitrifying bacteria are reported in the range: 1.06-1.08 (Takai *et al.*, 1997).

In practical terms, the effects of low temperatures on denitrification activity is relevant to UK groundwater (where a representative temp. = 10 °C) and decreasing temperature (e.g. <15 °C) appears to lengthen the 'start up' time to denitrification and reduce the amount of denitrification overall (e.g. Dawson and Murphy, 1972). In addition, the influence of temperature on denitrification rate may be relatively exaggerated outside of the 'optimal' range for bacterial activity of 25-35 °C (Metcalf and Eddy, 2003). For example, Jianping *et al.* (2003) noted that denitrification activity

was largely unaffected by temperature increases within the range 20-35 °C, however, their experimental data revealed significantly less activity at 15 °C and 40 °C. It is thought that high temperatures may denature the protein structures of the enzymes whilst low temperatures may inhibit enzyme activity, slowing metabolism overall. The affects of temperature may be ameliorated in certain types of biocommunity, such as biofilms, where the structure of the extra cellular polymers encapsulating and binding the biofilm may have protective, insulating properties (Jianping *et al.*, 2003).

Although denitrification rate is slowed in the low temperature range, it is not inhibited altogether. Indeed, the relative sensitivity of biodenitrification to low temperatures has been described as 'weak' (Cao *et al.*, 2002; Welander and Mattiasson, 2003) and there are several examples of groundwater denitrification in the 10-15 °C temperature range (Janda *et al.*, 1988; Hamon and Fustec, 1991; Bates and Spalding, 1998). Indeed, denitrification processes are stable in this temperature range over relatively long periods of time (Welander and Mattiasson, 2003).

The efficiency of denitrification is determined by the incumbent microbial community, which is in turn influenced by environmental conditions as described. For heterotrophic biological denitrification to be applied as an alternative NO_3^- removal method to ion exchange, it must produce water of a high quality to comply with stringent drinking water regulations. The first approach adopted to engineer biological denitrification for drinking water, was the adaptation of conventional wastewater treatment processes.

1.6 BIOLOGICAL DENITRIFICATION REACTORS: PUMP AND TREAT APPLICATION

Bioreactors have been used for the treatment of wastewater for more than 100 years (Langwaldt and Puhakka, 2000). Wastewater treatment plants commonly have integrated nitrification and denitrification processes for complete N removal, where a single reactor may be exposed to alternating nitrification and denitrification phases, or separate nitrifying/denitrifying reactors are coupled together. Various designs of bioreactor are used in WWTPs, depending on economic and practical criteria such as location, type of wastewater and degree of N removal required. Reactors may vary according to: flow characteristics, carbon source/supply method, the nature of the microbial community and the method by which the wastewater requiring treatment is brought into contact with the microbial community. The principles of wastewater treatment have been applied in groundwater clean up to remove NO_3^- and other compounds, particularly organic contaminants arising from industrial pollution. For example, biological reactors may be brought to a contaminated site, the polluted

groundwater pumped from the aquifer and treated in the bioreactor before being discharged back to the aquifer. This 'pump and treat' approach may additionally used to treat groundwater intended for potable supply. Pump and treat reactors are designed according to the nature of the contamination, fulfilling technical, environmental and economic requirements. Factors particular to groundwater remediation include:

- Comparatively low contaminant concentration at outset, which may present problems for biomass accumulation, resulting in a relatively long start up phase;
- High remediation yield requirement, in order to meet regulatory water quality standards;
- Varying environmental conditions, which treatment processes must be able to accommodate;
- The remediation time scales typically last a number of years, thus the treatment facility must be amenable to long term operation. The remediation process must therefore be environmentally sustainable and operationally durable, whilst remaining economically sound over the long term.

A summary of aerobic reactor designs that can be adapted for groundwater treatment is given in Table 1.2. For drinking water denitrification, many different reactor processes have been proposed, but processes involving a fixed biofilm, such as fluidised-bed, packed-bed and biofilters are common (Mateju *et al.*, 1992).

The pump and treat approach to drinking water denitrification has been pioneered in Europe, and particularly in France and Germany. In 1983, two different types of commercial processing facility; BIODENIT and NITRAZUR were opened in France. The BIODENIT process consists of four, fixed-bed denitrification reactors and four post-denitrification units. Contaminated groundwater is denitrified in reactors fed with ethanol and phosphate, based on sand filter principles; water flows downwards through a clayey medium under a slight pressure. The denitrified water is polished using aeration, a sand and activated carbon filter to remove solids and organics and finally disinfected using ozonation and chlorination (Green and Shelef, 1994). In the NITRAZUR process, contaminated groundwater is passed through an up-flow reactor filled with a granular biomass carrier (BIOLITE). Similarly to the BIODENIT process, ethanol is used as the OC substrate and phosphate is added. Post-treatments included aeration, filtration through granular activated carbon (GAC) and chlorination. Nitrite accumulation problems were not observed, although biofouling was observed in the BIODENIT plant. This was resolved through the introduction of a

backwashing cycle, typically carried out for 1 hour following 48 hours of denitrification (Green and Shelef, 1994).

Table 1.2: Summary of aerobic bioreactor designs that can be applied to pump and treat groundwater treatment (Langwaldt and Puhakka, 2000)

REACTOR DESIGN	OPERATIONAL SUMMARY
Trickling Filter - supported bacterial growth	The bacteria are grown on a matrix formed by the support material (e.g. plastic, rock, pumice). Water is allowed to trickle through from the top of the filter to the bottom (under gravimetric force). The filter is aerated from the bottom by an air blower or ambient air. This type of bioreactor has been used in full scale operations but is not commonly reported in scientific literature.
Rotating Biological Contactor - supported bacterial growth	Bacterial cells grow on the surface of rotating disks, which are partially immersed in water and partially in contact with the atmosphere. When the discs are rotated, the bacteria are alternately brought into contact with the water to be treated and the air. This type of bioreactor is not commonly used in the case of groundwater treatment.
Upflow Fixed-Film Reactor - supported bacterial growth	As with the trickling filter, the bacteria are grown on an inert support material, which forms a matrix within the bioreactor. The water being treated is pumped upwards through the matrix; if aerobic conditions are necessary then oxygen is provided by air sparging. This treatment in particular is widely used for the cleanup of contaminated groundwater; it has been successfully implemented at bench, pilot and full scale.
Fluidized-Bed Reactor - supported bacterial growth	In this type of bioreactor, the biomass is grown on a granular support material, which is held in a fluidized bed. The influent contaminated groundwater is diluted with re-circulation water and passed through the bed at a high re-circulation rate. Aeration may be added to the influent line. The advantage of this scheme is that high biomass concentrations may be achieved. Aerobic treatments are successfully used for groundwater cleanup but anaerobic treatments have currently only been used at bench scale.
Activated Sludge Process - bacteria grown in suspension	The essential component of this process is an aeration basin where the activated sludge may mix with the contaminated water and air is injected into the basin. In addition, there is a settling tank where sludge is separated from the effluents and recycled back into the aeration basin, leaving the treated water to be removed.
Upflow Anaerobic Sludge Blanket Reactor - bacteria grown in suspension	Upflow anaerobic sludge blanket reactors are similar to the activated sludge process. Suspended granular biomass degrades contaminants by production of methane and carbon dioxide. These reactors require elevated temperatures (≥ 28 °C) and high organic carbon concentration.

Presently, the BIODENIT and NITRAZUR processes are economically feasible and have demonstrated good long-term operational stability. The excellent results at full scale have propagated the application of both processes throughout France (Mateju *et al.*, 1992). In Germany, the DENIPOR process has also been in operation since the 1980s. Denitrification is carried out in a fixed-bed filter, packed with buoyant carriers and supplied with ethanol and phosphate. The denitrified water is filtered through an

aerated bed of anthracite and sand and subsequently chlorinated. The DENIPOR process is also operating at full scale in plants throughout Germany (Green and Shelef, 1994).

Heterotrophic biological denitrification in reactors of this nature represents one of the most attractive drinking water treatments to date. High quality potable water may be achieved with denitrification and post-treatments. Disadvantages include the persistence of NO_2^-, although this is an inherent risk to most denitrification processes, potential biofouling, which causes pressure increase within the reactor and creates preferential flow channels in the biological support matrix. Finally, biological reactor denitrification requires continual maintenance of the reactor to control environmental conditions such as temperature and pH and additionally to manage the biomass and prevent biofouling. Investments in biological denitrification reactors, particularly with regard to the immobilisation/support of the microbial biomass, continue to optimise the process.

However whilst the biological reactor process has been under development in the last decade, an additional biological denitrification technology has evolved based on the same principles, alternatively utilising the aquifer itself as a natural 'bioreactor.' *In situ* biological denitrification (ISBD) has not experienced the same level of attention as biological reactor denitrification (Dahab, 1993) although the approach could offer significant operational advantages.

1.7 *IN SITU* BIOLOGICAL DENITRIFICATION IN AQUIFERS

1.7.1 General Description

By the 1980s, pump-and-treat biological denitrification had become an established NO_3^- removal treatment for contaminated groundwater resources, however, the economical implications of constructing and maintaining the necessary treatment facilities proved prohibitive, particularly for small, rural communities (Hamon and Fustec, 1991). As a less expensive alternative, the concept of using a portion of the aquifer, adjacent to the abstraction well, as a natural bioreactor for denitrification was proposed (Janda *et al.*, 1988; Mercado *et al.*, 1988).

Heterotrophic denitrification may occur naturally in soils, sediments, and wetlands and has been widely studied in these environs (Hamon and Fustec, 1991; Dahab, 1993; Nutall *et al.*, 1999). Natural denitrification has also been observed in aquifers, but is poorly documented by comparison (Bates and Spalding, 1998; Korom, 1992).

It is assumed that endogenous heterotrophic denitrification is extremely slow in aquifers, owing to nutrient and OC limitation, and natural denitrification in aquifers is thus regarded as insignificant (Mateju *et al.*, 1992; Nutall *et al.*, 1999; Devlin *et al.*, 2000). However, bacteria capable of denitrification are indigenous to most groundwater environments and according to Korom (1992), the range of bacteria that can inhabit aquifers is diverse and large populations may exist.

Under proposed ISBD schemes, an OC substrate is injected into the aquifer via an injection borehole adjacent to the abstraction well or using the abstraction well itself. The OC stimulates growth of the indigenous bacterial community, resulting in enhanced respiration rates. Once the DO in the aquifer has been consumed, NO_3^- will be consumed as the next preferred terminal electron acceptor and denitrification can occur. Conceptually, a potential advantage of ISBD is the minimisation of post-denitrification treatments (Mercado *et al.*, 1988). The granular matrix of the aquifer should exert some filtering action over the denitrified groundwater, retaining the microbial biomass, and also adsorbing some of the soluble organics. Furthermore, the groundwater could be re-oxygenated as it passes from the zone of denitrification activity into the increasingly aerobic region of the abstraction well. The OC injection would theoretically create a zone of biological activity in the immediate vicinity of the injection borehole, as depicted in Figure 1.4. The majority of denitrification activity should occur in the region closest to the injection borehole, and diminish with increasing distance from the injection point, as the OC concentration decreases. Pioneers of ISBD also proposed that residual organics and turbidity produced in the denitrification zone should be further biodegraded, filtered or adsorbed by the aquifer matrix, in a second zone, concentrically formed around the zone of denitrification (Mercado *et al.*, 1988). Finally, the denitrified water is denoted as a third zone, which provides the potable resource (Figure 1.4). This zoned scheme forms the basis of the various abstraction facilities engineered for ISBD (Mercado *et al.*, 1988).

1.7.2 ISBD Operational Schemes

In situ biological denitrification plants may be categorized into four operational designs, which are based on the development of denitrification zones, as depicted in Figure 1.4 and subsequent capture of the denitrified water via an abstraction well. The common operational schemes are summarised in Table 1.3. In some ISBD schemes, the abstraction well is operated intermittently, to allow denitrification to proceed in a batch-like process. Alternatively, the groundwater flow induced by pumping at the abstraction well can be used to maintain the boundaries of the bioreactor in a flow-through process.

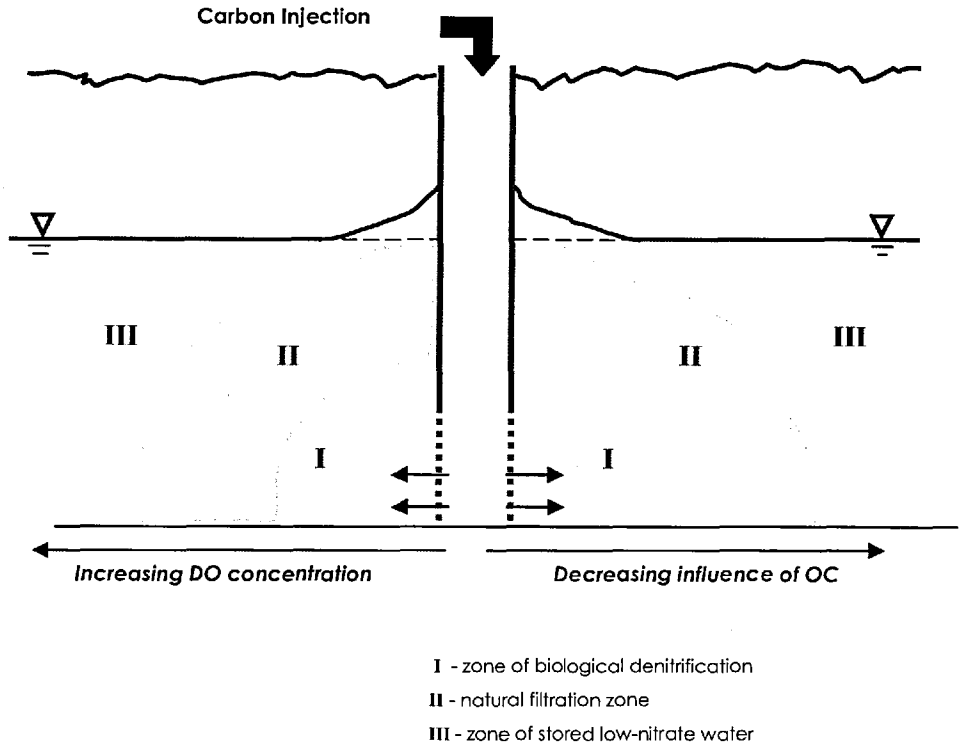


Figure 1.4: Cross sectional schematic of ISBD carbon injection well, illustrating conceptual zones of denitrification activity (adapted from Mercado *et al.*, 1988)

Table 1.3: Operational schemes for ISBD (adapted from Mercado *et al.*, 1988; Dahab, 1993)

ISBD DESIGN	SUMMARY
Dual-purpose well	A single well is used alternately for recharge of aquifer with the OC amendment and pumping of treated water. The system is operated intermittently, to allow time for the denitrification reaction following recharge, before abstraction operation, after which, recharge may continue. An extra well may be required to provide recharge water with which to mix the OC amendment. Owing to the intermittent operation of individual dual- purpose wells, several are necessary to permit continuous operation and to provide adequate yield of denitrified water.
Horizontal doublet system	Two separate wells of the same depth are located 10-20 m apart. One well is used to inject the OC, the other for abstraction of denitrified water. Supplementary wells are used to provide polluted water with which to mix the OC amendment.
Vertical doublet system	This system is similar to the horizontal doublet design, except that the wells are drilled to different depths and the reaction zone is created in the vertical, rather than the horizontal plane. The injection well is the shallower well and denitrified water is abstracted from the deeper well. The wells are in close proximity to one another or may both be situated in a single borehole of sufficiently large diameter. As with the horizontal doublet system, an extra recharge well is required to provide mixing water for the OC substrate.
Daisy system	A single abstraction well is encircled by several injection wells. Carbon is injected into the aquifer and is diluted by the polluted groundwater as it flows towards the central abstraction well (the OC may be mixed with recirculation water prior to injection). Denitrification activity develops in plumes in the direction of flow – towards the abstraction well (see Figure 1.5). Denitrification in this instance is largely governed by the number and spacing of the injection wells and on the lateral dispersion of the substrate.

Every scheme has various advantages and disadvantages; although empirical data on the performance of each scheme is limited, the Daisy System has emerged as the favourite approach. The Daisy System has lower projected costs, based on the simplicity of design; facilitates mixing between denitrified and non-denitrified groundwater at the abstraction well, allowing denitrified water to re-oxygenate; and the denitrification efficiency can be maintained under various discharge rates, without the need for changes to the number of injection boreholes (Mercado *et al.*, 1988). The Daisy scheme is designed to operate in a flow through manner and the conceptual configuration of the denitrification plumes that develop are characteristic of the petals of a daisy flower, as depicted in Figure 1.5.

Under the Daisy System design, the pumping of the abstraction well creates a circular zone of flow from the aquifer into the abstraction well. Injection boreholes are situated within the radius of the 'zone of influence' of the abstraction well, so that the OC amendment is injected into the flow path of the contaminated groundwater and transported towards the abstraction well. The reaction zones defined in Figure 1.4 develop in the direction of flow, giving the overall system configuration a daisy-like pattern. The success of the system depends largely on the geometry of the OC dispersion plumes and the mixing of OC with contaminated groundwater is critical under this scheme, compared to the other plant designs, where a pulse of OC is essentially passed between 2 adjacent points in the horizontal or vertical plane, or a single well is used.

A summary of reported ISBD trials is given in Table 1.4; from the table, it is apparent that little advance in field-scale ISBD has been made since the early examples of ISBD in the late 1980s (Kruithof *et al.*, 1985; Janda *et al.*, 1988; Mercado *et al.*, 1988) and more recent examples of ISBD in the late 1990s still report biofouling and NO_2^- problems (Khan and Spalding, 1998). The only well-documented application of full-scale ISBD is the NITREDOX plant, which has been applied in at least one location in Austria, as reported by Braester and Martinell (1988).

The NITREDOX system, based on the daisy design, was developed into a commercial process by 'Vyrmetoder AB.' It utilises two concentric rings of injection boreholes around a central abstraction well; the OC is injected into the outer ring of the well, creating a zone of denitrification, and the inner injection ring is used for air sparging and release of gaseous products. The second 'oxygenation' ring allows residual NO_2^- to be re-oxidised to NO_3^-, the less harmful N-oxide, and N_2 gas produced by denitrification may be stripped off. In addition, any dissolved Fe or Mn ions may be precipitated out as oxides at this stage, thereby removing them from the drinking

water product. The injection of OC is operated in a cyclic pattern between adjacent injection wells and incorporates a re-circulation element to dilute the substrate. NITREDOX plants have reportedly been used to reduce NO_3^- concentrations of > 100 mg l^{-1} to < 25 mg l^{-1}, with NO_2^- concentrations < 0.02 mg l^{-1} (Braester and Martinell, 1988). No incidence of biofouling or NO_2^- persistence were reported, although other authors have observed that various water quality aspects pertinent to drinking water supply, such as microbial quality, were not reported (Mateju *et al.*, 1992). Recent information on NITREDOX plants could not be found and so it remains unclear whether the plants continued to be successful over the long term. The only other full scale ISBD for drinking water denitrification reported in the scientific literature is the daisy system developed by Janda *et al.*, (1988) in the Czech Republic.

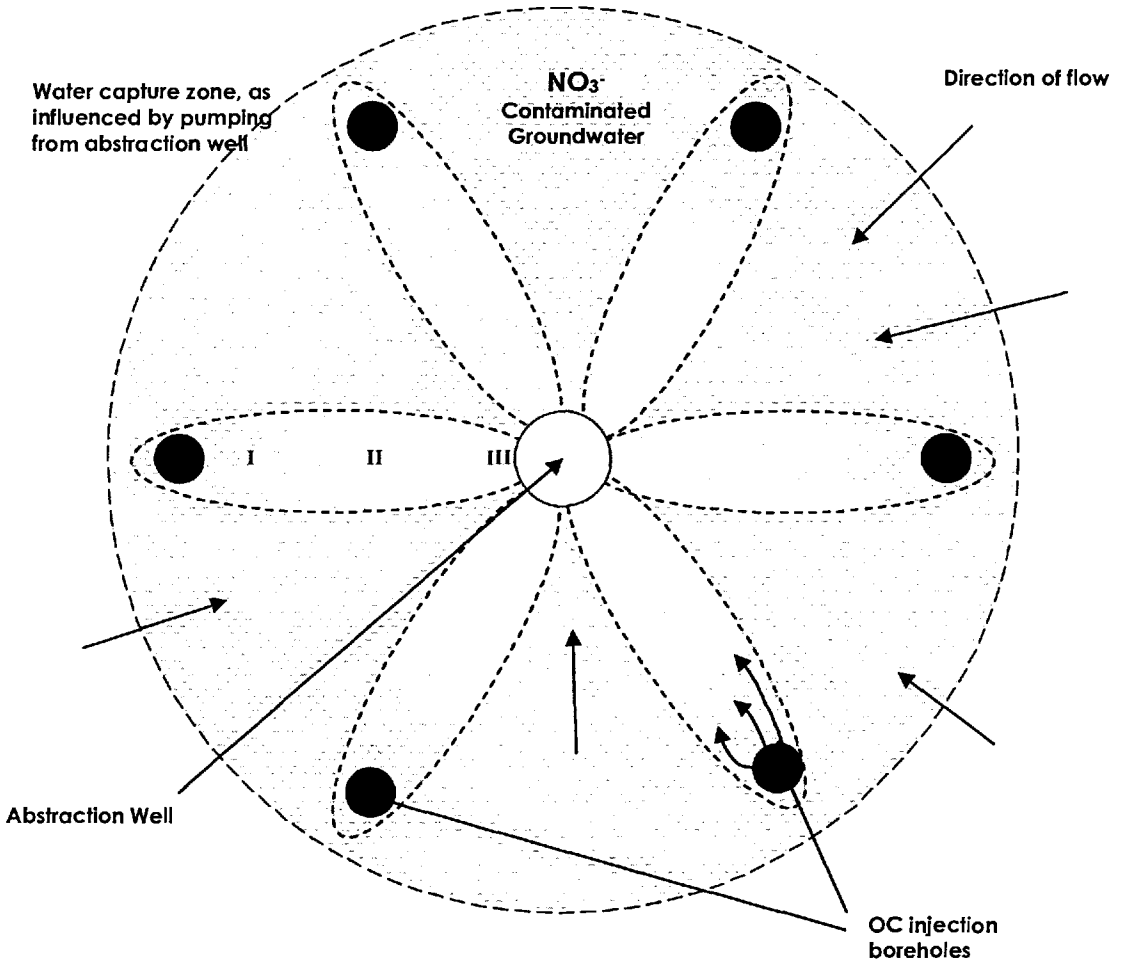


Figure 1.5: Conceptual zones of denitrification in a Daisy-scheme ISBD facility: I = zone of denitrification activity; II = zone of filtration; III = NO_3^- free zone (adapted from Mercado *et al.*, 1988)

Table 1.4: Summary of reported ISBD facilities published between 1988 and 2004 *(Note: 'cnt' = continuous, 'recirc' = recirculation)*

Reference, treatment & location	Aquifer type	Carbon substrate	Operational Details			Initial NO_3^--N mg l^{-1}	% NO_3^- reduced	Observations
			injection regime	injection boreholes	abstraction rate			
Kruithof *et al.* (1985) horizontal doublet design, Netherlands	phreatic	methanol	cnt. & pulsed	min of 3 boreholes @ 10-25m radii	-	19	30 – cnt. 50 - pulsed	This method was found to remove NO_3^- but NO_2^- accumulated. Also, clogging of aquifer was reported. Intermittent methanol dosing did not ameliorate clogging in this instance.
Braester *et al.* (1988) Daisy system, Austria	sand & gravel	ethanol or methanol	cyclic pumping	16 boreholes @ 18m radii from abstraction well	5 Ml/day	23	75	See section on NITREDOX method for further information.
Mercado *et al.* (1988) Daisy system, Israel	hetero-genous	sucrose	pulsed	2 operational boreholes @ 100m depth, 15-25m radii, rate = 0.048-0.1 ML/day	1.2-1.4 Ml/day	14	10	Clogging was experienced in one of the wells, rendering it inoperable and low denitrification yield was observed in the other borehole. Local clogging of substrate injection wells could be reduced by intermittent substrate injection. Aquifer denitrification efficiency depended on hydrodynamic dispersion and local hydrogeological conditions. The authors recommended the use of more injection wells.
Janda *et al.* (1988) Daisy system, Central Bohemia	fine gravel & sand	ethanol with 5 % methanol	cnt. – with and without recirc.	4 boreholes @ 17m depth and 12-15m radii	0.5 Ml/day	25-27	20-30 no recirc. 30.50 with recirc.	Initially, the trial was operated without the recirculation of groundwater. Under this regime, inadequate mixing was demonstrated with very high carbon concentration in some parts, causing breakthrough, whilst in other parts, groundwater was flowing through the system without exposure to carbon. The change in operation to include the recirculation of some of the treated water through the aquifer improved overall efficiency. More boreholes were also recommended to further improve efficiency.
Hamon & Fustec, (1991) Daisy system, France	shallow alluvial aquifer	ethanol	cnt. & pulsed	15 boreholes @ 25m radii	0.7 Ml/day	23	70 for both cnt. & pulsed	This was a successful field demonstration, lowering local NO_3^- concentration to below the EC limit. Increasing no. of boreholes increased homogeneity of the clean up. Clogging was limited by pulsing carbon supply (1 hour on/1 hour off), or, when pumping was continuous, using limiting carbon concentration). Recirculation of some of treated water also ameliarated clogging risks.
Khan & Spalding, (1998) Daisy system , US	sand & gravel	ethanol	cnt. & pulsed	8 boreholes @ 12m depth and 12m radii, rate = 0.065 Ml/day	6 Ml/day	40	35 - cnt. 90-100 - pulsed	This operation proved successful at pilot scale. The continuous regime gave higher denitrification efficiency than the pulse regime, but it also led to complete biofouling after 10 days. This could perhaps have been prevented by recirculation of treated water through the system to dilute the high NO_3^- water. This system also used an inner oxidation ring @ 6m radius (presumably to oxidise any residual NO_2^-).
Chevron *et al.* (1998) line of injection boreholes, France	chalk	ethanol	-	clusters of 3 boreholes, boreholes 3m apart	-	226-565	80	Natural in-situ denitrification was evident prior to remediation in this instance, but was carbon limited. The pulse injection regime used depended on the fissuring of the chalk. Denitrification was achieved in long term operation (450 days). Improvements to the system could have reduced this time period. Rates of denitrification were achieved when trace metals were supplied in conjunction with the carbon substrate.

Mateju *et al.* (1992) reported that this plant had been successfully operating for more than three years with no recorded incidents of biofouling or NO_2^- persistence, although the microbial counts were very close to the maximum permissible. Again, updated information on the Czech ISBD plant is scarce in the literature.

The first wave of ISBD trials, including Janda *et al.* (1988); Mercado *et al.* (1988) and Hamon and Fustec (1991) provided encouraging results for ISBD, however, the process was not sufficiently optimised or manageable to be applied on a wider scale. Indeed, the most recent guidelines for ISBD in the US, published in 2002 for aquifer remediation, continue to cite biofouling as a problem (ITRC, 2002). In their review of biological denitrification, Mateju *et al.* (1992) concluded that ISBD application was problematic and that solutions were difficult to find, owing to the low level of control over essential denitrification parameters under aquifer conditions. Optimisation of the ISBD process requires an understanding of how the critical parameters of denitrification, as defined in Section 1.5, influence denitrification in the aquifer environment. It is understood that biological denitrification in aquifers is additionally affected by the hydrology and geology specific to the ISBD site, therefore, detailed investigation of the immediate site hydrogeology is necessary (e.g. Mateju *et al.*, 1992). However, a more general understanding of the interactions between the geochemistry of the aquifer and the microbial community under OC enhancement is also necessary.

1.7.3 Aquifer Biogeochemistry

Denitrification can be described as a microbially mediated redox reaction whereby NO_3^- is degraded to N_2, and acts as a TEA, as previously reviewed in Section 1.4. There are a number of TEAs that may be used in microbial respiration; O_2, NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^{2-} (e.g. Smith, 2002). From a microbial perspective, the terminal electron accepting process (TEAP) which produces the highest amount of energy for the organism is used preferentially and so, assuming that the TEAs are present in equal amounts, the order in which they will be consumed follows a thermodynamic hierarchy, as described in Table 1.5.

In aquifer environs, the TEAs may be present in various concentrations according to the geological composition and hydrology. Dissolved O_2 and NO_3^- typically enter aquifers during recharge events; Fe and Mn are present in rock minerals such as oxides/hydroxides, carbonates and sulphides; and sulphate occurs in inland groundwaters via mineral dissolution or as a result of seawater intrusion in coastal aquifers (Smith, 2002). The CO_2 (or HCO_3^-) produced as a result of these microbial

processes may be further used as an electron acceptor itself, during the process of methanogenesis.

In aquifers, the thermodynamic ordering of these TEAPs induces a spatial gradient of microbial oxidation-reduction activity that develops in the direction of groundwater flow (Smith, 2002). A succession of TEAPs occurs as the more energetically favourable TEA species become depleted, illustrated in Figure 1.6. The redox (Eh) of the aquifer becomes progressively more reducing with the succession towards methanogenesis, also in the direction of flow.

Table 1.5: Hierarchy of terminal electron accepting processes following aerobic respiration (adapted from Chapelle, 2001)

Process	Oxidation/Reduction Reaction	Potential Standard Free-Energy (kj per mole of H)
Denitrification	$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	224
Mn (IV) reduction	$MnO_2 + H_2 \rightarrow Mn(OH)_2$	163
Fe (III) reduction	$2\ Fe(OH)_3 \rightarrow 2\ Fe(OH)_2 + 2H_2O$	50
Sulphate reduction	$SO_4^{2-} + 4H_2O + H^+ \rightarrow HS^- + 4H_2O$	38
Methanogenesis	$HCO_3^- + 4H_2 + H^+ \rightarrow CH_4 + 3H_2O$	34

Groundwater Eh may therefore provide a qualitative indication of the antecedent TEAPs, and the metabolic capacity of the aquifer (Smith, 2002). However, whilst the distribution of TEAPs is largely determined by microbial activity, it is also significantly influenced by aquifer geochemistry and hydrological flow/transport processes (Ludvigsen *et al.*, 1998; Smith, 2002). Several studies have found that more than one TEAP may occur simultaneously in leachate plumes, for example, depending on the availability of TEA species and the heterogeneity of the aquifer, which may harbour localised zones of differing redox potential. Nonetheless, it appears that each TEAP zone is dominated by one process (Ludvigsen *et al.*, 1998; Groffman and Crossey, 1999).

Microbially mediated degradation of OC therefore has a significant effect on the geochemical transformations in the aquifer including: depletion of TEAs O_2, NO_3^- SO_4^{2-}; reductive dissolution of iron and manganese (hydr)oxides; precipitation of carbonate and sulphide minerals and changes to alkalinity and pH. The various biogeochemical processes are interrelated, with one process initiating another and so on. The introduction of an extraneous OC substrate into a relatively pristine aquifer environment therefore has consequences for the overall ecology of the aquifer, potentially changing the characteristics of the aquifer irreversibly (Dahab, 1993). In

this regard, engineered ISBD processes attempt to control the biogeochemistry within a defined reaction zone, determined by the process design, as discussed in Section 1.7.2.

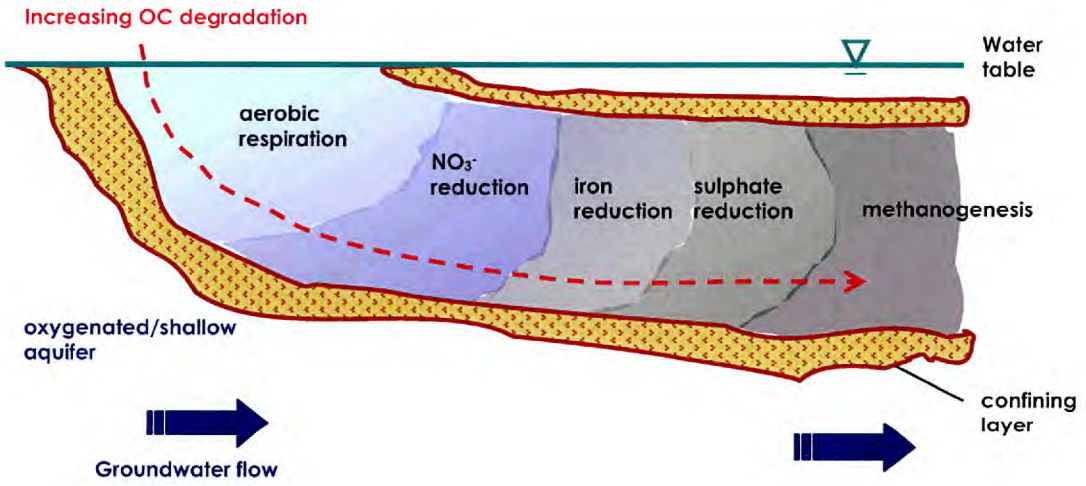


Figure 1.6: Spatial distribution of TEAPs in a pristine aquifer (OC limited), controlled by microbial degradation of OC (adapted from Korom, 1992; Smith, 2002). The red arrow indicates the increasing oxidation of the OC substrate and decreasing redox (Eh) of the groundwater.

An idealised ISBD process should maintain denitrification as the dominant TEAP in the reaction zone and be potentially controlled by the OC injection. The OC dose can be controlled so as to deplete DO and NO_3^-, allowing complete denitrification and thereby minimising potential NO_2^- accumulation, as discussed in Section 1.5.2. To preserve the redox status and prevent alternative TEAPs from arising, it is equally important not to over-dose the system with OC, as this would encourage increasingly reducing conditions and initiate alternative TEAPs.

1.8 EFFECTS OF ISBD ON AQUIFER AND GROUNDWATER

1.8.1 Biogeochemical transformations

The onset of the alternative TEAPs: *Fe-, Mn- and* SO_4^{2-} reduction within the reaction zone of an ISBD plant would have damaging ramifications for the quality of abstracted groundwater and may also interfere with operational facilities. For example, the reduced product of SO_4^{2-} would be hydrogen sulphide (H_2S), as described in Table 1.4. This reduced S-species is aesthetically undesirable as it emits a strong 'rotten egg' odour and may taint the water. Reductive dissolution of Fe and

Mn from the poorly crystalline mineral Fe^{3+} and Mn^{4+} forms, to the soluble Fe^{2+} and Mn^{2+} species, increases the concentration of these ions in groundwater. As a result, the concentration of Fe^{2+} and Mn^{2+} in abstracted water may rise, impairing the quality and potentially tainting the taste and odour of the water (e.g. Coates and Achenbach, 2002).

A more serious problem involving mobilised Fe^{2+} is the re-oxidisation to Fe^{3+} in the more aerobic zone of the ISBD abstraction well. Ferric ion (Fe^{3+}) forms (hydr)oxides and precipitates out of solution, with deleterious operational consequences. The development of solid Fe minerals at abstraction facilities is a commonly observed phenomenon, causing the casing of the abstraction well to become clogged so that it may no longer draw groundwater from the aquifer. Furthermore, the water quality becomes impaired and corrosion problems may arise in the distribution network (Coates and Achenbach, 2002). Indeed, ferrous iron (Fe^{2+}) is one of the most common problems for water abstraction facilities globally, and remediation of iron-fouled wells is a major industry (McLaughlan, 1996; Coates and Achenbach, 2002). The volume of biogenic Fe^{2+} mobilised in the groundwater is relatively small, however, and a greater portion is retained in solid form. For example, forming carbonate, sulphide or silicaceous minerals: siderite ($FeCO_3$), pyrite (FeS_2), biotite ($[(Mg, Fe^{2+})_3(Si_3Al)O_{10}(OH)_2]K$); or bonding to the surface of crystalline Fe^{3+} oxide minerals (Coates and Achenbach, 2002). Similarly, the reduced Mn^{2+} ion may be retained in the solid phase of the aquifer by adsorbing to other minerals or forming oxides (e.g. Mn_3O_4) or carbonates (e.g. $MnCO_3$) (Gounot, 1994).

The dissolution and precipitation of minerals in the ISBD reaction zone is also related to the pH, as well as the Eh of the environment (McLaughlan, 1996). Ordinarily, groundwater pH is expected to be in the circum-neutral range necessary for optimal denitrification activity, as discussed in Section 1.5.4. Changes in pH occur as a result of microbial activity; denitrification is commonly associated with increasing alkalinity (pH), as bicarbonate (HCO_3^-) or hydroxide (OH^-) may be reaction products (Korom, 1992; ITRC, 2002). As a general observation, mineral phases of Fe and Mn tend to be insoluble under alkaline conditions (e.g. Stumm and Morgan, 1981). In order to maintain the pH at natural aquifer conditions, a phosphate buffer may be added in combination with the OC injection during bioremediation (Rust *et al.*, 2000), however, many authors report that the natural buffering capacity of the aquifer rock is sufficient to compensate for changes in pH (Dahab, 1993; ITRC, 2002). Furthermore, Nutall *et al.* (1999) reported that aquifer pH returned to pre-denitrification conditions following a single well ISBD trial.

Clearly, an important aim of ISBD would be to minimise the inherent disruption of the aquifer's biogeochemical ecology caused by the OC injection, whilst still being able to facilitate denitrification. Ultimately, undesirable changes in the chemical equilibrium of the aquifer will impinge on the quality of the denitrified water abstracted from the ISBD plant.

1.8.2 Water Quality

As potable water, groundwater is conventionally regarded as a relatively clean resource, compared to surface water sources. The high quality of groundwater historically has meant that very few, if any post-abstraction treatments were necessary. Biological denitrification improves the quality of the water with regards to NO_3^- contamination, but can impair the bacterial and chemical quality of the water. Denitrified water may have low O_2 content, depending on the amount of O_2 diffusion in the zone nearest the abstraction well and the presence of residual OC from the substrate injection. Dissolved Mn and H_2S have also been observed at the abstraction well, as has OC residuals. Most authors therefore advocate the use of filtration and aeration as post-treatments (Mercado *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993). Disinfection, however, is the most common requirement for groundwater treated by ISBD, as high counts of bacterial cells are frequently reported (e.g. Dahab, 1993).

1.8.3 Microbial Biofouling

Microbial cells adhere to any submerged surface in a natural aquatic environment, initially associating with the surface via weak forces of attraction before cementing more tenaciously to the surface with the aid of extracellular polymeric substances (EPS) that extend from the cell. As more cells accumulate and extrude EPS, the fibres lend structure to the assemblage, and a biofilm is formed. Additionally, suspended solids become trapped in the EPS and contribute to the biofilm structure (Characklis and Cooksey, 1983). Biofilms are spatially and temporally heterogeneous structures and the ecology of biofilms is complex. The volume of a biofilm depends principally on the rate of bacterial growth and product formation, relative to decay processes such as cell death and biofilm detachment. In turn, these processes are influenced by environmental conditions such as fluid dynamics and nutrient availability. Biofilms are harnessed for several useful water treatment processes, including denitrification in pump-and-treat reactors. The term 'biofouling' is used to describe the undesired formation of biofilms or the overgrowth of beneficial biofilms (Characklis and Cooksey, 1983). Biofilm morphology in porous media, such as aquifer matrices and various water treatment reactors is considerably different to the formation of biofilms on flat surfaces and is poorly understood in comparison (Paulsen *et al.*, 1997). On flat

surfaces, biomass essentially forms a continuous film, which may increase in thickness, depending on nutrient loading and diffusivity in the biofilm and fluid dynamics such as turbulence. In contrast, biofilm development in porous media is thought to be more web-like in structure, maintaining a large surface area and minimal depth (Paulsen *et al.*, 1997). However, generalisations should be avoided, as several contrasting reports of biofilm formation in porous media have been made. Some authors have observed continuous biofilm formations, similar to more 'classical' biofilm morphology, whilst others described the formation of biofilm aggregates in pore throats, lending a discontinuous, 'patchy' formation overall (Rittman, 1993). Again, the factors influencing these distinct types of morphology are detailed and complex, including: nutrient loading, size of constituent particles within the porous medium and fluid conditions (Rittmann, 1993). In aquifers, it is proposed that biofilm distribution and morphology will be similar to that observed in drinking water treatment biofilters, because the particle size and nutrient loading of the porous media is similar, compared to wastewater treatment biofilters, where the medium size is larger and the substrate loading is much higher (Rittmann, 1993).

In aquifers, clogging of the porous matrix is attributed to the proliferation of biomass and consequently, accumulation of suspended solids trapped in the EPS, binding of gas bubbles in the EPS and accumulation of clay within the EPS (Rinck-Pfeiffer *et al.*, 2000). In the first wave of ISBD field trials in the 1990's, clogging severely hampered operation in most examples, even though the applications were for drinking water purposes, where it can be assumed that the nutrient load was comparatively low (e.g. Mercado *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993; Abdelouas *et al.*, 1999). Although a common problem, biofouling is scarcely researched and predictive mathematical/computer models generally fail to account for the interaction between the biological phase and physical and chemical contributors, such as filtering action and dissolution/precipitation of minerals. Moreover, laboratory studies using actual materials taken from the aquifer, rather than synthetic porous media and water, are rare (Baveye *et al.*, 1998; Rinck-Pfeiffer *et al.*, 2000).

In terms of biological clogging, the thickness/density of the biofilm growth is thought to be related to physical factors such as sheer stress and turbulence in the fluid phase (Characklis and Cooksey, 1983; Alves *et al.*, 2002), although the proliferation of cells and production of EPS is ultimately caused by the nutrient supply (e.g. Acuna *et al.*, 2002). The production of EPS is critical in aquifer biofouling, as the fibrous web formed may occupy a volume many times greater than the bacterial cells themselves (Thompkins *et al.*, 2001). However, increased EPS production is not simply a function of increased cell number, but is also influenced by other factors such as the growth

phase of the cell and nutrient availability. For example, more EPS may be produced during the log phase of cell growth than by stationary phase cells, and C-limitation may retard EPS formation whilst N-limitation typically promotes EPS synthesis (Characklis and Cooksey, 1983). Furthermore, EPS production does not necessarily contribute to clogging, since EPS extruded from bacterial cells can take on different forms; closely associated capsules that surround the cell, or loosely associated 'slimes' that may extend away from the cell. Vandevivere and Baveye (1992) observed that in saturated porous media inoculated with different variants of an environmental bacteria, non-mucoid strains did not produce any detectable EPS, capsule-producing strains had little effect on the hydraulic conductivity (analogous to clogging) whilst slime-producing strains had a large impact on hydraulic conductivity. In this case, the reduction in hydraulic conductivity was tentatively attributed to the obstruction of flow channels with slime (Vandevivere and Baveye, 1992).

The restriction of flow channels by increasing biofilm volume is the mechanism by which aquifers may become clogged. Decreased cross sectional area of pores and, to a lesser extent, increased frictional resistance of the rough biofilm surface contribute to reduce the permeability of the porous media and hence, reduce the hydraulic conductivity (Rittmann, 1993; Baveye *et al.*, 1998). Under low substrate loading conditions (e.g. aquifers), where biofilm formation is thought to be discontinuous, detachment processes play a large role in dispersing the biofilm, where the biofilm aggregates can be transported and deposited at a different pore throat (Rittmann, 1993). The patchy biofilms that develop in relatively homogenous media such as sandstone affect the flow distribution throughout the matrix by diverting flow from areas of initially high permeability to areas of initially low permeability (Paulsen *et al.*, 1997). When the porous medium becomes completely clogged, hydraulic conductivity becomes degraded until flow ceases. In ISBD applications, clogging events almost universally occur in the region adjacent to the nutrient injection point, where biomass proliferation is greatest and clogging may be exacerbated by physical phenomena such as filtering of suspended solids. To a lesser extent, clogging may also occur at the discharge point, although this is more likely to be caused by chemical dissolution/precipitation of minerals (Dahab, 1993; Rinck-Pfeiffer *et al.*, 2000) (refer also to Section 1.8.1 on well fouling).

To combat biological fouling at nutrient injection wells, a commonly adopted strategy is the intermittent injection of organic substrate in combination with recycled water from the abstraction well (e.g. 10 % of total discharged volume) (e.g. Mercado *et al.*, 1988; Baveye *et al.*, 1998). This strategy promotes increased distribution of the organic substrate into the aquifer matrix. As a result, less substrate is consumed in the vicinity of the injection borehole and the biomass is less concentrated. The increased

travel of the substrate front into the aquifer matrix facilitates an increased biologically active zone and can promote higher contaminant degradation rates (Peyton, 1996). However, it is not clear whether substrate pulsing prevents clogging from occurring completely, the implication being that hydraulic conductivity may still be severely reduced at a point further away from the injection well (Baveye *et al.*, 1998). Indeed, Hamon and Fustec (1991) had to terminate ISBD trials under comparatively low nutrient loading, owing to aquifer clogging, even under intermittent substrate injection. Alternatively, various biocides have been employed to temporarily relieve clogging, in soils and sediments including chemical treatments such as hydrogen peroxide, chlorine, sodium hypochlorite and various acids (Baveye *et al.*, 1998), although such treatments would clearly have to be compatible with drinking water requirements (Hamon and Fustec, 1991). In some instances biofilm sloughing, defined as the random detachment of a large section of biofilm (Characklis and Cooksey, 1983), has been observed to reverse decreasing trends in hydraulic conductivity. Biofilm sloughing is poorly understood, but has been attributed to a number of factors including: increased sheer stress acting on the biofilm in the reduced pore volume; forces exerted by gaseous N_2 bubbles in denitrifying biofilms; accumulation of toxic metabolites; decomposition of biomass that weakens the adhesive forces in sessile colonies; shifts in microbial flora. However, as previously described, biomass sloughing may equally decrease permeability by drifting to an alternative location and re-establishing (Baveye *et al.*, 1998).

Biofouling is therefore, a complex and dynamic phenomenon, which is mutually dependent on biological, physical and chemical clogging elements. At present, biological clogging cannot be reliably predicted in a given situation (Baveye *et al.*, 1998), and proposed strategies for minimisation and amelioration are not completely guaranteed. Biofouling may severely compromise bioremediation schemes including ISBD, and is a subject for continued research.

1.9 DEVELOPMENTS IN ISBD

Field trials in the late 1980s to early 1990s (e.g. Janda *et al.*, 1988; Mercado *et al.*, 1988; Hamon and Fustec, 1911; Dahab, 1993), revealed that ISBD had several shortcomings, including presence of organic residues and bacterial cells in the denitrified water and severe clogging around the injection wells. During the last decade, there have been few reported advances in ISBD technology. Guidelines for ISBD (field scale) of contaminated aquifers have been published in the US, but this publication seems to be more geared towards remediation of contaminant plumes

within large sections of aquifer, rather than ongoing treatment of drinking water using an abstraction facility as a bioreactor (ITRC, 2002). Indeed, much of the work on aquifer denitrification is conducted from a leachate-remediation perspective where the NO_3^- served as a reductant for organic pollutant plumes (e.g. Martienssen and Schops, 1997; Spence *et al.*, 2001). It appears that the majority of research over the last decade has involved laboratory column studies and bioreactors, focussed almost entirely on process optimisation with the primary objective of maximising denitrification yield. Various attempts have been made to ascertain the affects of DO, pH, temperature and carbon substrate on denitrification activity (as described in Section 1.5). Carbon substrate in particular has come under scrutiny, with much attention devoted to the appraisal of different kinds of substrate, C/N ratios and optimisation of injection regimes (e.g. Her and Huang, 1995; Abdelouas *et al.*, 1999; Mosheni-Bandpi *et al*,. 1999; Rajapakse and Scutt, 1999; Devlin *et al.*, 2000; Gomez *et al.*, 2000). The spate of laboratory experiments has lead to a better definition of the parameters controlling denitrification including denitrification enzyme activity and particularly reaction kinetics (e.g. Almeida *et al.*, 1995; Burns *et al.*, 1996; Glass and Silverstein, 1998). This has lead to a flurry of models on biological denitrification in laboratory columns (e.g. Lu *et al.*, 1999; Bourrel *et al.* 2000: Cao *et al.*, 2002; Strong and Fillery, 2002).

The literature reviewed for this Chapter would suggest that research has moved towards refined biological denitrification reactor processes for drinking water treatment and autotrophic processes have also gained increased attention. Follow-up publications from the authors of early ISBD field trials (Janda *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993) were not evident from the literature searches for this review. The initial problems encountered with ISBD, such as aquifer clogging and impacts on water quality may have deterred research from this approach to drinking water treatment. However, now that biological denitrification is increasingly better documented, it would be pertinent to readdress the potential application of ISBD for drinking water treatment since it is estimated that ISBD is still economically favourable compared to pump-and-treat, bioreactors, with cost-savings of a factor of five predicted (Abdelouas *et al.*, 1999).

1.10 SUMMARY

In-situ bioremediation technology has been used for several years in the clean-up of point source pollution in the subsurface environment, particularly with regards to hazardous chemical and oil spills. The degradation of contaminant plumes is mediated by indigenous microbial communities, often involving the injection of TEAs

to remediate OC plumes. However, application of the same *in situ* bioremediation principles for NO_3^- removal has been approached with scepticism. Since groundwater contaminated with NO_3^- is still a relatively pristine resource, there is a reluctance to introduce extraneous substances into the aquifer and alter the existing equilibrium (Dahab, 1993). Likely perturbations resulting from ISBD include changes in DO, pH, redox potential, and mineralogy. Furthermore, the quality of the denitrified water under heterotrophic schemes may be altered with respect to bacterial count, turbidity and residual organics and post treatments such as filtration, disinfection and oxygenation may be necessary. The potential risk posed by biofouling, however, is probably the most potential limitation to the further development and application of ISBD.

However, biological denitrification has undergone considerable process optimisation and the interaction between biological, physical and chemical elements in the aquifer is increasingly better defined. With the increased catalogue of knowledge, ISBD could provide a competitive technology for drinking water treatment. The cost-savings using ISBD are not disputed and the process may be easily automated with fewer process engineering/control and maintenance requirements compared to pump-and-treat technologies or conventional NO_3^- removal processes such as IE. In countries with large rural areas such as the US and France, ISBD could be particularly advantageous to small communities with smaller budgets for municipal water supply treatments (Hamon and Fustec, 1991) and in the UK, a regional Water Utility is has expressed an interest in ISBD for NO_3^- removal at a small rural town supply well, where the groundwater has been affected by local farming land use (refer to Chapter 2, Section 2.2.4.

To promote the further investigation of ISBD at field scale in the UK, research into the quality of denitrified water produced under UK conditions is required, particularly with regards to groundwater temperature and mineral composition of UK aquifers. In addition, the risk of biofouling must be quantified and the affect of ISBD on the microbial ecology of the aquifer better defined. To this end, a laboratory research programme has been undertaken to demonstrate the practicality of biological denitrification and assess denitrified water quality under UK-representative conditions. The principal focus of the research program was to understand and control the biochemical dynamics of NO_2^- production and quantification of the hydraulic conductivity in the aquifer material, in relation to biological clogging. Attention was also given to the potential affects of TEAPs and DNRA on water quality, and the occurrence of OC residual. To facilitate the further development of microbial ecology concepts in relation to ISBD, novel DNA techniques were applied to

characterise the indigenous denitrifying community from three different geological rock types, grown using two different carbon substrates. The experimental rational to achieve these objectives is described in the following Chapter.

CHAPTER 2

EXPERIMENTAL RATIONALE AND OBJECTIVES

2.1 DEVELOPMENT OF EXPERIMENTAL OBJECTIVES

2.2 RATIONALE FOR MICROCOSM TRIALS

2.2.1 MC TRIAL I: Laboratory Microcosm optimisation with Glucose supported Denitrification

2.2.2 MC TRIAL II: Denitrification using Sodium Acetate

2.2.3 MC TRIAL III: Denitrification in conjunction with Alternative Electron Accepting Reactions

2.2.4 MC TRIAL IV: Denitrification using Greensand Aquifer Materials

2.1 DEVELOPMENT OF EXPERIMENTAL OBJECTIVES

As demonstrated in the literature review, previous research on ISBD is process-orientated; experimental studies have been dominated by denitrification kinetics and process efficiency, especially regarding the electron donor (e.g. Hamon and Fustec, 1991; Dahab, 1993; Devlin *et al.*, 2000). Much work on biological denitrification has been published for wastewater treatment purposes, regarding the affects of alterations in key parameters such as dissolved oxygen, temperature and pH on process outputs (e.g. Dawson and Murphy, 1972; Glass and Silverstein, 1998; Oh and Silverstein, 1999). Such information may be readily applied to biological reactors for 'pump-and-treat' biological denitrification of groundwater for drinking, but is not so directly applicable to *in situ* biodenitrification (ISBD). Moreover, there is a scarcity of detailed quantitative information on the long term operational success of ISBD, and its impacts on the local aquifer conditions and abstracted groundwater has apparently deterred Water Utilities in the UK from pursuing ISBD as a NO_3^- removal treatment.

Therefore, to better define the effects of ISBD on groundwater quality and aquifer characteristics, with regards to potential application in the UK, the following hypothesis were investigated:

1. That NO_2^- accumulation could be minimised by the selection of a substrate other than glucose, previously proposed for use in UK ISBD trials;

2. That DNRA would not occur as a denitrification pathway, under the C/N ratios used for ISBD;

3. That alternative electron accepting processes such as iron, manganese and sulphate reduction would not compromise the quality of the abstracted water;

4. That the quality of the denitrified water would not be compromised by bacterial contamination resulting from enhanced biomass growth stimulated by organic carbon (OC) injection, commonly observed in biological denitrification reactors.

In addition, this project sought to define a quantitative relationship between biofouling and nutrient loading concentrations (i.e. NO_3^- and OC). Therefore, methods of soil biomass quantification, including: measurement of ATP (adenosine tri phosphate) and EPS (extracellular polymeric substances; and molecular, DNA-based techniques such as PCR (polymerase chain reaction) and DGGE (denaturing gradient gel electrophoresis) were developed for use with sandstone material.

2.2 RATIONALE FOR MICROCOSM TRIALS

The experimental hypotheses outlined above were addressed by observing water quality and sandstone characteristics in denitrifying laboratory microcosms. Microcosms are typically employed in the study of biochemical aquifer processes because they are considered sufficiently representative of aquifer conditions and afford precise control over experimental parameters and surveillance of experimental outputs (Obenhuber and Lowrance, 1991; Hamon and Fustec, 1991; Rinck-Pfeiffer *et al.*, 2000). Various configurations of microcosm assembly are documented, essentially comprising a cylindrical container, filled with aquifer material with inlet/outlet ports to facilitate flow-through capability. Additional ports for sampling and 'on-line' monitoring devises may also be incorporated.

The experimental programme presented here was based on quantitative measurements of biological denitrification in flow-through microcosms using a Triassic sandstone matrix and indigenous bacterial community. The microcosms were continually supplied with 'feed' solutions and microcosm effluents were sampled throughout operation. Changes between influent feed and effluent (denitrified water) were analytically quantified. Nitrate and various OC substrates were supplied to the microcosms in pure water, representing the contamination and treatment, respectively. Biological nutrients (essential for bacterial growth) were incorporated to ensure that denitrification was not inhibited by nutrient deficiency. Feed solutions were simple in composition, to avoid obscuring the denitrification data and to aid continuity throughout the trials. All microcosm treatments were performed in duplicate microcosms and controls were also included. The microcosm trials were managed as discreet experiments, but the data obtained were consolidated to facilitate integrated interpretation. The rationale for individual trials and selection criteria for specific analytical methods are discussed in the following Section.

2.2.1 MC TRIAL (I): Laboratory Microcosm optimisation with Glucose supported Denitrification

The principal objective of this trial was to demonstrate the performance of the experimental system. The microcosm apparatus used for all trials was previously custom-built and used in denitrification modelling studies carried out by Imperial College and WRc plc (Cartmell *et al.*, 1997; Tompkins *et al.*, 2001). However, as part of this PhD research, alterations to the equipment and experimental methods were made, including:

- Modifications to the influent delivery and effluent collection equipment;
- Incorporation of pressure transducers;
- Incorporation of custom-built redox probes;
- Changes to nutrient preparation and storage;
- Introduction of a more comprehensive range of analytical measurements: NH_4^+, NO_2^-, SO_4^{2-}, filterable Fe and Mn, bacterial counts, biomass determinations

The operation of the revised microcosm apparatus was assessed by a short denitrification experiment using glucose as the OC substrate, according the previous experimentation with the microcosm apparatus (Cartmell *et al.*, 1997; Tompkins *et al.*, 2001). The daily observational record of the trials together with the data collected enabled the sampling and analyses strategy to be optimised and refined for future trials and provided a set of reference data.

A secondary aim of this trial was towards optimising the OC dose, (refer to Chapter 1). The carbon dosage regime for this experiment was based on the stoichiometry for the reaction of glucose with DO and subsequently the reaction of glucose with NO_3-N. This dosage was supplemented by 10% for biomass assimilation, based on conservative estimates of biomass assimilation (e.g. ITRC, 2002) to arrive at an estimated optimum OC dose. Two further doses were also selected for experimentation; a 'low' dose, at 50 % of this optimum; and a 'high' dose, which had twice the OC allowance for [denitrification + deoxygenation], approximating to 177 % of the optimum.

2.2.2 MC TRIAL (II): Denitrification using Sodium Acetate

The principal aim of the second experiment was to evaluate a non-fermentable carbon substrate, sodium acetate, as an alternative to glucose for ISBD applications. Glucose is generally regarded as a universally bioavailable substrate for microbial growth, and is typically used as a model substrate for microbial degradation activity. However, it has

been repeatedly demonstrated that fermentable substrates such as Sucrose and Glucose are not suitable for ISBD applications, and are linked with biofouling and significant NO_2^- production (described in Chapter 1). In spite of the majority of evidence disapproving the use of Glucose in ISBD applications, preliminary investigations on UK ISBD, jointly funded by the Environment Agency, Severn Trent Water and Hanson Waste Management, (Cartmell *et al.*, 2000), continued to specify the use of glucose. Apparently this was on the basis that its injection into the saturated zone would be potentially authorised (Dr. S. R. Smith, pers. comm.).

Therefore, as part of the investigations for this PhD, microcosm trials were used to demonstrate the feasibility of alternative OC substrates, with regards to denitrification yield and quality of denitrified water.

Sodium acetate and ethanol were considered as alterative substrates but only one could be selected as it was also desirable to include a glucose regime as a comparison. Ethanol was rejected as previous comparison of these two substrates had favoured acetate (Abdelouas *et al.*, 1999), with biofouling experienced in the ethanol experiment. Additionally, ethanol is less practical for ISBD as it has more COSHH (Control of Substances Hazardous to Health) regulations for handling and storage (i.e. its high flammability) and is also less economical.

The OC dosing regime for the glucose and sodium acetate was based on the reaction stoichiometry, as described for MC Trial I, substituting acetate for glucose where necessary.

2.2.3 MC TRIAL (III): Denitrification in conjunction with Alternative Electron Accepting Reactions

In this microcosm trial, the potential for alternative electron processes to compete for electrons from the OC substrate was assessed, since it is known that reduction of Mn and Fe may overlap with denitrification as TEAPs (refer to Chapter 1.7). Terminal electron accepting substances Fe^{3+}, Mn^{4+} and SO_4^{2-} (TEA's) which may be present in the relatively pristine aquifers used for potable water, were incorporated during the experiment, in addition to OC and NO_3^-.

Under natural UK groundwater conditions, Fe and Mn are present as insoluble minerals contained in the aquifer rock, whereas SO_4^{2-} typically disassociates from minerals such as calcium sulphate (Gypsum) or magnesium sulphate (Epsomite) and is present as the

soluble SO_4^{2-} ion in groundwater (Kimblin, 1995). In this regard, three different types of sandstone; Triassic sandstone, Lower Greensand and Devonian sandstone were used in the microcosms to supply a gradient of mineral Fe and Mn contents, as described in Table 2.1. Conversely, SO_4^{2-} was dissolved into the microcosm feed solution from solid calcium sulphate, a common source of soluble SO_4^{2-} in aquifers (Czerewko *et al.*, 2003). Sulphate concentrations in groundwater vary but concentrations in the range 60-70 mg l^{-1} are reported in UK aquifers (Thornton *et al.*, 2000; Goody *et al.*, 2002). In this experiment, a median concentration of 65 mg SO_4^{2-} l^{-1} was used.

Sodium acetate was used as the OC substrate, at a dose of 35 mg C l^{-1} based on the denitrification results of MC Trial II.

Table 2.1: Iron and manganese contents of three test sandstones used during Trial III (measurements are of total metals extracted from solid rock mass)

Sandstone Type	Total Fe (mg kg^{-1} dry wt)	Total Mn (mg kg^{-1} dry wt)
Triassic sandstone (Burntstump, Nottingham)	2446	80
Lower Greensand (Westerham, Kent)	11108	76
Devonian sandstone (Forest of Dean, Gloucestershire)	16756	455

2.2.4 MC TRIAL (IV): Denitrification using Greensand Aquifer materials

This experiment was carried out in conjunction with preliminary hydrogeological investigations for a full scale ISBD trial for the water utility, Wessex Water, at the Fovant Public Water Supply facility in Wiltshire (NGR: SU 013286). This is an important, isolated local drinking water supply source with an abstraction licence of 2.07 Ml d^{-1} , but the groundwater has exhibited increasing NO_3^- concentrations since the mid 1970s, (Figure 2.1), and has recently exceeded the EU MAC for NO_3^- (11.3 mg NO_3-N l^{-1}).

The Fovant site is situated on a minor Upper Greensand aquifer, where the Greensand layer comes to the surface, but is underlain by an aquiclude of impermeable Gault clay. The abstraction boreholes draw water entirely from the Upper Greensand aquifer, which varies between 25-40 m thick. The sandstone in the vicinity of the site is a mixture of glauconitic sand and sandstones with hard, cemented sandstone, tending towards clay-rich sandstone at a depth of >25 mbgl. There is a significant degree of fissuring at the

site, but the fissuring varies greatly, leaving relatively homogenous zones (Source: Wessex Water).

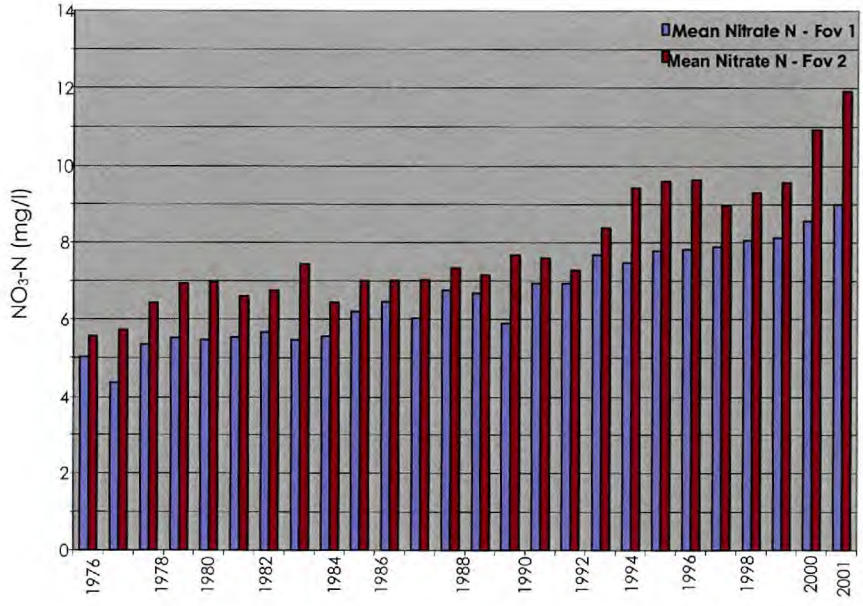
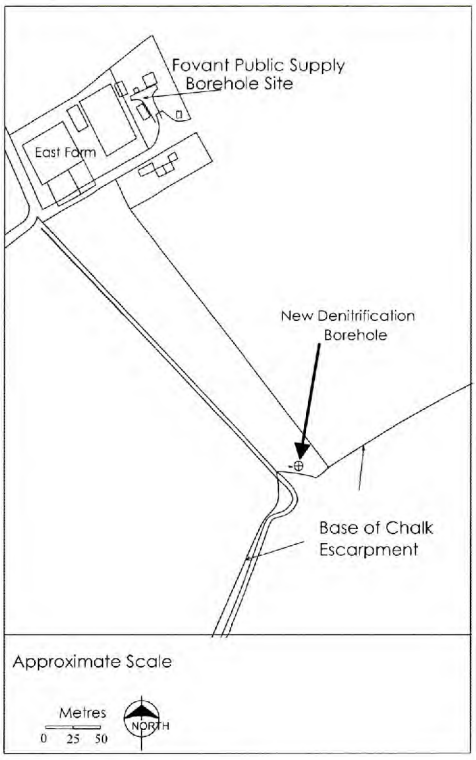


Figure 2.1: Nitrate concentration in groundwater abstracted from two operational boreholes at the Fovant public water supply, during the period 1976 – 2001 (Source: Wessex Water)

The existing abstraction facility, located at a secure compound on mixed arable farmland has two operational boreholes and a twin tower GAC plant for pesticide removal (there is an associated wash out pit for regeneration of the GAC filters and pump/generator houses). For the purposes of the ISBD trial, a new borehole (BH 4) was drilled 350m to the south east of the abstraction compound in the Upper Greensand, as shown in Figure 2.2, enabling the ISBD trials to continue without disruption to ordinary supply (the cone of depression for BH4 should not overlap with cones of depression for existing operational boreholes). The borehole was drilled at the foot of a chalk escarpment which runs in a northeast-south west direction and blocks the southerly drainage from the test borehole and groundwater flow at the site is in a north-easterly direction, away from the escarpment. The test borehole itself is 40m deep, penetrating the Upper Greensand layer of the aquifer.

(a)



(b)



(c)



Figure 2.2: Location of ISBD Test BH4 at Fovant Site **(a)** Schematic demonstrating location of ISBD BH in relation to existing abstraction facility, **(b)** Finished BH with manhole covering, showing aquifer material from drilling processes as green coloured mud and **(c)** View of chalk escarpment from bottom of track leading from East Farm up to test site, showing drilling apparatus for smaller observation borehole.

The laboratory microcosm experiment described here was also reported in conjunction with hydrogeological surveys and flow-modelling work as part of the preliminary investigations into the feasibility of an ISBD plant at the Fovant abstraction facility (Clark *et al.*, 2004). Laboratory studies are frequently used prior to full scale aquifer remediation treatment schemes to gain information about the potential impacts of the treatment on the groundwater and the aquifer environment. Data from preliminary laboratory work is typically used in the design of full-scale treatments tailored to the particular hydrogeological parameters of the site.

The objectives of the laboratory trial were to optimise the OC dose, forecast the quality of the treated water, and to assess potential biofouling of the aquifer material. Particular attention was paid to NO_2^-, a significant denitrification product in previous laboratory experiements (refer to Chapters 4 and 5) and also to soluble Fe and Mn, since the Upper Greensand aquifer contains a mixture of iron containing minerals such as Glauconite.

Prior to the laboratory work, glucose was proposed as the carbon substrate based on previous drinking water treatment experiences of Wessex Water and it was therefore a prerequisite that glucose be used in the microcosm trials. However, acetic acid was also studied as an alternative. Acetic acid has been previously shown as a highly efficient substrate for ISBD, and is one of only two authorised substrates for drinking water treatment (biological reactors) in France (Discussed in Chapter 1). It was chosen for this trial, however, mainly on the basis of the experimental results from the previous microcosm trial (III), in which sodium acetate had been used successfully, but had lead to significant increases in pH. The increased pH was attributed to an accumulation in hydroxyl ions (OH^-), according to the reaction:

$$5CH_3COO^- + 8NO_3^- \rightarrow 4N_2 + 10CO_2\ 8H_2O + 13OH^- \quad \text{(ITRC, 2002)}$$

It was anticipated that acetic acid, which has an analogous functional group to acetate (CH_3OO^-), could give similar results without increasing the pH to the same extent; for example, consider the reaction between acetic acid and NO_3^- as defined by Mateju (1992):

$$5CH_3COOH + 8NO_3^- \rightarrow 4N_2 + 8HCO_3^- + 2CO_2 + 6H_2O$$

Furthermore, the use of acetic acid would avoid the introduction of sodium ions (Na^+) into the aquifer that is inherent when sodium acetate is used as the substrate and it is

envisaged that its use would be licensed by the Environment Agency. Details of sandstone and groundwater collected from the site are given in Chapter 3.

All microcosm experiments are listed in Table 2.2, with a summary of the determinands investigated in each microcosm trial is given in Table 2.3.

Table 2.2: Summary of operational details for all microcosm experiments

Microcosm Trial No.	Duration of trial (d)	Operational Details					Comments
		Microcosm Identitity No.	Sandstone type	Nitrate input conc. (mg N l^{-1})	Organic Carbon substrate	Nominal input DOC conc. (mg C l^{-1})	
I	22	1	Triassic Sandstone	15	Glucose	22.0	Sterile control
		2	Triassic Sandstone	15	-	0.0	Autotrophic control
		3 & 4	Triassic Sandstone	15	Glucose	22.0	mid regime
		5 & 6	Triassic Sandstone	15	Glucose	11.0	low regime
		7 & 8	Triassic Sandstone	15	Glucose	39.0	high regime
II	84	1	Triassic Sandstone	15	Sodium Acetate	22.0	Sterile control
		2	Triassic Sandstone	15	-	0.0	Autotrophic control
		3 & 4	Triassic Sandstone	15	Glucose	22.0 (d1- d60); 27.0 (d64 - d70); 32.0 d72 - d78); 37.0 (d79 - d84)	Optimised regime for glucose C
		5 & 6	Triassic Sandstone	15	Sodium Acetate	19.0 (d1- d60); 24.0 (d64 - d70); 29.0 d72 - d78); 34.0 (d79 - d84)	Optimised regime for acetate C
		7 & 8	Triassic Sandstone	15	Sodium Acetate	11.0 (d1- d51)	Low regime acetate (similar to MC 5 & 6 in trial I) - discontined early as no denitrification was occuring
III	55	1	Weathered Greensand	15	Sodium Acetate	35.0 (d1- 37); 50.0 (d 38- d55)	Sterile control
		2	Devonian Sandstone	15	Sodium Acetate	35.0 (d1- 37); 50.0 (d 38- d55)	Sterile control
		3 & 4	Devonian Sandstone	15	Sodium Acetate	35.0 (d1- 37); 50.0 (d 38- d55)	High metal mineral content in sand (Fe and Mn)
		5 & 6	Weathered Greensand	15	Sodium Acetate	35.0 (d1- 37); 50.0 (d 38- d55)	Medium metal mineral content in sand (Fe and Mn)
		7 & 8	Triassic Sandstone	15	Sodium Acetate	35.0 (d1- 37); 50.0 (d 38- d55)	Low metal mineral content in sand (Fe and Mn)
IV	32	1	Greensand	15 ± 3	-	-	Autotrophic control
		2	Greensand	15 ± 3	-	-	Autotrophic control
		3 & 4	Greensand	15 ± 3	Glucose	35.0	Optimised glucose regime
		5	Greensand	15 ± 3	Glucose	28.0	Low glucose regime
		6	Greensand	15 ± 3	Glucose	44.0	High Glucose regime
		7 & 8	Greensand	15 ± 3	Acetic acid	35.0	Optimised acetic acid regime - to compare with MC 3 & 4

Table 2.3: Summary of experimental determinands measured at various times during Trials I-IV

Experimental Determinand	Trial I	Trial II	Trial III	Trial IV	Method/Protocol (details in Chapter 3)	Rationale
NO_3^- N	✓	✓	✓	✓	Autoanalyser – colorimetric reaction	To indicate NO_3^- consumption
NO_2^- N	✓	✓	✓	✓	Autoanalyser – colorimetric reaction	To determine NO_2^- production as a potential water contaminant
NH_4^+	✓	✓	✓	✓	Autoanalyser – colorimetric reaction	To determine whether DNRA reaction competes with true denitrification
DOC	✓	✓	✓	✓	Persulphate oxidation	To determine consumption of OC substrate
SO_4^{2-}			✓		Autoanalyser – colorimetric reaction	To determine SO_4^{2-} reduction as a potential TEAP
Dissolved (filterable) Fe			✓	✓	Graphite Furnace Atomic Adsorption Spectrophotometry / *External laboratory*	To determine Fe^{3+} reduction as a potential TEAP
Dissolved (filterable) Mn			✓	✓	Graphite Furnace Atomic Adsorption Spectrophotometry / *External laboratory*	To determine Mn^{4+} reduction as a potential TEAP
pH	✓	✓	✓	✓	pH Electrode	To determine quality of treated water
DO		✓			DO Probe	To determine quality of treated water
Redox	✓	✓	✓	✓	ORP Electrode	To monitor progression of redox conditions within microcosms as indicator of TEAPs
Turbidity	✓	✓	✓	✓	Nephelometric method	To determine quality of treated water
Bacterial quality of effluent			✓	✓	Heterotrophic Plate Count / Total Viable Cell count *(external laboratory)*	To determine quality of treated water
Pressure	✓	✓	✓*	✓	Electronic transducers	To assess potential clogging of aquifer material within microcosm
Dye breakthrough curves	✓	✓	✓	✓	Sodium fluorescein dye breakthrough	To assess potential clogging of aquifer material within microcosm
ATP		✓	✓	✓	Cold aqueous extraction + Bioluminescence Reaction	To assess potential clogging of aquifer material within microcosm
EPS-c		✓*	✓*	✓	Centrifugation with Formaldehyde extraction + Dubois colorimetric method for Sugar determination	To assess potential clogging of aquifer material within microcosm
EPS-p		✓*	✓*	✓	Steaming extraction + Modified Lowry colorimetric method for protein determination	To assess potential clogging of aquifer material within microcosm
Microbial Diversity		✓	✓		Extraction by bead-beater, PCR amplification and DGGE of DNA, sequencing at external laboratory	To assess feasibility of methods on environmental-type samples of low anticipated biomass and to characterise microbial community

* *Denotes that data for these determinands was lost due to failure in the experimental procedures, details given in Chapter 6*

CHAPTER 3

GENERAL MATERIALS AND METHODS

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3.1 MICROCOSM APPARATUS

The flow-through microcosm apparatus used in these experiments was previously used for biological denitrification trials conducted by WRc plc and Imperial College (Thompkins *et al.*, 2001; Cartmell *et al.*, 2000; Cartmell, 1997). Individual microcosm sections made from borosilicate glass (Soham Scientific, Ely UK) were assembled into a completed microcosm using silicone grease and plastic brackets, thus forming a sealed biological reactor, as depicted in Figure 3.1. Eight microcosms were assembled for each experiment and filled with sand (as described in section 3.3), before being transferred to an incubator (Sanyo MIR253, Japan) where they were maintained in darkness at constant temperature (10 °C), to represent groundwater temperature conditions.

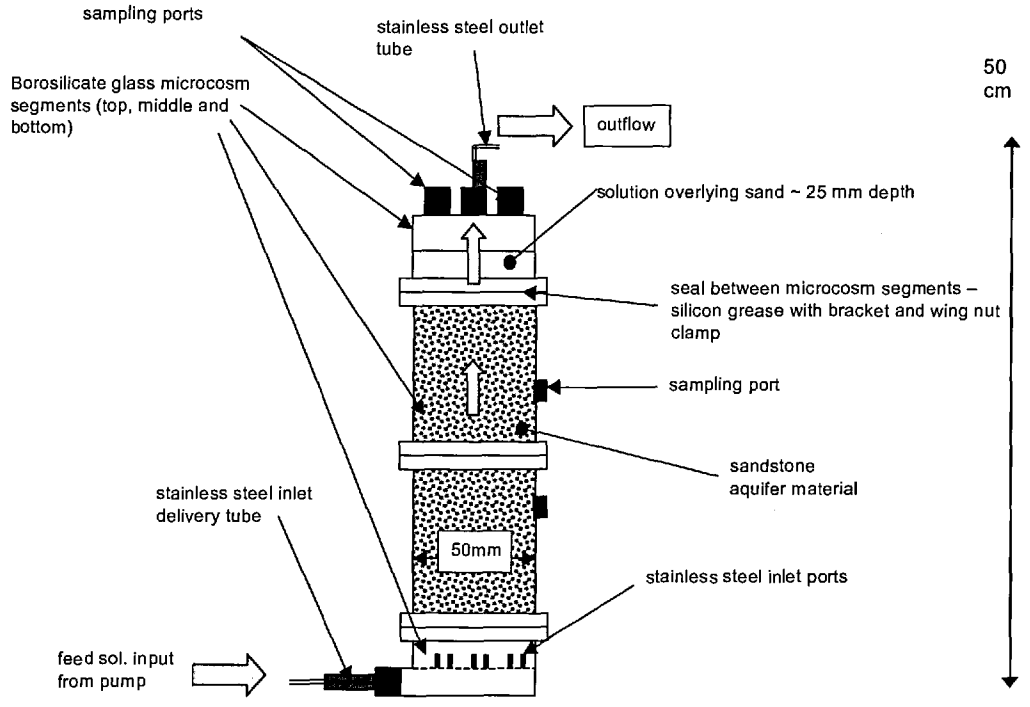


Figure 3.1: Schematic of microcosm assemblage (not scaled)

The solutions used to feed microcosms were held outside the incubator in 5 l vessels covered with black plastic to exclude light, at the ambient temperature (20-22 °C). Feed solution was delivered to each microcosm via black Tygon tubing (Tygon R-3400) through stainless steel inlets (I.D. approx. 1 mm). The Tygon tubing was cleaned and disinfected regularly with Virkon to prevent biofilm accumulation. A peristaltic

pump (model 505S, Watson and Marlow, Cornwall, UK) on the feed line was used to pump feed solution into a chamber at the base of the microcosm and subsequently into the sandstone matrix via a series of stainless steel tubes, designed to optimise dispersion into the sandstone matrix (Cartmell, 1997). The upwards direction of flow minimised the formation of preferential flow channels within the microcosm (Cartmell, 1997). Tygon tubing was also used at the top of the microcosms to remove treated effluents from a pool of solution overlying the sand column to collection bottles (2 l Nalgene plastic bottles,) situated on a shelf above the microcosms in the incubator, as shown in Figure 3.2.

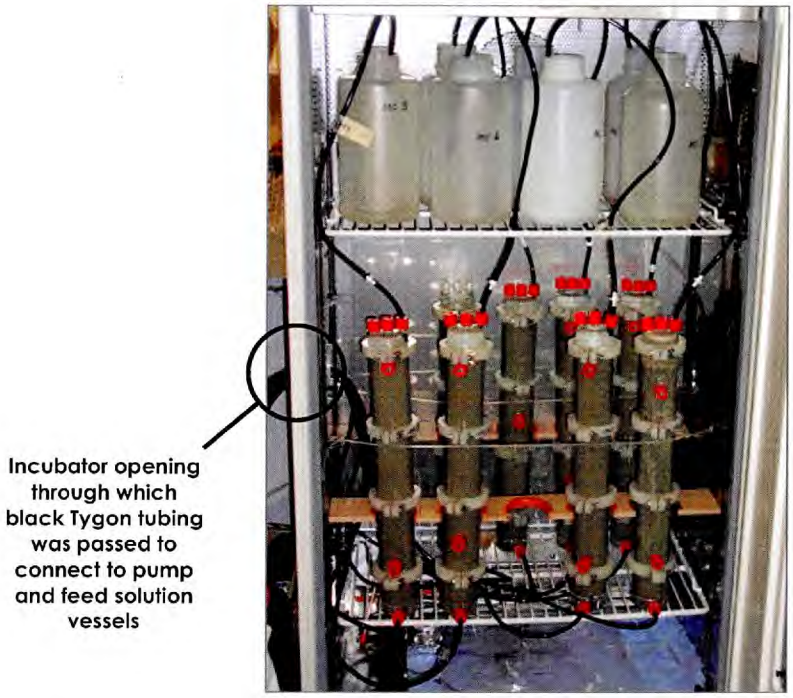


Figure 3.2: Microcosms assembled in incubator showing location of effluent collection bottles

Under flow-through operation, the physical flow characteristics of the system resembled a constant-head permeameter

3.2 PUMP FLOW RATE

When the microcosm equipment was designed and built, Cartmell (1997) tested a range of flow rates (0.13; 0.42; 1.55 ml min^{-1}) and reported that all would allow microcosm denitrification to proceed. In these experiments, a flow rate of 1.45 ml min^{-1} (2.088 l d^{-1}) was employed to enable replenishment of microcosm feed and collection of effluent on a 24 hourly basis. The pump flow rate (U) may be compared to aquifer flow velocities (U_x), (which describe the distance a unit of groundwater travels per unit time), using the equation:

$$U_x = U/(a\ n_e) \qquad \textit{equation 3.1}$$

Where : U_x = flow velocity

U = microcosm pump flow rate ($m^3\ d^{-1}$)

a = cross sectional area

n_e = volumetric porosity

The corresponding aquifer flow velocity, for a laboratory pump rate of 1.45 ml min^{-1} is 2.87 m d^{-1}, a relatively fast aquifer flow velocity but still within the range of observed flow rates for UK aquifers (Cartmell, 1997). Under this flow regime, however, the hydraulic residence time within the microcosms was approximately 7 h which was practical for operational and sampling purposes.

3.3 AQUIFER MATERIALS

3.3.1 Triassic Sandstone

The sandstone used in Microcosm Trials I, II, and for two microcosms of Trial III was Triassic sandstone collected from the Triassic Sherwood Sandstone Group aquifer in the UK Midlands. This is a major aquifer and the geological formation is comprised of fine to medium grained, plain and cross-laminated red and brown sandstones that are intercalated with mudstones and silt (Thornton *et al.*, 2000). Sandstone was collected from Burntstump Quarry, Nottingham (NGR: SK 589 502), a site that has been extensively studied (Blakey and Towler, (1988), Lewin *et al.*, (1994), Young *et al.*, (1994) as cited in Thornton *et al.*, 2000). Sandstone from this site had also been used in the previous microcosm experiments of Cartmell (1997) and Thompkins *et al.* (2001).

Burntstump Quarry is a working site and so fresh rock was removed directly from the rock face and transported by quarry personnel to a safe area where it was transferred to clean black bags and stored in cool boxes at ambient temperature. It was not possible, therefore to collect the stone under sterile conditions, but efforts were made to minimise contamination by handling the sandstone with clean utensils and by wearing laboratory gloves. On return to the laboratory, the sandstone was stored in tact (in the black bags) in a refrigerated cold store at 4 °C until use.

The Burntstump sandstone is un-cemented and friable (Thornton *et al.*, 2000) and consolidated portions were prepared for microcosms use by crumbling into loose sand, sieving through 500 µm and washing in pure laboratory water (Purite, reverse osmosis water). This removed the silt and fine particulate fractions which had been shown to wash out of the microcosms, interfering with turbidity measurements (this

process did not appear to hamper biomass establishment). The sandstone was left to drain in a fume cupboard and used to fill the microcosms whilst moist, as shown in Figure 3.3. Samples of the washed and unwashed samples were sent for specialised qualitative X-ray diffraction (XRD) analysis at the Natural History Museum, to characterise the mineralogy of the sandstone.

3.3.2 Lower Greensand and Devonian Sandstone

In Microcosm Trial III, two alternative sandstones were used; Lower Greensand and Devonian Sandstone. The Lower Greensand was collected from a commercial sandpit situated on the Lower Greensand of the Folkestone Beds, in Kent, UK (NGR: TQ 418 535). The greensand at this location is weathered and has a surface coating of various iron oxides and was mixed with small pebbles of ironstone. The sandstone was in free sand form, of a medium-coarse grain size, and a collection was made from a resting sand pile, after removing the surface layer. The sand was collected and stored following the procedure described for Triassic sandstone collection. Lower Greensand was prepared for experiments by sieving and washing, similarly to the Triassic sandstone (prepared sand shown in Figure 3.3) and packed into the microcosms whilst moist.

The Devonian sandstone was collected from George Read Quarries, Gloucestershire, UK (NGR: SO 616 150). The sandstone quarried from this region is a hard, red, cemented calcareous sandstone, typically used as a building material, and so a portion of freshly quarried rock had to be collected from the face and cut into small 'boulders' by quarry personnel. The sandstone was transported to the laboratory and stored as previously described.

When preparing the Devonian sandstone, it was too hard to be crumbled into sand by hand and was therefore crushed into pieces of approximately four inches diameter using a hydraulic press and then milled using a rock milling machine. The milling machine pressurises the rock between two jaws which effectively cause the rock to implode or crumble. The milling was a graded process which produced small, irregular fragments of rock, approximately 1-2 cm diameter after being passed through the machine once (shown in Figure 3.4), and subsequently a fine, powder-like dry sediment following a second pass through the mill (Figure 3.3). It was impossible to obtain a material of sand-like grain size, and so the sediment fraction was used in the microcosm experiments, because the fragmented rock produced after one round of milling was too large and would have had resulted in a matrix porosity that was too high.

The Devonian sandstone dry sediment was collected and moistened to form wet sediment, as shown in Figure 3.4, which was packed into the microcosms and allowed to settle so that excess pore water came to the surface.





Figure 3.3: **(a)** Prepared Triassic Sandstone for Microcosm Packing **(b)** Prepared Lower Greensand **(c)** Ironstones removed during sieving

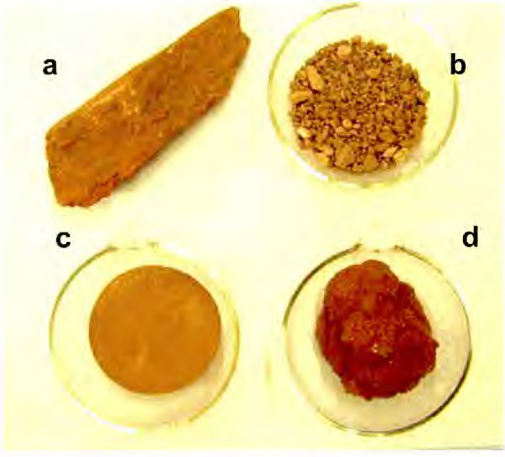


Figure 3.4: Process of Devonian Sandstone preparation; **(a)** crushed rock for milling; **(b)** milled rock after 1 round; **(c)** milled rock after 2 rounds; **(d)** wet Devonian sandstone sediment – ready to pack microcosm

The Devonian sandstone dry sediment was collected and moistened to form wet sediment, as shown in Figure 3.4, which was packed into the microcosms and allowed to settle so that excess pore water came to the surface.

The physical characteristics of the processed Devonian sandstone were different from the native rock but this was of little relevance as the geochemical composition of the rock was the principal experimental interest.

3.3.3 Upper Greensand from Field Trial Site

Upper Greensand was collected from a drilling site at Fovant (refer to section 2.2.4) in core form, obtained from the saturated zone at a depth of 21-26 mbgl (further details are given in appendix 1). The cores were transported from the site to the laboratory in the plastic casing used by the drilling contractor, where they were opened and the core sections were washed to remove loose debris. The lithological profile of the core was relatively heterogeneous (Figure 3.5), ranging from friable green sandstone at the top of the core (21 mbgl) to more consolidated pebbles of weakly cemented calcareous sandstone in the middle of the core, finishing in hard cemented, calcareous sandstone at the bottom of the core (26.85 mbgl) which remained undisturbed by the drilling process (refer to appendix 1 for lithological log). The cores were re-cased and refrigerated until use.

The microcosms were packed with mixed sandstone taken from various sections of the core to make the microcosm matrix as geo-chemically representative as possible of the rock profile through the core. The hard consolidated sandstone had to be milled, as previously described, but owing to the clay content of the rock, which plugged the machine, only one round of milling was possible. The pebbles produced were subsequently dried in a fume cupboard to increase the brittleness, ground by hand with a pestle and mortar and finally the various fractions of sandstone were combined to provide a consistent and homogenous matrix. Samples were sent to the Natural History Museum for XRD analysis, as described for the Triassic sandstone.

The porosity of Triassic sandstone and Lower Greensand (which were not mechanically ground) once packed into the microcosms was calculated as described by Charbeneau (1999):

$$n = 1 - (\rho_b / \rho_s) \qquad \textit{equation 3.2}$$

Where : n = porosity

ρ_b = bulk density of sandstone (mass/volume)

ρ_s = Sandstone particle density (taken as 2.65 g cm^{3-1}, (CRC, 1992)

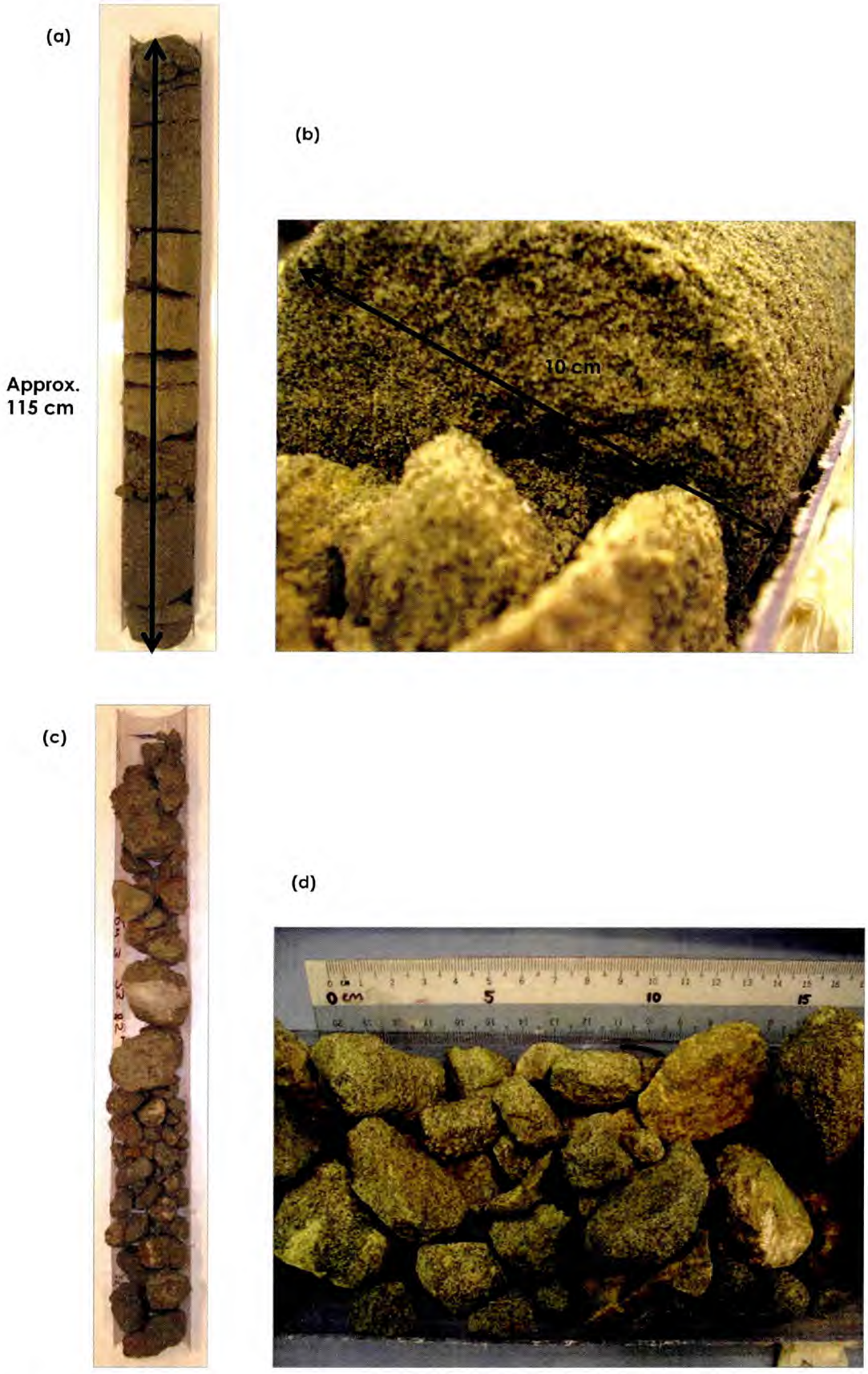


Figure 3.5: (a) washed down core from 21-22.5 mbgl showing cross section in **(b)**; **(c)** washed down core from 22.5 - 23.85 mbgl showing details of sandstone pebbles in **(d)**. *Continued overleaf*

(e)



(f)



(g)



(h)



Figure 3.5 cont'd: (e) washed down core from 23.85 – 25.35 mbgl increasingly consolidated sections in **(f)**; **(g)** washed down core from 25.35 – 26.85 mbgl showing hard, cemented sections left in tact by drilling in **(h)**

3.4 MICROCOSM OPERATION

3.4.1 General Description

Packed microcosms were transferred to an incubator connected to the influent tubing. Reverse osmosis water was pumped into the microcosms with the ports at the top initially left open to allow air to escape. Before the microcosms were completely filled with water, oxidation-reduction-potential probes (ORP) (Sensorex, CA, US) and pressure transducers (RS Components, UK) were fixed into microcosm ports to complete the apparatus set up. ORP probes were either immersed into effluent overlying the sand column or into the interstitial microcosm solution via the side ports and sealed into place using screw caps containing rubber o-rings. Similarly, the pressure transducers were immersed in the interstitial microcosm water filling the side ports as demonstrated in Figure 3.6. The microcosms were sealed completely before commencing pumping.

Under flow through operation, the peristaltic pump on the feed line was sufficient to maintain flow and the effluent line at the top of the microcosm remained opened to allow effluent to collect in the Nalgene bottles as previously described. The volume of effluent collected daily was measured before being discarded or sampled. The microcosm feed solutions were replaced each day and there was a period of up to 4 h d^{-1} where the pump was turned off to maintain the system.

3.4.2 Determination of Hydraulic Conductivity in the Microcosms

Hydraulic conductivity was calculated according to the Darcy expression:

$$K = Q l / A h_L \qquad \textit{equation 3.3}$$

Where :

K	=	hydraulic conductivity
Q	=	flow rate
A	=	cross sectional area
h_L	=	head loss
l	=	length

The flow rate in the microcosms and the dimensions of cross sectional area and length were constant throughout all experiments and therefore head loss was the only variable which was determined experimentally. The head loss (h_L) in the system was calculated as the difference in pressure head (P_1-P_2), measured at the top and bottom of the sand column, as depicted in Figure 3.7. The pressure head at P_1 and P_2 was measured using pressure transducers (0-5 psi) which were connected to a multi

channel data logger. The electronic output (mV) from the data logger was captured on computer using the TRIAX data acquisition software (Toll, 1999) and converted to pressure units (mm water). The system monitored pressures within the microcosms continually, producing real time output on screen, and storing data at 6 h intervals. Under typical flow conditions, each transducer measurement (mm) was indicative of the elevation height between the transducer and the effluent outlet (refer to Figure 3.7).

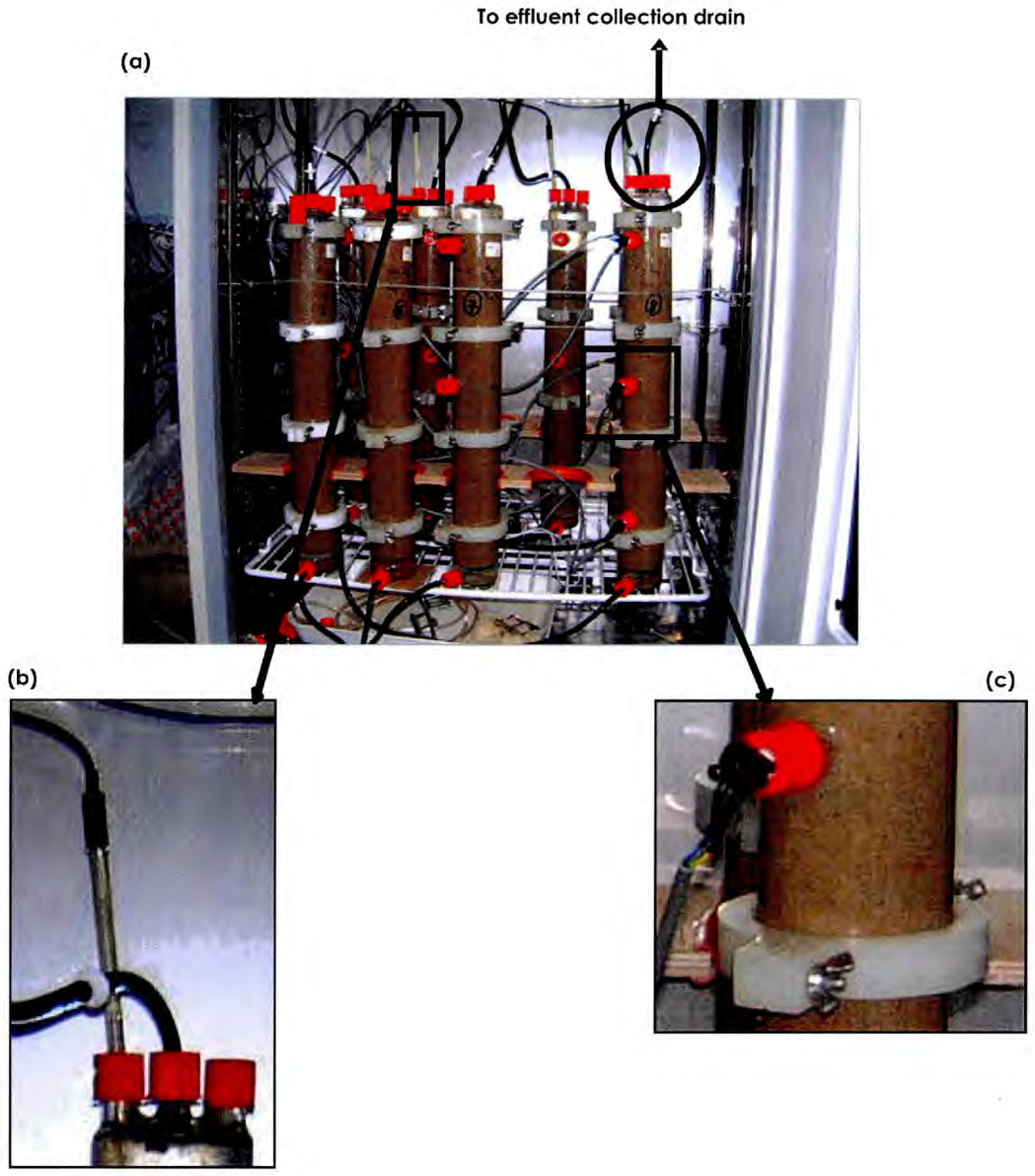


Figure 3.6: **(a)** Microcosms set up in incubator; **(b)** ORP probe located in top port; **(c)** Pressure transducer in side port

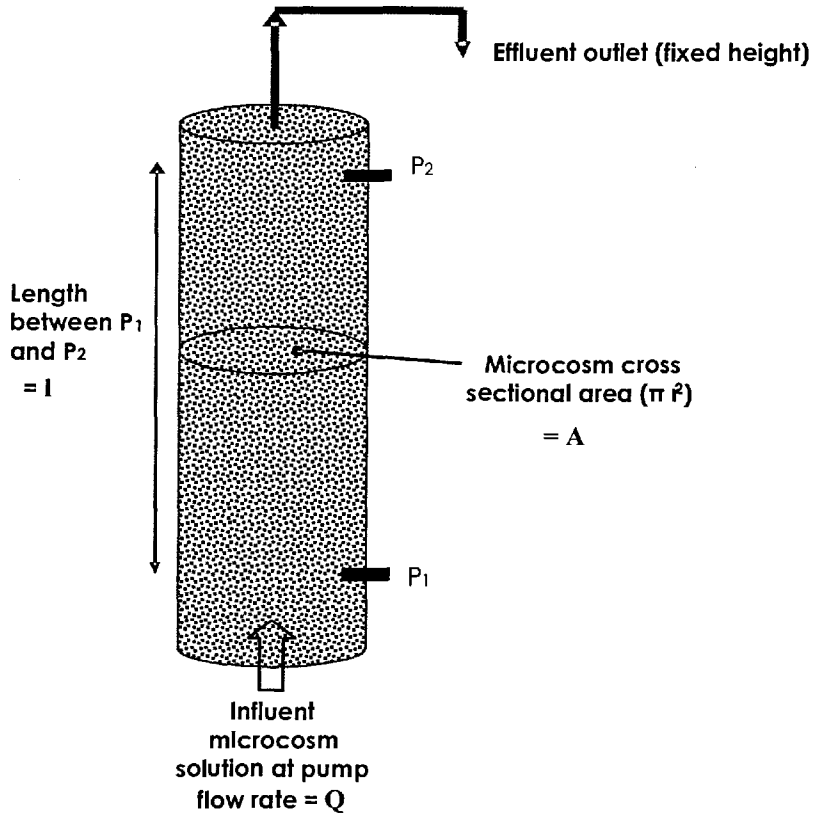


Figure 3.7: Schematic representation of microcosm sand column showing parameters for calculation of hydraulic conductivity

3.5 MICROCOSM FEED SOLUTIONS

3.5.1 Trials (I), (II) and (III)

In Trials I, II and III, all microcosm feed solutions were prepared in RO water and included $NaNO_3$ and nutrients throughout, with additions of OC as appropriate. Mercuric chloride was used as a sterilising agent in abiotic control microcosms.

Concentrated stock solutions of NO_3^-, OC, $HgCl_2$ and nutrients were prepared on a weekly basis and stored at 4 °C, and were used to spike 5 l batches of microcosm feed solution to the nominal concentration required. Feed solutions were not sterilised as the volumes required made it impractical to filter-sterilise and autoclaving caused precipitation of nutrient minerals. However, microbial growth in the feed solutions was minimised by replacing every 24 h with fresh solutions in sterilised containers.

Sodium nitrate was used as the source of NO_3-N, at a nominal concentration of 15 mg NO_3-N l^{-1} and $HgCl_2$ was used at a concentration of 100 mg Hg l^{-1} (Cartmell, 1997).

Both glucose and sodium acetate were used as OC substrates used in the Trials (I)-(III). Details of the nutrient additions (all microcosm feeds) are given in Table 3.1.

Table 3.1: Final concentration of nutrients in microcosm feed solutions (adapted from Schlegel, 1991)

NUTRIENT	CONCENTRATION (mg l^{-1})	TRACE NUTRIENT	CONCENTRATION (µg l^{-1})
$K_2HPO_4.3H_2O$	1.0	$ZnCl_2$	0.14
$MgSO_4.7H_2O$	0.4	$MnCl_2.4H_2O$	0.2
$FeSO_4.7H_2O$	0.02	$CoCl_2.6H_2O$	0.04
$CaCl_2.2H_2O$	0.02	$NiCl_2.6H_2O$	0.2
NH_4Cl	0.1	$CuCl_2.2H_2O$	0.04
		$NaMoO_4.2H_2O$	0.125

3.5.2 Trial (IV)

In contrast to the previous microcosm experiments, trial IV received raw groundwater taken from the Fovant Public Water Supply. Batches of groundwater were collected in 25 l carboys on two occasions during the experiment and stored in a cold store (4 °C) prior to use. The concentration of NO_3^- in the water was approximately 9 mg NO_3-N l^{-1}, which was adjusted to 15 mg N l^{-1} using $NaNO_3$. Glucose or acetic acid was added as necessary, but no nutrients were incorporated, since the water was from a natural source.

3.6 SAMPLING PROCEDURES

During the first week of each experiment, samples were collected from the microcosm effluent on a daily basis and thereafter ever other day (except Trial I, where sampling was reduced to once per week); samples of the microcosm feed solutions were always taken in conjunction with the effluent samples. The microcosm sandstone was sampled after experiments in individual microcosms had terminated, when the columns were dismantled.

The sampling method and subsequent handling of samples were determined according to analytical protocol (described in section 3.7). Details of samples, including manipulation and storage are given in Table 3.2. Redox measurements were made twice per week by connecting a pH meter (Fisherbrand Hydrus Benchtop 500) to the ORP probes, which were sealed into the microcosms.

Microcosm effluent was sampled from the effluent collection bottles, which were shaken beforehand to mix. Sub samples of 250 ml were filtered through 0.45 µm (to remove biomass), into clean glass beakers. From the filtrate, 2 sets of effluent samples were stored in 60 ml plastic bottles; an undiluted set and a set of 1/10 dilutions (prepared in RO water). These samples were stored at –20 °C prior to N ($NO_3^-/NO_2^-/NH_3$) and DOC analysis (filtration/dilution was necessary for analytical purposes). The remaining filtered effluent was examined immediately to determine general water quality parameters (pH, DO, turbidity, conductivity) and then discarded. The samples of microcosm feed solution were treated similarly, but without filtration or dilution.

Samples of interstitial microcosm solution for Fe and Mn analysis were collected throughout microcosm Trials III and IV. During Trial III, the microcosms were allowed to drain (under gravitational force) into sample bottles, however this could take over an hour. During Trial IV, therefore, samples were collected using a syringe to gently draw the interstitial solution out from the microcosm side port. Under the drainage method (trial III), acid-rinsed glass Drescher bottles (100 ml), flushed with O_2-free N_2 gas were connected to the side ports of the microcosm using silicon tubing. Clamps were employed on the inlet/outlet of the bottle to minimise the entry of air into the bottles. When the silicon tubing was securely screwed into the microcosm side port, the clamps were released and interstitial solution drained into the Drescher bottle, displacing the N_2 gas from the outlet. Once sufficient sample had been collected (50 ml), the clamps were closed, the bottle removed and the microcosm side ports re-sealed. Once collected, the sample was subsequently syringe-filtered through 0.45 µm (Whatman) and acidified with concentrated nitric acid (to concentration of 1 % $^v/_v$). Acidified samples were stored at ambient conditions until analysis (< 1 month storage time).

Samples of effluent collected for heterotrophic bacterial plate counts during trial III were stored in sterile 50 ml Sterilin centrifuge tubes before plating. The storage period was < 4 h under refrigeration at 4 °C.

Sandstone samples were taken once the microcosm had been drained (under gravitational force, < 5 h drainage time). Sandstone from top, middle and bottom sections of the microcosms were transferred into clean glass beakers and mixed to homogenise. Approximately 4 cm depth of sand between each sampled section of the microcosm was discarded to separate the sand into discreet, representative regions. Homogenised sandstone was weighed into clean, labelled plastic bags and stored in the freezer (-20 °C). Additionally, 5 g samples from each section were weighed into foil containers for determination of dry weight.

3.7 ANALYTICAL PROTOCOLS

3.7.1 Dissolved Oxygen, pH and Conductivity

Dissolved oxygen, conductivity and pH were measured using calibrated laboratory meters: DO meter 970, Jenway, UK; Conductivity meter 470, Jenway, UK and a Hydrus Benchtop 500 pH meter, Fisherbrand, Fisher Scientific UK. In each case, probes were calibrated according to the manufacturer's instructions and sample readings were taken by immersing the respective probes into a stirred sample and manually recording the readings.

3.7.2 Turbidity

Turbidity was measured using a HACH 2100A Turbidimeter, according to the nephelometric method: 2130 B (APHA, 1995). The Turbidimeter was calibrated throughout proceedings using Gelex Turbidity Standards.

3.7.3 Dissolved Organic Carbon

Effluent samples, filtered through 0.45 µm to remove un-dissolved carbonaceous matter (necessary for dissolved OC measurement) and feed solution samples were acidified (< pH 2.0) with orthophosphoric acid and analysed by the UV radiation persulphate oxidation procedure (SCA, 1979) on a Dohrmann DC80 Carbon Analyser. Briefly, acidified samples were sparged with O_2 gas in the presence of UV light to remove inorganic carbon and the remaining organic carbon content was oxidised with persulphate to CO_2. The converted CO_2 content of the sample was quantified with an infra red detector. The instrument, calibrated using potassium acid phthalate standards, retained its calibration between analytical runs but as a quality control (QC) procedure, a series of RO water and 10 mg l^{-1} C standards were analysed prior to each set of samples. In addition, two 10 mg l^{-1} standards were analysed for every 10 samples on the sample carousel and the coefficient of variation was typically < 1 %.

3.7.4 Nitrate, Nitrite and Ammonia

Nitrate, NO_2^- and NH_4^+ were analysed colorimetrically on a Skalar SAN plus Analyser (Skalar). Samples for auto-analysis were filtered at the sampling stage to remove biological debris. In addition to the filtering step, it was necessary to prepare a complete set of dilutions (1/10) for the effluent samples, to ensure that NO_2^- concentrations were within the analytical detection range of the instrument. Samples were simultaneously analysed for NO_3^-, NO_2^- and NH_4^+ according to the following colorimetric reactions:

- NO_2^- reacts quantitatively with sulphanilamide under acidic conditions to form a diazonium compound. This reacts with α-naphthylethylenediamine dihydrochloride to form a red-purple azo dye which is measured spectrophotmetrically at 540 nm (method: 4500-NO_2^-B; APHA, 1980).
- NO_3^- is reduced to NO_2^- using a cadmium column and then measured as above (method: 4500-NO_3^-F; APHA, 1980). Therefore, the NO_3^- channel on the instrument is actually measuring the sum of the NO_3^- and NO_2^- present in the sample, referred to as the total oxidised nitrogen (TON). In samples containing NO_3^- and NO_2^-, the NO_3^- channel must be operated in conjunction with the NO_2^- channel and the final NO_3^- concentration was obtained by subtracting NO_2^- from TON concentrations.
- NH_4^+ analysis is based on a modified Berthelot reaction: NH_4^+ was chlorinated to monochloramine and then reacted with salicylate to form 5-aminosalicylate. The resulting blue-green colour was measured at 660 nm (Krom, M., 1980; Searle, P. L., 1984; cited in Skalar SAN plus Analyser Manual).

The Skalar was calibrated at each analytical run and the methods included quality control measures such as blank-wash correction and drift check over the duration of the run (Skalar SAN plus Analyser Manual).

3.7.5 Sulphate

Sulphate was also measured colorimetrically using the Skalar SAN plus Analyser in conjunction with the N analysis. Briefly, samples were passed through an ion exchange column to remove multivalent metal ions and then mixed with an alcoholic solution of $BaCl_2$ + methyl thymol blue indicator (grey colour) at pH 2.5-3.0, whereon the SO_4^{2-} in the sample forms $BaSO_4$. The pH of the combined solution was then increased to pH 12.5-13.0 with NaOH solution, where, under alkaline conditions, excess Ba^+ (not reacted with SO_4^{2-}), chelates with the methyl thymol blue. The chelated methyl thymol blue forms a blue colour whereas the un-chelated methyl thymol blue is grey. The intensity of the grey colour was measured at 460 nm to quantify the amount of sulphate, according to a standard calibration (US Dept. of Agriculture, 1962). Quality control measures as described for N analysis were also employed during SO_4^{2-} analysis.

3.7.6 Dissolved (Filterable) Iron and Manganese

Filtered acidified samples were analysed for Fe and Mn by graphite furnace atomic absorption spectrophotometer (GFAAS). The acidification preserved the sample and the filtration ensured that dissolved (filterable) rather than 'Total' Fe/Mn was quantified (method 303B; APHA, 1995).

Table 3.2: Summary of sampling details

Sample Type / Analysis Type	Trial I	Trial II	Trial III	Trial IV	Sampling frequency	Sample Manipulation/Storage
Eff, FS: pH	✓	✓	✓	✓	Every other day	Measured immediately
DO		✓				Measured immediately
Eff, FS: Turbidity		✓	✓	✓	Twice weekly	Measured immediately
Eff, FS: NO_3^- NO_2^- NH_3	✓	✓	✓	✓	Every other day	Samples filtered through 0.45 µm, extra set 1/10 dilutions made and all processed samples frozen (-20 °C) in clean plastic bottles
Eff, FS: DOC	✓	✓	✓	✓	Every other day	Samples filtered through 0.45 µm, extra set 1/10 dilutions made and all processed samples frozen (-20 °C) in clean plastic bottles
Eff, FS: SO_4^{2-}			✓		Every other day	Samples filtered through 0.45 µm, extra set 1/10 dilutions made and all processed samples frozen (-20 °C) in clean plastic bottles
Eff: Bacterial plate Count			✓	✓	Once → twice weekly	Samples collected in sterile plastic bottles and *(i)* plated immediately or *(ii)* incubated for 5 days then plated (5 day plate count test)
IS: ORP (*in situ* probe)	✓	✓	✓	✓	Every other day → twice weekly	ORP probe located in microcosm
IS: Pressure Transducer	✓	✓		✓	Continuous measurements	Data stored electronically using data logger and PC software
IS: Iron / Manganese			✓	✓	Once → twice weekly	Samples collected under O_2 limited conditions, acidified with nitric acid (final concentration 1% v/v) immediately and stored in glass volumetric flasks at ambient conditions (dark) until analysis (stored for < 1 month)
Sandstone:						Samples collected in clean plastic bags and:
ATP			✓	✓	At end of trial	Analysed on the same day
EPS			✓	✓		Frozen (-20 °C) until analysis
DGGE		✓	✓			Frozen (-20 °C) until analysis

Key: Eff = microcosm effluent IS = interstitial microcosm solution FS = microcosm feed solution

The GFAAS was performed using a Perkin Elmer Zeeman 5100 PC Atomic Absorption Spectrophotometer with accompanying graphite furnace, sample carousel and software. The methods are as defined by the Perkin Elmer WinLab Software manual which accompanies the instrument (Perkin Elmer, Conneticut, US).

In addition, sample collected during microcosm trial IV were sent to an external laboratory for analysis (ICP method, Alcontrol Laboratories, UKAS Lab. No. 0996, Bradford, UK).

3.7.7 Enumeration of Effluent Bacteria

To quantify the bacterial content of the microcosm effluents, heterotrophic plate counts (HPC), the reference method for estimating numbers of live heterotrophic bacteria during water treatment (Boulos, 1999) were carried out. Effluent samples were cultured on R2A agar (OXOID, UK), using a spread-plate technique and incubated at 20 °C for two days and five days (HMSO, 1994). Counts were reported as colony forming units per ml of inoculum (CFU ml^{-1}).

During microcosm trial IV, effluent samples were sent to an external laboratory and analysed for total viable counts (TVCs). This is also an HPC method, where total viable counts are expressed as colony forming units per ml of inoculum (CFU ml^{-1}) over 3 days at 22 °C (Alcontrol Laboratories, UKAS Lab. No. 0996, Bradford, UK).

3.7.8 Sandstone Dry Weights

Representative samples of homogenised sandstone were weighed into disposable foil tins (6 cm diameter, 4 cm depth) and dried in a forced-air oven to a constant weight at 105 °C. The moisture content (g) was taken as the mass lost during the drying period, leaving the dry matter content (g) remaining.

3.7.9 Sandstone Adenosine Tri-phosphate (ATP) Quantification

The ATP content of microcosm sandstone samples was determined using an extraction and analysis procedure based on Zelles *et al.* (1985). For the extraction; 5 g (fresh wt) of sandstone was weighed into a sterile centrifuge tube (50 ml capacity) containing 2.5 g clean/dry glass beads (1.5 mm diameter). Subsequently, 24 ml of ice-cold TRIS-EDTA reagent (0.4 mM TRIS + 5 mM Di-sodium EDTA) was added and the sample was mixed using a combination of vortex-mixing on a Whirli Mixer (Fisher Scientific) and shaking by hand over a period of 1 minute. Ice-cold TCA reagent (1 ml of [0.5 M Trichloroacetic acid + 0.25 M Di-sodium hydrogen phosphate]) was added to the solution and shaken for a further minute. Finally, the centrifuge tubes were placed in an ultrasonic bath (PUL125, Kerry, UK) and subjected to 1 minute of

ultrasonic vibration (50 hertz). The ATP in the sandstone is extracted into the solution during this procedure and consequently, the tubes were centrifuged (Gallenkamp) at 3000 rpm for 5 minutes (4 °C) and the extracted ATP-supernatant was decanted. The original sandstone was discarded and the supernatants were again centrifuged (3000 rpm over 5 mins) to remove any remaining turbidity that could interfere with the light-capture process in the luminometry instrumentation.

An enzymatic luminescence reaction was used to quantify ATP content in the extracted samples, using Celsis Bioluminescence Reagents (Celsis, The Netherlands) and a luminometer (Bio-Orbit 1251, WKB Wallac, Finland). Extracted sample (100 µl) was pipetted into a cuvette containing 100 µl of Celsis LuminEX™ reagent. The cuvette was then secured in the instrument sample chamber and 50 µl of the light-generating reagent, Celsis LuminATE™, was injected directly into cuvette from a glass syringe (Hamilton). The instantaneous light emission was measured for a period of 2 minutes and the integrated relative light units (RLU) output over this time was recorded. The RLU output for each sample was converted to ATP concentration using a standard curve prepared from a Celsis ATP Standard. Reagent blanks were included for QC purposes.

3.7.10 Extracellular Polymeric Substance (Carbohydrate and Protein) Quantification

Two different extraction methods were used to extract EPS from sandstone samples; regular centrifugation with formaldehyde (RCF) to extract EPS-carbohydrates (EPS-c) and steaming extraction for EPS-protein (EPS-p), after Jia *et al.* (1996) and Zhang *et al.* (1999) respectively.

For the EPS-c determinations, sandstone samples were washed in RO water to remove interstitial solution which may have contained glucose (where glucose was used as OC substrate) and left to drain on open filter papers (Whatman no. 1). Sub-samples (5 g) of the moist sand were transferred to Oakridge centrifuge tubes and 5 ml of extractant solution (8.5 % sodium chloride solution + 0.22 % formaldehyde), was added. The tubes were shaken by hand for 1 minute and centrifuged (MSE Hi Spin 21, Fisons, UK) for 30 minutes at 12,000 rpm. The supernatant was filtered through 0.22 µm (to remove biomass) and the filtered supernatant was analysed for carbohydrate content.

The carbohydrate content was determined using a phenol/sulphuric acid reagent (Dubois *et al.*, 1956). Briefly, 2 ml of sample was mixed with 1 ml of 5 % phenol solution (5 % $^{w}/_{v}$ in RO water) in a glass boiling tube. Working in a fume cupboard, 5 ml of concentrated sulphuric acid was added rapidly to the sample suspension. The tubes

were allowed to stand for 10 minutes before being transferred to a water bath at 25 °C to cool. Once at ambient temperature, the absorbance of the sample suspensions was measured at 480 nm (Pye Unicam SP8-100 Ultraviolet Spectrophotometer). Sample measurements were compared to a standard curve prepared with glucose solutions.

For the EPS-p determinations, two methods were used: initially, a quiescent extraction with EDTA was used for samples collected at the end of microcosm trial III; alternatively, a steaming extraction was used for samples obtained from trial IV. During the EDTA extraction, 5 g samples of microcosm sand (fresh wt) were weighed into sterilyn centrifuge tubes with 5 ml of 2 % EDTA solution. The tubes were shaken by hand for 1 minute, refrigerated for 3 hours (4 °C) and then centrifuged (20 minutes, 14,000 rpm). The supernatant was decanted and filtered through 0.22 µm and the protein content of the filtrate was determined using a protein assay kit (Sigma Aldrich, Poole, UK) based on the Folin-Ciocalteu colorimetric method (Lowry *et al.*, 1951). The protein (peptide) content of the sample was complexed with an alkaline cupric tartrate reagent and precipitated with a solution of deoxycholate. The precipitation was pelleted using a microfuge (10 minutes at 13,000 rpm) and re-suspended in Folin-Ciocalteu reagent. The absorbance of the samples were quantified at 750 nm (Pye Unicam SP8-100 UV-VIS spectrophotometer) using a standard curve prepared from bovine serum albumin (BSA).

For the steaming method, 5 g samples of microcosm sand were weighed into Oakridge centrifuge tubes with 5 ml of RO water and then steamed in an autoclave for 10 minutes (80 °C, pressure = 1 bar). The tubes were centrifuged whilst still hot (10 minutes at 8000 rpm, 15 °C) and the supernatant (protein extract) was decanted and filtered (0.22 µm). As described for the EDTA extraction method, the protein assay was conducted using Sigma Aldrich reagent kit, although the precipitation step was omitted. Instead, the alkaline cupric tartrate reagent was added to the sample and then mixed directly with the Folin-Ciocalteu reagent. Sample absorbance was measured at 750 nm as described previously.

3.7.11 Polymerase Chain Reaction and Denaturing Gradient Gel Electrophoresis

This procedure involved 5 phases:

I. Extraction of DNA from the sandstone and purification;
II. Amplification of DNA using polymerase chain reaction (PCR) techniques;
III. Electrophoresis of PCR-products (amplified DNA) on agarose gel;
IV. Electrophoresis of selected PCR-products on denaturing gradient gel electrophoresis (DGGE);

V. Visualisation of the gel and excision of DNA bands for sequencing.

Contamination/degradation of samples was minimized by working in a confined area swabbed with ethanol, using sterile consumables and importantly, laboratory gloves were worn throughout. The PCR work was carried out inside a class II laminar flow cabinet.

(i) DNA Extraction and Purification

Frozen sandstone samples were defrosted under ambient conditions and 0.5 g portions were weighed into plastic vials containing 0.5 g of glass beads for bead-milling (Hybaid, Middlesex, UK). The sandstone/beads were suspended in 500 µl of sodium hydrogen phosphate (0.12M/pH 8.0) and 700 µl of PCIA reagent (phenol:chloroform:isoamyl alcohol; 25:24:1) was added. Sample suspensions were subsequently lysed using a bead-beater (Hybaid Ribolyser) at 6.5 m s^{-1} for 45 seconds.

The lysed samples were then centrifuged (Biofuge Pico, Heraeus, Jencons, UK) at 13,000 rpm for 10 minutes to produce an upper aqueous layer, containing DNA, and a phenolic layer, containing cellular 'impurities' and the sand/bead pellet. The aqueous upper layer (containing DNA) was removed by pipette into a fresh eppendorf, mixed with 500 µl PCIA and re-centrifuged. This process was repeated and following the third centrifugation sequence, the upper aqueous layer containing the extracted DNA was pipetted into a fresh eppendorf and polyethylene glycol (PEG; PEG 8000 30% $^{w}/_{v}$ in 1.6 M sodium chloride) was added (volume of PEG = 2 * volume of DNA extract).

The extracts were mixed and refrigerated (4 °C) for one h to allow DNA to precipitate, leaving impurities in the aqueous layer. The precipitated DNA was pelleted by centrifugation (15 minutes at 13,000 rpm) and the supernatant was removed. The pellet was re-suspended in 200 µl Tris-EDTA buffer (pH 8.0), 20 µl of 3M sodium acetate (pH 5.2) and 2 volumes of ice-cold absolute ethanol. The suspension was mixed and cooled for 10 minutes at –20 °C, after which it was centrifuged (10 minutes at 13,000 rpm). The supernatant was removed by pipette and excess ethanol allowed to evaporate from the purified DNA pellet, before it was re-suspended in 50 µl Tris-EDTA buffer to give the final DNA extract suspension.

DNA extracts were checked for concentration and purity using a spectrophotometer (Unicam 8625 UV/VIS). Briefly, a 1/100 dilution of the DNA was prepared in Tris-EDTA buffer and its absorbance at 260 and 280 nm was recoded.

The concentration of the DNA was calculated as:

Absorbance $_{260}$ * 50 * dilution factor (100) = concentration DNA (µg ml^{-1})

The purity of the DNA was taken as:

Absorbance $_{260}$ / Absorbance $_{280}$

Optimally, the concentration of DNA for PCR should be > 20 µg ml^{-1} with a purity ratio of ≥1.8.

(ii) DNA Amplification using PCR

A nested PCR procedure was used to amplify the DNA due to the relatively small amount of DNA extracted from the sandstone samples. The DNA extracts were diluted (1/10) to reduce the quantity of impurities in the samples (which could interfere with the PCR). The first PCR was completed using the universal primer pair 'pA/pH' (1500 base pairs) to amplify a large section of the DNA. Briefly; 1µl of diluted DNA extract was mixed with 49 µl PCR 'mix,' (a master mix of constituents required for the replication DNA in the PCR procedure, including; buffers, nucleotide bases, primers and Taq DNA polymerase) (Bioline, London, UK). In addition, positive and negative controls were included as samples. Mineral oil was pipetted over the suspension to prevent evaporation during the PCR incubation. The samples were loaded into a Perkin Elmer DNA Thermal Cycler for amplification (thermal cycles described in Table 3.3). The products from the first round of PCR were checked for viability using agarose gel electorphoresis before two subsequent rounds of PCR were carried out using primer pairs:

- Vr/Vf (200 base pairs + GC clamp) – which gives an easily visible banding pattern on the DGGE gel, necessary for image analysis and statistical comparison of samples;
- pD/pF (500 base pairs) – which gives a poorer banding pattern on the DGGE gel but amplifies longer nucleotide sequences so is, therefore more effective for sequencing.

Both of these PCR primer pairs incorporate a 'GC-clamp' into the amplified DNA (necessary for the DGGE stage, to anchor the un-dissociated DNA fragments in the polyacrylamide gel). The procedure for these PCR rounds was essentially the same as described for the first PCR round, with the pA/pH primer pair substituted for the Vr/Vf and pD/pF primer pairs respectively. An additional sterilisation step (using UV light) was incorporated at this stage, to sterilise all materials used in the PCR preparation

processes including the constituents of the PCR reagent mix, with the exception of the DNA primer pairs, which cannot be treated with UV as this would denature the DNA of the GC-clamp component. The extra sterilisation step is necessary when using nested PCR, to prevent amplification of contamination, which may interfere with subsequent DGGE. As previously described, the products of these secondary PCRs were examined on agarose gel prior to DGGE. Details of primers and PCR cycles are given in Table 3.3.

(iii) Agarose Gel Electrophoresis

Agarose gel was prepared by dissolving 1 g of agarose powder in 100 ml of 1x TAE buffer (2M Tris-EDTA, pH 8.3, Eppendorf Scientific Inc., NY, US) on a hot plate. When the gel had cooled to approximately 60 °C, 2µl of ethidium bromide, a molecular stain that binds to DNA and fluoresces under UV light, was added and the gel was poured into a mould to set. Two series of 25 sample-wells were set into the gel by inserting plastic combs in the liquid-gel, to be removed when the gel had solidified.

Once set, the agarose gel was transferred to the electrophoresis cell (wide mini sub cell; Biorad, Hemel Hempstead, UK) and submerged in 1x TAE buffer (the electrolyte). Samples of PCR-products (5 µl) were mixed with 2 µl of 'loading buffer' (a molecular stain; Sigma Aldrich, Poole, UK) and pipetted into the wells on the agarose gel. The wells at the end of each row were filled with a marker for PCR products (a 100 base-pair ladder; Sigma, Poole, UK) mixed with the loading buffer. The marker provides a positive DNA banding on the agarose gel and gives an indication of the size of the PCR product (i.e. number of base pairs). The electrophoresis was conducted for approx. 40 minutes at 100 V (Power Pac 300 Supply; Biorad, Hemel Hempstead, UK), after which it was examined under UV light using a dual intensity transilluminator (UVP, San Gabriel, CA, US). Viable samples and controls showing DNA 'bands' were selected for further analysis using DGGE.

(iv) Denaturing Gradient Gel Electrophoresis

A D-gene DGGE system (Bio-Rad, Hercules, CA, US) was used to conduct the DGGE analysis with a 10% polyacrylamide gel containing a DNA denaturant (7 M urea + 40% $^{v}/_{v}$ formamide in 1 x TAE buffer). The concentration gradient of denaturant in the gel was 30-60% and in accordance with health and safety regulations, preparation was carried out by laboratory personnel licensed to handle polyacrylamide. The solidified gel (0.75 mm thick, 16 * 16 cm) was set between 2 glass plates with spacers along the vertical edges and fixed into a plastic frame, which holds the gel upright in the DGGE tank and connects to electrodes, forming the 'core' of the DGGE cell. Once prepared, the core was placed in the plastic DGGE tank, filled with buffer (1x TAE)

and pre-heated to 65 °C. The samples wells in the top of the gel were flushed out with buffer from the tank to remove debris such as fragments of gel, which might block the path of the mobilised sample. Two gels were prepared; one gel for the DNA extracts amplified using Vr/Vf primers, and one for the DNA amplified using pD/pF primers.

The PCR-products selected for DGGE were mixed with an equal volume of loading buffer (11 µl) and loaded into the cell wells on the gel with DNA markers at the end of the sample row to act as a reference when visualising the gel under UV. The DGGE was run at 65 °C using 50 V (Power Pac 300 Supply; Biorad, Hemel Hempstead, UK) for approximately 16 h. The current was terminated when the dye of the loading buffer had migrated completely through the gel and dispersed into the TAE buffer in the cell from the bottom end of the gel.

(v) Visualisation of the gel and excising of DNA bands for sequencing

When the electrophoresis was complete, the core was removed from the tank to a shallow plastic tray to stain the gel. The glass plates were released from the clamp and the apparatus was removed leaving the exposed gel resting flat on one remaining glass plate (necessary to support the gel) in the staining tray. The gel was stained for 30 minutes using SYBR green I (Sigma, Poole, UK; diluted $1/10^3$ in 1x TAE) in the dark. The stained gel was subsequently viewed on a UV transilluminator (UVP, San Gabriel, CA, US) and photographed using Polaroid image analysis equipment (CU-5 GRI, Great Dunmow, Essex, UK). Finally, the gel was transferred onto a UV light box and selected DNA bands were excised from the gel, placed in eppendorf vials filled with 50 µl of Tris-EDTA buffer (pH 8.0) and sent for sequencing by a specialist laboratory at The University of Newcastle upon Tyne.

The DGGE banding patterns from the photographic images of the gel were analysed using the Quantity One® 4.1.1 software package (Bio-Rad, Hercules, CA, US), discussed further in Chapter 7.

Table 3.3: Summary of Primers and PCR Amplification Thermal Cycles used during amplification of 16S RNA gene

Primer Pair	Sequence	Reference	PCR Amplification Cycles
pA	AGAGTTTGATCCTGGTCAG	Edwards *et al.*, (1989)	Initial Denaturation: 3 min, 95 °C Denaturation: 1 min, 95 °C Annealing: 1 min, 55 °C } No. of cycles = 30 Extension: 1 min, 72 °C Final Extension: 10 min, 72 °C
pH	AAGGAGGTGATCCAGCCGCA		
Vr	CCTACGGGAGGCAGCAG	Muyzer *et al.*, (1992)	Initial Denaturation: 3 min, 95 °C Denaturation: 1 min, 95 °C Annealing: 1 min, 65 °C (reduced by 1°C every second cycle) } No. of cycles = 24 Extension: 1 min, 72 °C Denaturation: 1 min, 95 °C Annealing: 1 min, 53 °C } No. of cycles = 15 Extension: 1 min, 72 °C Final Extension = 10 min, 72 °C
Vf	ATTACCGCGGCTGCGGG		
pD	CAGCAGCCGCGGTAATAC	Edwards *et al.*, (1989)	Initial Denaturation: 3 min, 95 °C Denaturation: 1 min, 95 °C Annealing: 1 min, 55 °C } No. of cycles = 30 Extension: 1 min, 72 °C Final Extension: 10 min, 72 °C
pF	CATGGCTGTCGTCAGCTCGT		

CHAPTER 4

DENITRIFICATION RESULTS

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4.1 OVERVIEW

The denitrification results from all trials are presented graphically in this chapter together with results from statistical analyses. The results are organised by trial in consecutive order (I, II, III, IV); corresponding raw data and associated manipulations are tabulated in Appendix 2. Owing to the volume of data, the individual Trial Sections (4.2 - 4.5) give an overview of the empirical data and statistics. The results are subsequently interpreted and discussed in greater detail, in the General Discussion at the end of this Chapter, Section 4.6.

4.1.1 Data Management

The denitrification results, describing C and N data, are based on concentrations measured from single bulk samples of effluents collected over 20-24 hr period, and the microcosm feed solutions used to supply the microcosms during this time. The coefficient of variation for both NO_x^- and DOC analyses was < 1 % and instruments were calibrated before each batch of analysis, with a quality check performed by analyzing 2-3 samples in triplicate.

Throughout the trials, denitrification was diagnosed by a decrease in effluent NO_3^- and DOC concentration over time, the transient presence of NO_2^-, and an overall reduction in the concentration of total oxidised N (TON [NO_3^- + NO_2^-]), as reviewed in Chapter 1. Effluent TON is a more definitive measure of the amount of denitrification occurring in the microcosms, than measuring NO_3^- loss alone, because it encompasses NO_3^- and NO_2^- removal. Losses in TON were attributed to denitrification and not N assimilation, as this had previously been demonstrated as being very low (< 2 %) (Obenhuber and Lowrance, 1991) and additionally, NH_3-N was provided as a microbial nutrient in the microcosm feed solutions.

Nitrous oxide, an intermediate product of the denitrification reaction, was not considered relevant to the water quality theme of this research and was not quantified in these experiments. Ammonia-N analysis was performed on microcosm effluents but was not detected in the microcosm effluent and therefore, is not included in the results presented here, but considered in the General Discussion (Section 4.6).

The term 'denitrification' refers to the loss of TON from the system (it is also assumed for the purposes of these trials that the end product of denitrification is N_2 gas) and the terms NO_3^- and NO_2^- refer to NO_3–N and NO_2–N throughout. Denitrification yield is also considered here in terms of % Denitrification Efficiency, a parameter commonly

reported in literature (e.g. Her and Huang, 1995). This is calculated from cumulative mass data over specified experimental periods.

To assess the amount of influent OC that was used in denitrification, the mass of DOC consumed within the microcosms was calculated, taken as the difference between influent and effluent DOC (mg). This 'mass of DOC consumed' was subsequently proportioned between the following categories of microbial activity:

- DO removal
- Denitrification
- NO_3^- reduction to NO_2^-
- Biomass assimilation
- Undetermined

A combination of empirical data from the trials and estimated values (based on reaction stoichiometry and published literature) were used to divide the mass of DOC consumed between these categories (details of data manipulation in Table 4.1). Together with the mass of DOC measured in the effluent, the results provided a conceptual hypothesis of how the OC fed into the microcosms might be utilised biochemically. The results summarize the cumulative data from respective microcosms, over the whole trial, excluding the first 4-7 days, when an initial period of equilibration has been allowed (data are tabulated in Appendix 2).

4.1.2 Description of Statistical Analysis

The experimental data were statistically scrutinised using the general linear model (GLM), which could accommodate the unbalanced experimental design of the microcosm trials (pers. comm. Dr. E. Kulinskaya, Statistical Advisory Service, Imperial College, London). Two different parameters were tested separately:

(i) Denitrification concentration, (conc. of TON removed from the system);
(ii) NO_2^- presence, (conc. of effluent NO_2^-).

The null hypothesis (H_0) throughout was that there was no significant difference between the means of treatment groups (5 treatment groups per trial) at the 95 % confidence level, ($\alpha = 0.05$).

The data from the microcosm tests was considered in terms of treatment type, each treatment consisting initially of two data sets from replicate microcosms, which were subsequently pooled for the statistical analysis.

Table 4.1: Details of data manipulation used to partition the C mass balance into conceptual categories of input OC utilisation

Utilisation Category	Description	Calculations
DO removal	The amount of DOC that would be used to remove 10 mg l^{-1} DO (this was used as a standard for all experiments, based on laboratory measurement of feed solution DO which ranged from 9-11 mg DO l^{-1})	Based on the assumption that the microcosm feed solutions contained 10 mg l^{-1}, the amount of DOC required to remove the DO was set at 3.75 mg C l^{-1}, according to the stoichiometric reaction between the OC substrate and DO (corresponding to 7.83 mg d^{-1})
Denitrification	The amount of DOC required to satisfy the observed denitrification (overall loss of TON)	Calculated by multiplying the mass of NO_3^- denitrified (TON loss) by a factor of 1.07, which represents the stoichiometric C/N requirement for complete denitrification
$NO_3^- \rightarrow NO_2^-$ reduction	DOC requirement to reduced NO_3^- to NO_2^-, based on the amount of in the effluent	Calculated by multiplying the mass of NO_2^- present in the microcosm effluent by a factor of 0.43, which represents the stoichiometric C/N requirement for reduction of NO_3^- to NO_2^-
Effluent DOC	DOC measured in microcosm effluent, accounting for background residual (refer to Section 4.6.2)	Measured effluent DOC samples converted from concentration to mass data. The residual DOC that was present throughout the Trials was accounted for as the lowest residual DOC recorded, and this amount was subtracted from the measured DOC to give the effluent DOC used in the mass balances
Estimated biomass fraction	The estimated amount of DOC assimilated into the microcosm biomass	Estimated as 15 % of the observed DOC consumption, based on values in literature (ITRC, 2000), where the observed DOC consumption = difference between nominal DOC input and DOC residual in effluent
Unaccounted for	Mass of DOC that was consumed but could not be accounted for by the factors listed here (DO removal, denitrification, $NO_3^- \rightarrow NO_2^-$ reduction, effluent, biomass fraction)	Calculated as the difference between the observed DOC consumption and the sum of the DOC requirement for [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ + biomass estimate]

The GLM procedure was carried out using the MINITAB (14) software package, and the factors used to define the GLM were:

- Treatment type
- Day of experiment
- Microcosm number (a random factor nested within treatment type)

The effect of time ('day of experiment') on the observation data was not of statistical interest, but was included in the model to increase the proportion of variance in the data sets explained by the GLM ANOVA. Where the GLM determined that there was a significant difference between treatments, pair-wise Tukey tests were performed for to determine which treatments were significantly different from each other.

4.2 TRIAL I

The objectives of this trial were to achieve denitrification in the modified microcosm apparatus and to measure NO_2^- in the effluent. Nitrite was not monitored in the previous work using the experimental apparatus (Cartmell, 1997), which examined only NO_3^- removal in relation to C supply. The microcosm (MC) identification codes for the experiment are as follows:

- **MC 1** Sterile control ($HgCl_2$)
- **MC 2** Autotrophic control (no OC)
- **MC 3** } Glucose treatment; 22 mg C l^{-1}
- **MC 4** }
- **MC 5** } Glucose treatment; 11 mg C l^{-1}
- **MC 6** }
- **MC 7** } Glucose treatment; 39 mg C l^{-1}
- **MC 8** }

The nominal NO_3^- input concentration was 15 mg N l^{-1} in all microcosms. The results for trial I are presented in Figures 4.1 to 4.3

4.2.1 Control Treatments (MC 1 and MC 2)

In both the sterile and the autotrophic controls (MC 1 and 2), the concentration of NO_3^- in the effluent remained at the influent value and the diagnostic presence of NO_2^- in the effluent was not observed (Figure 4.1 a, b). The DOC concentration in the effluent of the sterile control also remained at the influent concentration. Therefore, in both control treatments, biological denitrification was absent. Data from the controls were not considered further in this chapter except in the statistical examination of results.

An unexpected observation was the consistent presence of some form of DOC in the autotrophic control (MC 2), at ± 4 mg C l^{-1}. This residual DOC had originated from the

microcosm itself, as it was not present in the microcosm influent. This is discussed further in the General Discussion (Section 4.6).

4.2.2 Glucose Treatments: 22 mg C l^{-1} (MC 3 and MC 4)

The concentration data for these treatment replicates (MC 3 and 4), demonstrate that influent NO_3^- was reduced over the duration of the trial (21 days), decreasing from the influent value ± 15 mg N l^{-1} to ≤ 4 mg N l^{-1}. Moreover, a concomitant NO_2^- increase is observed in both microcosms (Figure 4.1 b, c). Nitrate reduction commenced during the first week, but NO_3^- and NO_2^- were detected in the effluent at the end of the trial suggesting that the denitrification reaction had not progressed to completion (where N_2 gas is the final product). A loss of TON in the third week of the trial (day 19 and 21) was observed compared to the first 8 days (Figure 4.2 a, b), but NO_2^- remained persistent. Therefore, it appears that although some denitrification was detected, significant of NO_2^- in the effluent occurred, accounting for >50 % of the observed TON in samples taken after d 8.

The DOC profile for this treatment shows that the DOC was consumed to ± 4 mg C l^{-1} in the effluents of both replicates (Figure 4.1 b, c). In MC 3, DOC declined with time from the nominal input of 22 mg C l^{-1} to < 4 mg C l^{-1} by day 21. The same concentration decrease was more immediate in MC 4, falling to < 4 mg C l^{-1} by day 6 and remaining at this value for the duration of the experiment.

A carbon mass balance comparing the consumption for different biological processes is presented in Figure 4.3 a, b. The proportion of influent DOC used in biological activity was estimated as 68 % in MC 3 and 76 % in MC 4 and just under one third of the DOC supplied to the microcosms was recovered in the effluents. It is unlikely that the DOC is unutilised glucose, which is a highly available microbial substrate, since there was NO_x^- also remaining. Of the 68-76 % influent DOC used in biological activity, only 28-29 % was involved in denitrification-type reactions [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction]. Consequently, 'alternative' biological activity within the microcosms apparently successfully competed for, and consumed the greater share of the influent DOC, even when biomass assimilation was considered.

4.2.3 Glucose Treatments: 11 mg C l^{-1} (MC 5 and MC 6)

In the low-range glucose replicates, receiving 11 mg C l^{-1}, no NO_3^- reduction was observed in either replicate for the first week; thereafter, NO_3^- decreased by approximately 6 and 5 mg N l^{-1} in MC 5 and 6, respectively (Figure 4.1 d, e). This was associated with a concomitant an increase in NO_2^- concentration of approximately 5

mg N l^{-1} in both replicates. Furthermore, the NO_x^- profile observed for MC 6 showed a dynamic corresponding pattern in declining NO_3^- and increasing NO_2^- concentration followed by the opposite condition where NO_3^- increased and NO_2^- declined. Under flow-through conditions, such concentration dynamics are indicative of $NO_3^- \rightarrow NO_2^-$ reduction reactions, where NO_2^- is the end product and a decrease in effluent NO_3^- results in an increase in NO_2^- of an approximately equal magnitude. Conversely, when less NO_3^- is reduced, the concentration in the effluent increases again, with a concomitant decrease in NO_2^-. Consequently, overall, whilst the proportion of NO_3^- and NO_2^- in the effluent varies, there was no net loss of TON; hence complete denitrification to $N_{2\,(g)}$ was not achieved (Figure. 4.2 c, d).

The influent OC was rapidly consumed in both treatment replicates; the effluent DOC concentration fell to ± 4 mg C l^{-1} after day 3 (Figure 4.1 d, e) and remained at this value for the duration of the trial. The DOC mass balance suggested that 86 % of the influent carbon was consumed biologically in MC 5, and 74 % in MC 6 (Figure 4.3 c, d). Of this fraction, 38-40 % was consumed in denitrification-type reactions [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction]. However, DO removal accounted for 34-35 % of this and actual denitrification accounted for ≤ 1 %.

4.2.4 Glucose Treatments: 39 mg C l^{-1} (MC 7 and MC 8)

In the high-range glucose treatments (39 mg C l^{-1}), denitrification activity was observed after day 5 in both replicates (Figure. 4.1 f, g). The NO_3^- concentration had decreased to < 1.5 mg N l^{-1} at d 21, and the NO_2^- peaked at 8-9 mg N l^{-1} on d 8 and declined subsequently. This was more pronounced in MC 8, where the final NO_2^- concentration was 1.7 mg N l^{-1} compared to 4.7 mg N l^{-1} in MC 7 on d 21. The transient peak in NO_2^- indicated that denitrification had proceeded beyond the initial $NO_3^- \rightarrow NO_2^-$ reaction and that TON loss was occurring, which was confirmed by the TON data (Figure. 4.2 e, f). There was no decline in TON during the first 8 days of the trial; however, on d 10 and d 21, TON was reduced by more than 50 % to < 6.0 mg l^{-1}. The TON in these effluent samples was composed mainly of NO_2^-. Therefore, although 'complete denitrification to N_2 was observed, there was an imbalance in the efficiency of the processes of NO_3^- and NO_2^- reduction (discussed in Section 4.6).

The DOC concentration profiles for both treatment replicates show a decrease in effluent DOC to < 5 mg C l^{-1} which occurred over the duration of the trial (Figure 4.1 f, g). The proportion of influent DOC apparently consumed in biological activity was 69 and 80 % in MC 7 and 8, respectively (Figure 4.3 e, f). Of this percentage, 21 % was used in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] in MC 7 and 23 % in MC 8.

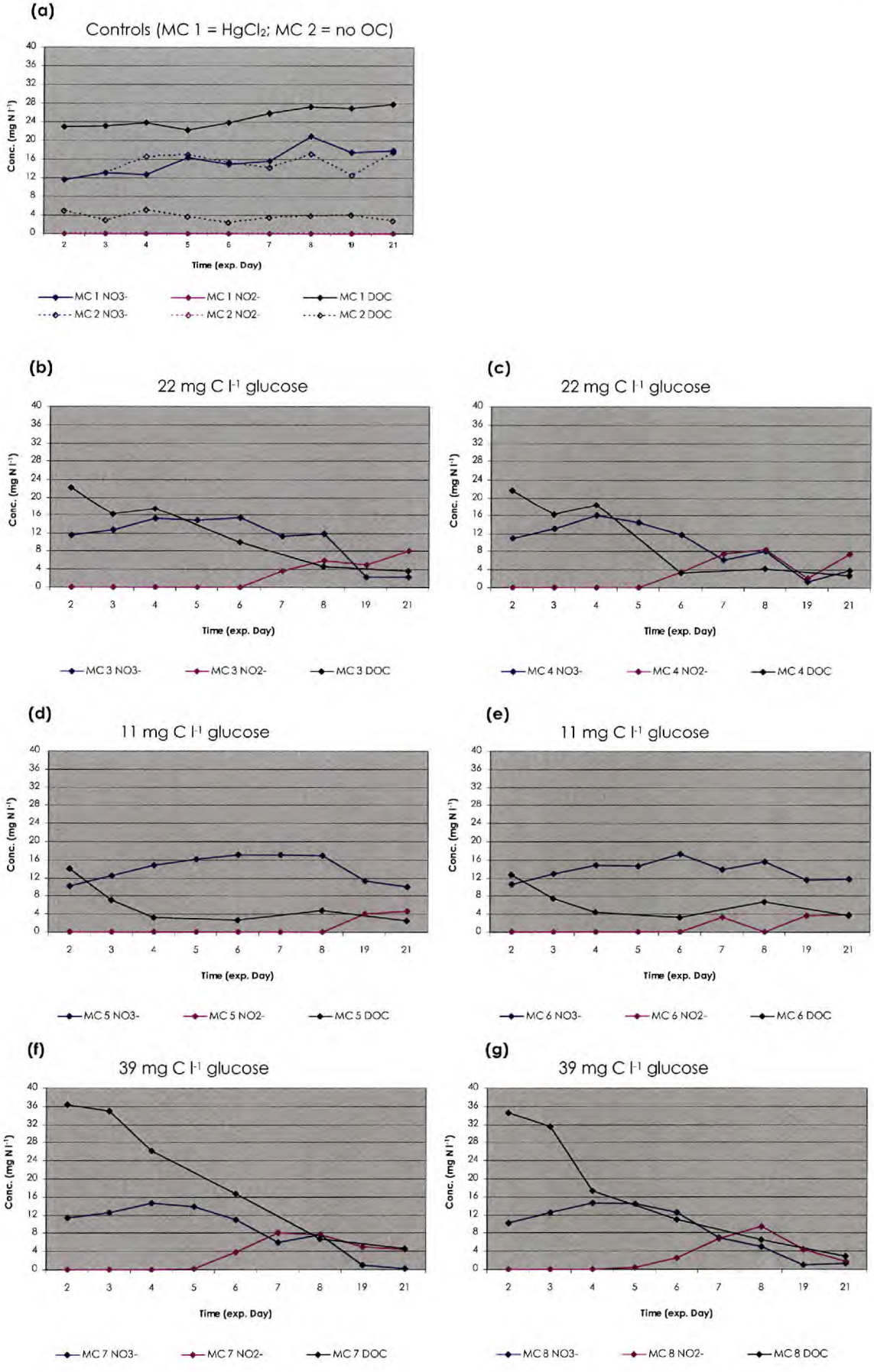


Figure 4.1 (a-g): Influence of glucose input: 22, 11 and 39 mg C l^{-1} on effluent concentrations of NO_3^-, NO_2^- and DOC during microcosm Trial I (concentration scale for DOC is in units: mg C l^{-1})

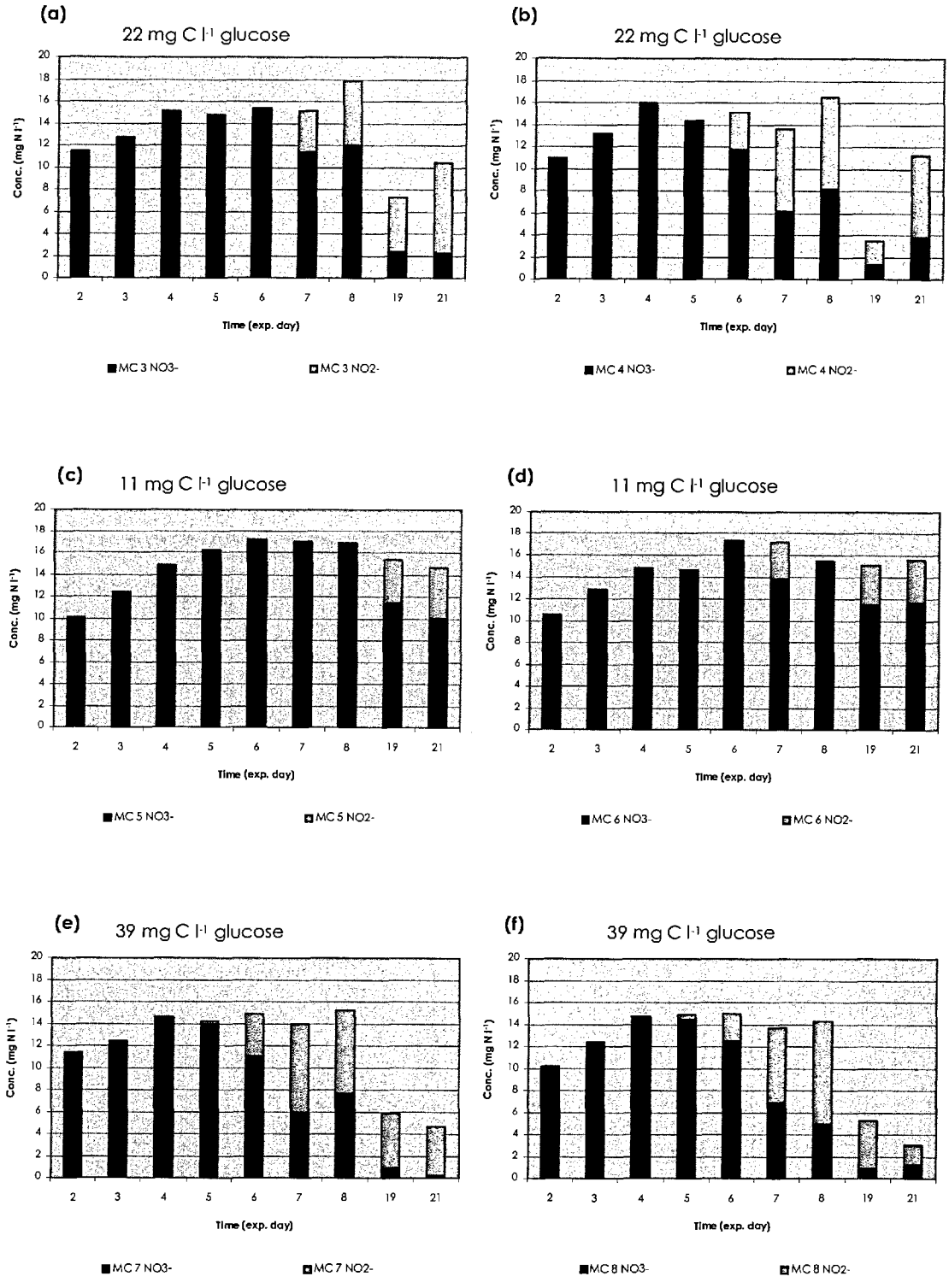


Figure 4.2 (a-f): Influence of glucose input: 22, 11 and 39 mg C l^{-1} on concentrations of NO_3^- and NO_2^- as portions of the effluent TON during microcosm Trial I

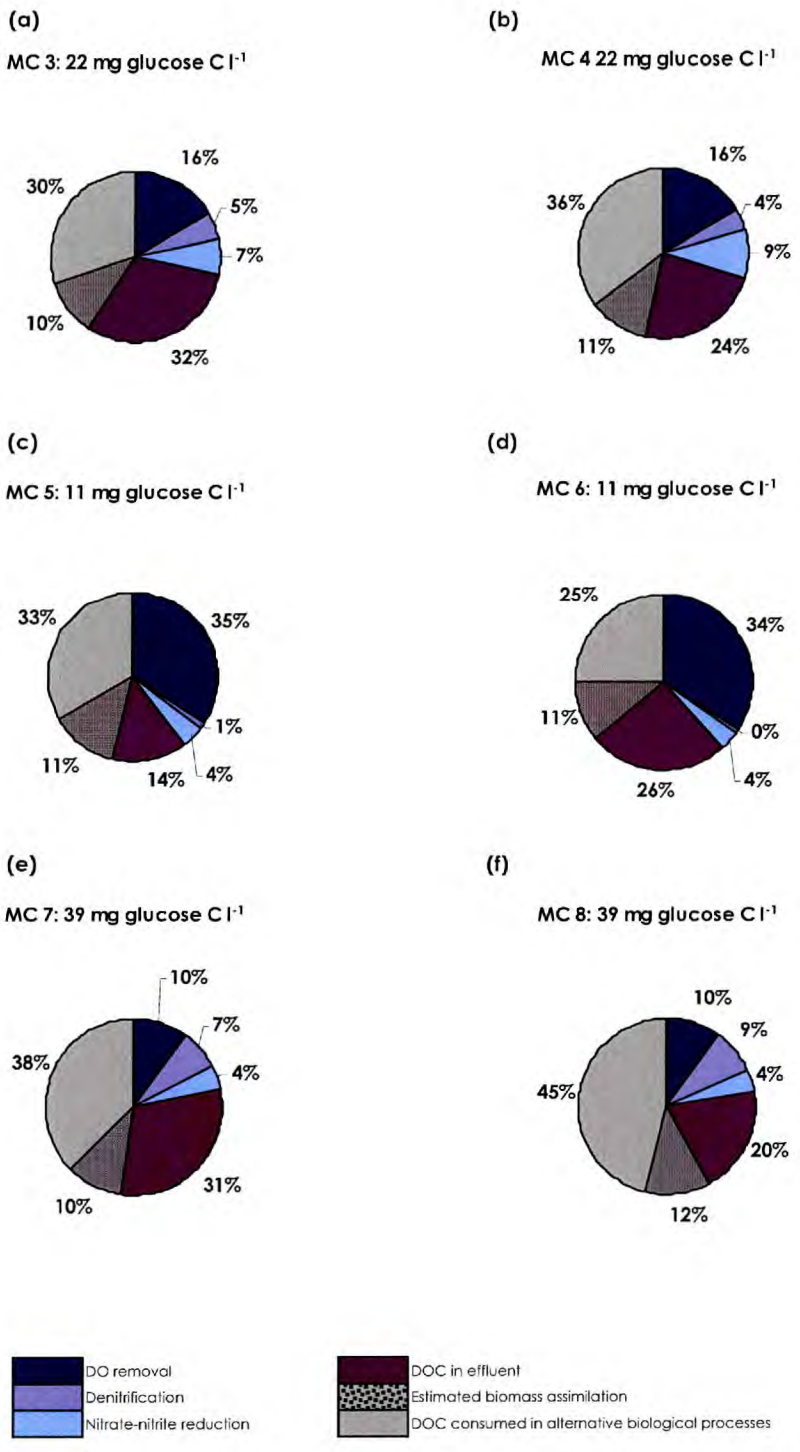


Figure 4.3 (a-f): Influence of glucose input: 22, 11 and 39 mg C l^{-1} on C mass balance and utilisation during microcosm Trial I

4.2.5 General Linear Model Results

The GLM to compare the effects of input glucose concentration on denitrification activity (TON removal) showed that there was no significant difference (P = 0.96) between treatments (controls, 22, 11 and 39 mg C l^{-1}) at the 95 % confidence level, (α = 0.05) (ANOVA results are presented in Appendix 2). A second GLM to compare the concentrations of NO_2^- persisting in the effluent also showed no significance between treatments at the 95 % confidence level (P = 0.135).

Although some NO_3^- reduction was observed in the treatment microcosms and denitrification activity was becoming established, the statistical test showed that this was not significant, given the nature of the statistical method, which pooled together the denitrification and NO_2^- concentration data sets respectively to get the treatment means. Therefore, given the relatively short duration of this experiment, the statistics were more influenced by the observations made during the period when no denitrification activity was observed.

4.2.6 Summary

- No denitrification activity was observed in the controls.
- NO_3^- reduction activity was observed in all active treatments; in the low-glucose treatment (11 mg C l^{-1}), NO_3^- was reduced to NO_2^- only and no overall denitrification was recorded; in the mid- and high glucose treatments (22 and 39 mg C l^{-1}), complete denitrification was observed, but there was a persistent NO_2^- residual. However, neither denitrification nor NO_2^- concentration were statistically significant over the short experimental duration.
- Increased influent OC concentration was associated with improved denitrification and less NO_2^- residual.
- Microcosm effluents contained a residual DOC of approximately 2.5 - 5.0 mg C l^{-1} in all treatment types by the end of the trial. Residual DOC was additionally observed in the effluent of the autotrophic control, which had not been fed OC.
- The proportions of influent OC used in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] were: 38-40 % in the low (11 mg C l^{-1}), 28-29 % in the mid-range (22 mg C l^{-1}) glucose treatments and 21-23 % in the high-glucose treatment (39 mg C l^{-1}).
- The effluent DOC, as a proportion of influent OC was: 30-42 %, 31-39 % and 23-34 % in the low-, mid- and high-glucose regimes, respectively.

4.3 TRIAL II

The objectives of this trial were to compare denitrification efficiency and NO_2^- accumulation in microcosms treated with two different substrate types: glucose and sodium acetate and, furthermore, to assess the effect of operational interruptions on denitrification. The microcosm (MC) identification codes are as follows:

- **MC 1** Sterile control ($HgCl_2$)
- **MC 2** Autotrophic control (no OC)
- **MC 3** } Glucose treatment; 22 mg C l^{-1}
- **MC 4** }
- **MC 5** } Acetate treatment; 19 mg C l^{-1}
- **MC 6** }
- **MC 7** } Acetate treatment; 11 mg C l^{-1}
- **MC 8** }

The nominal NO_3^- input concentration was 15 mg N l^{-1} in all microcosms. The results for trial II are presented in Figures 4.4 to 4.6. The denitrification results of this trial were more variable compared to the first trial, which had an essentially similar range of DOC influent concentrations. However, there were operational procedures that differed from the first trial, which must be considered. For instance, sampling was more frequent in this trial, providing a more detailed profile of denitrification dynamics. Furthermore, there were periods during this trial where the microcosms were not pumping and the interstitial solution of the microcosms remained standing. Care was taken to allow at least one day before sampling following a re-start. Finally, towards the end of the Trial, the influent DOC was increased in increments of 5 mg C l^{-1}, on days 60, 70 and 78 to increase denitrification activity in the microcosms. The microcosm stoppages and DOC increases are considered briefly here and in more detail in Section 4.6.

4.3.1 Control Treatments (MC 1 and MC 2)

Both the sterile- (MC 1) and the autotrophic (MC 2) control treatments inhibited denitrification. The NO_3^- remained at ± influent concentration and no NO_2^- was detected in the effluents (Figure 4.4 a, b). DOC concentrations in the sterile control also remained at the influent concentration, whilst in the autotrophic microcosm, a residual DOC of up to 4 mg C l^{-1} was observed. No further manipulations were carried out with the control data.

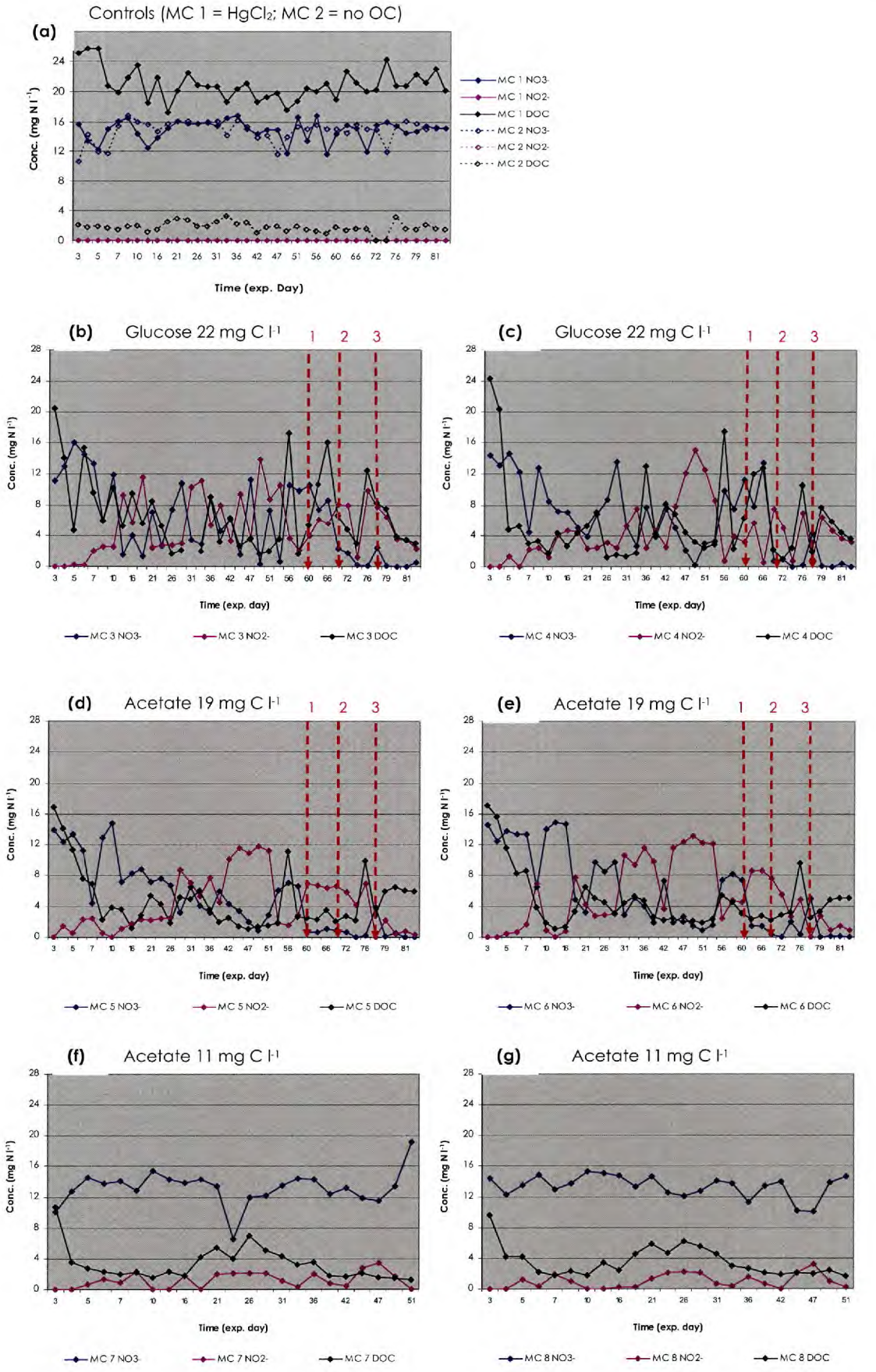


Figure 4.4 (a-g): Influence of OC input on effluent concentrations of NO_3^-, NO_2^- and DOC during microcosm Trial II, (concentration scale for DOC is in units: mg C l^{-1}). Dashed red lines and numbers indicate 5 mg C l^{-1} influent OC increases

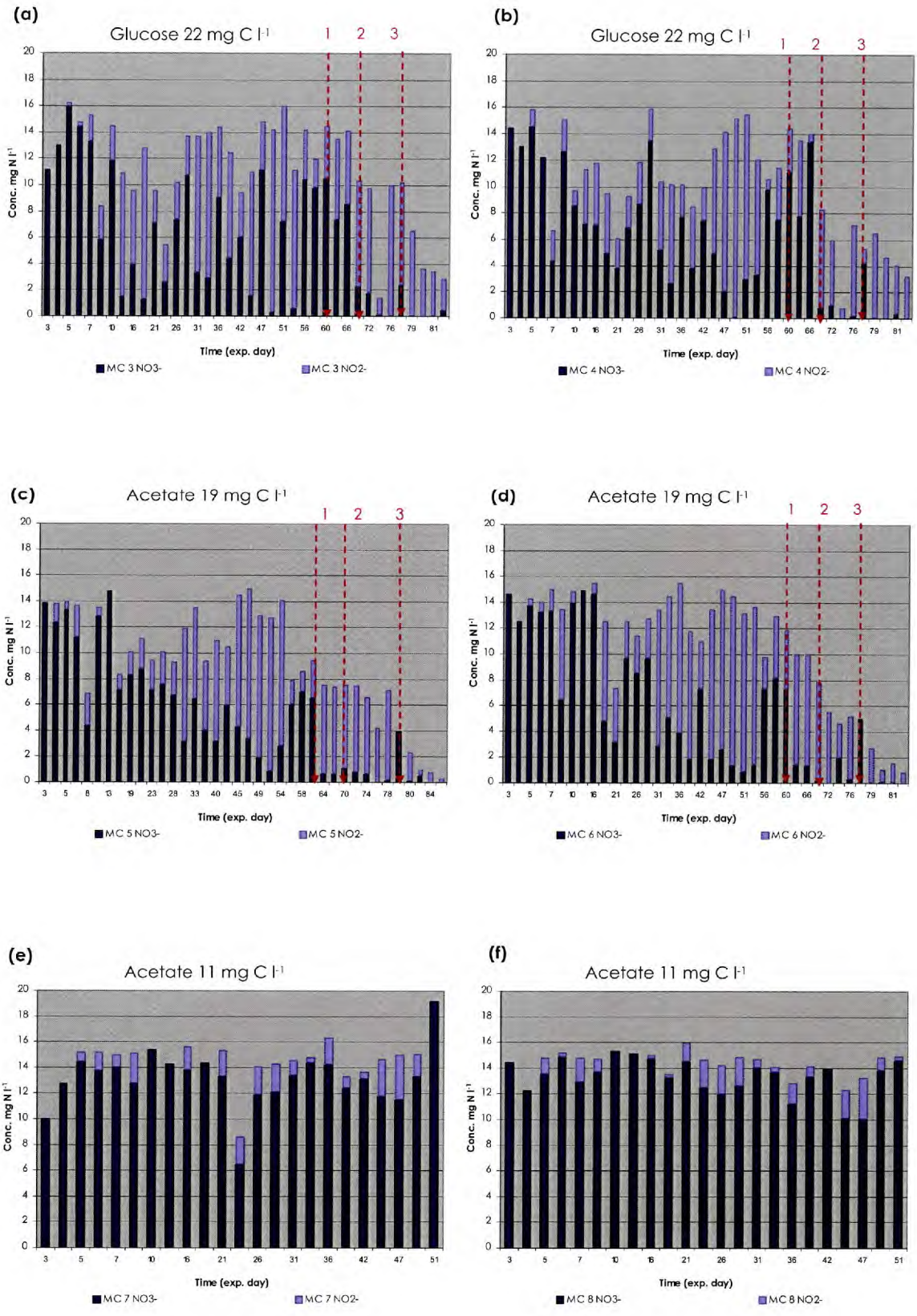


Figure 4.5 (a-f): Influence of OC input on concentrations of NO_3^- and NO_2^- as portions of the effluent TON during microcosm Trial II, dashed red lines and numbers indicate 5 mg C l^{-1} influent OC increases

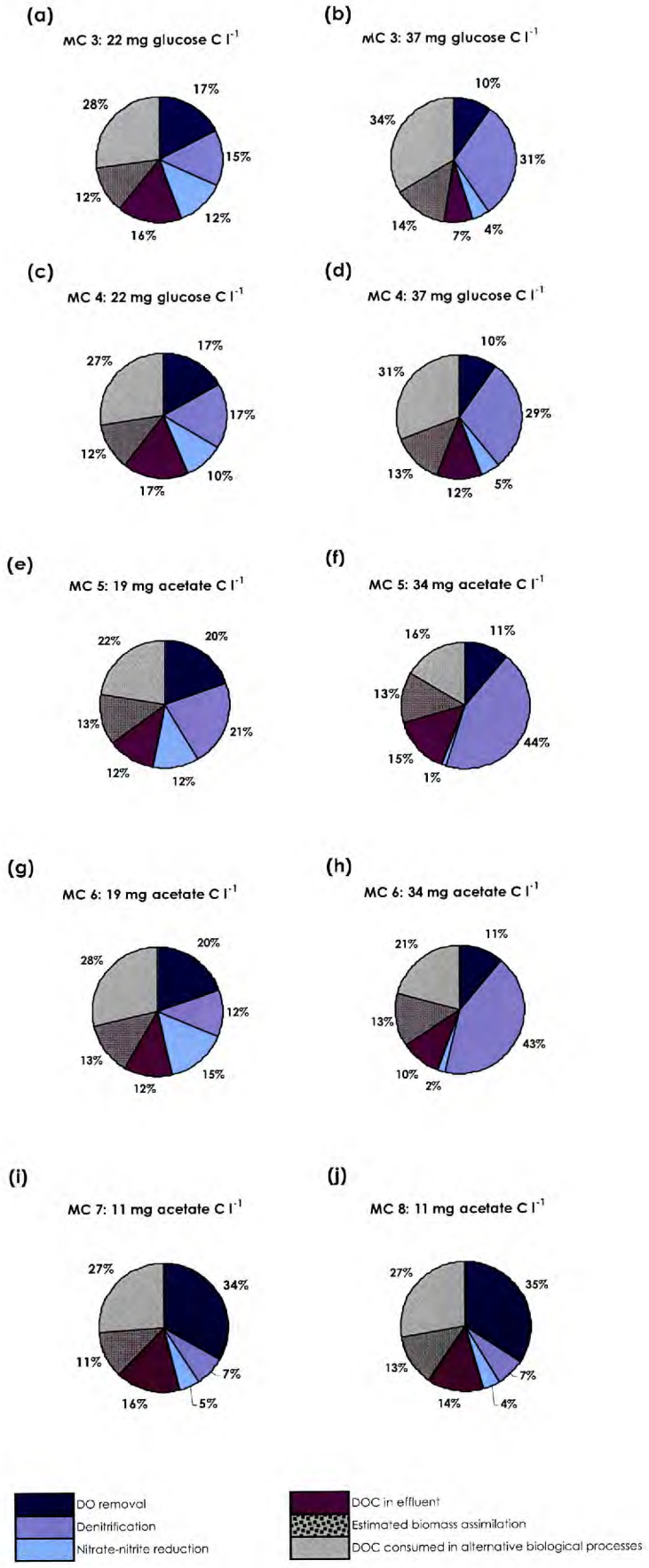


Figure 4.6 (a-j): Influence of OC input on C mass balance and utilisation during microcosm Trial II

4.3.2 Glucose Treatments: 22 mg C l^{-1} (MC 3 and MC 4)

The concentrations of NO_x^- and DOC for the glucose replicates (22 mg C l^{-1}) are presented in Figure 4.4. b, c. The NO_3^- concentration exhibited alternating maximum and minimum values, fluctuating by up to 13 mg N l^{-1} between consecutive sampling events. Following influent DOC increases on d 61 and 70, NO_3^- concentration declined and was undetectable in the effluent after the final influent OC increase (to 37 mg C l^{-1}) on d 78. The concentration of effluent NO_2^- also oscillated between consecutive samples, by up to 10 mg N l^{-1}. On d 49, NO_2^- peaked at 13.81 and 15 mg N l^{-1} in MC 3 and 4 respectively. The NO_2^- did not show any overall decline until day 78, after the final influent OC increase (to 37 mg C l^{-1}).

The TON concentrations for this treatment demonstrated the dynamic nature of the ratio of NO_3^- to NO_2^- in the effluents and of the TON. Between days 3 and 70 (when influent OC was increased to 32 mg C l^{-1}), there appeared to be a series of fluctuations in effluent TON, where peaks in TON coincided with increased NO_3^- concentration and the smallest TON values coincided with increased NO_2^- concentrations (Figure 4.5 a, b). The magnitude of the oscillations appeared to become more pronounced as the trial proceeded, until the second increase in influent OC on d 70, whereon NO_2^- became the dominant form of oxidised N in the effluent. Following the final influent OC increase on d 78, the microcosms appeared to sustain TON removal.

Thus, at the influent OC of 22 mg C l^{-1}, the effluent TON concentration was equivalent to the influent concentration, with NO_3^- and NO_2^- alternately constituting the greater portion (Figure 4.5). The increase in influent OC to 37 mg C l^{-1} induced complete denitrification, although a residual amount of NO_2^- remained. Interruptions to microcosm operation, on days 34, 54 and 61 did not apparently further impact the concentration profiles of NO_3^- and NO_2^-.

Concentrations of DOC in the effluents of the two replicates were also variable throughout the trial, although generally declined to approximately 4 mg C l^{-1} (Figure 4.4 b, c). Unlike the NO_x^- profiles, the DOC was more responsive to interruptions in microcosm operation. Following the first recess (day 34), effluent DOC increased by 7 and 11 mg C l^{-1} in MC 3 and 4 respectively, the second recess (day 54) produced concentration increases in excess of 13 mg C l^{-1}. The effect of the third recess was more obscure, as it coincided with the increases in influent OC, nonetheless, the sample taken after the third recess (on day 66) did exhibit a peak in DOC. These findings imply that periods of operational shut-down caused a transient decrease in

the utilisation of influent OC, potentially indicative of a decrease in microbial metabolism, although this did not seem influence NO_x^-.

The mass balances for DOC utilisation in Trial II are presented in Figure 4.6. These show C partitioning in the first phase of the trial, prior to the influent OC increases (d 8-60), and in the final phase of the trial, where influent DOC was increased to its maximum value (d 79-84), where applicable. Generally, increased influent OC resulted in an increased proportion of OC consumed in biological activity, relative to that observed in the effluent (MC 3 – MC 6). The amount of influent OC used in denitrification-related activities [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] was similar during the initial influent OC dose and the increased concentration doses (44 % and 44.5% respectively), however, the fraction of influent OC consumed in actual denitrification reactions increased (by 12 -16 %), demonstrating the greater efficiency under the higher dose.

4.3.3 Acetate Treatment: 19 mg C l^{-1} (MC 5 and MC 6)

Effluent NO_3^- concentrations for the acetate treatment (19 mg C l^{-1}) exhibited a fluctuating decreasing trend over the duration of the trial, stabilizing at a low value (< 1 mg N l^{-1}) after day 60, when influent OC was increased (Figure 4.4 d, e). The opposite pattern was observed for NO_2^- concentration. Thus, effluent NO_2^- content was variable, but generally increasing between d 4 and d 60. The dynamic nature of the concentration profiles was more pronounced in MC 6, where NO_3^- and NO_2^- concentration alternated in a series of high and low cycles, and the magnitude of the fluctuations increased over time. The TON results, however, showed that, although the concentration of NO_2^- in the effluent was increasing, there was an overall decrease in effluent TON and this was apparent prior to increasing the influent OC concentration, from day 47 onwards (Figure 4.5). These results imply, therefore, that denitrification was occurring in the microcosms at the initial acetate input concentration of 19 mg C l^{-1}. The subsequent incremental increases in influent OC apparently improved denitrification capacity, although the NO_2^- residual persisted in the effluent until the end of the trial, when the influent OC was raised to 35 mg C l^{-1}. Interruptions to microcosm operation on days 34, 54 and 64 had little effect on the overall N concentration dynamics, particularly given the general variability of the concentration profiles.

The DOC in the effluent fell below 4 mg C l^{-1} by d 8 and remained approximately at this concentration for the rest of the Trial. The operational interruptions coincided with only one incidence of increased effluent DOC, when the concentration measured in the effluent increased by 10 mg C l^{-1} was observed in MC 5 on d 56.

The DOC utilization in this treatment indicated that biological activity accounted for 88-90 % of the influent OC (Figure 4.6). Of this fraction, between 47-53 % was consumed in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] prior to raising the influent OC concentration and this increased to 56 % at the higher OC input. Specifically, the denitrification fraction increased by 23 % in MC 5 and 31 % in MC 6.

4.3.4 Low Concentration Acetate Treatment: 11 mg C l^{-1} (MC 7 and MC 8)

The effluent NO_3^- concentrations for the low acetate treatment (11 mg C l^{-1}), were consistently between 10-15 mg N l^{-1} (Figure 4.4 d, e). Concomitantly, NO_2^- fluctuated between 0-4 mg N l^{-1}. The TON data indicated relatively little quantitative loss of N in either treatment replicate (Figure 4.5 e, f), although there appeared to be an anomalous result in MC 7 on day 23. Therefore, the presence of NO_2^- in the effluent indicated that, at this low OC input concentration, the NO_3^- reduction only proceeded to NO_2^-.

Effluent DOC rapidly decreased to the background residual value of 2-4 mg C l^{-1} (Figure 4.4 f, g). A small increase in effluent DOC was observed on day 26, peaking at approximately 6 mg C l^{-1}. This did not coincide with any operational amendments or changes in NO_x^- activity, although it could be related to a decrease in OC uptake by non-denitrification microbial activity. This is given further consideration in Section 4.6.

The C mass balance (Figure 4.6) showed that 84-86 % of influent OC was consumed in biological activity, of which, 46 % was involved in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction]. The largest fraction of influent OC removal was consumed in DO removal however (34-35 %).

4.3.5 General Linear Model Results

The GLM showed that there was a statistically significant difference ($P = 0.02$) in the concentration of N denitrified between the five treatment groups in this trial. A significant difference ($P = 0.01$) in the concentration of effluent NO_2^- between the active treatments (glucose, acetate and low acetate) was also detected by the GLM. The mean concentrations of N denitrified and effluent NO_2^- for each treatment are summarised in Table 4.2.

The Tukey pair-wise comparison of N denitrified between treatments found no significant difference between the sterile and autotrophic control treatments. The concentration of N denitrified in all biologically active treatments statistically different

from the controls (P values are displayed in Table 4.3). Referring to the mean concentration values for N denitrified (Table 4.2), and the Tukey statistics, it is shown that the glucose (22 mg C l^{-1}) and acetate (19 mg C l^{-1}) treatments were not significantly different from each other but both gave a significantly higher concentration of N denitrified compared to the low acetate (11 mg C l^{-1}) treatment.

With regards to the effluent NO_2^- concentrations, Tukey comparisons of the active treatments (control treatments not included as no NO_2^- was detected) showed that effluents from glucose and acetate treatments had significantly higher effluent NO_2^- concentrations than the low concentration acetate treatment (P = <0.001) but were not significantly different from one another (P = 0.93). This is attributed to the limited denitrification activity observed in the low acetate treatment.

Table 4.2: Mean concentration values of N-denitrified and effluent NO_2^- for treatment regimes in Trial II

Treatment	Mean concentration of N denitrified during Trial (mg l^{-1})	Mean concentration of effluent NO_2^- during Trial (mg l^{-1})
Sterile control (MC 1)	0.47	0.00
Autotrophic control (MC 2)	0.34	0.00
Glucose 22 mg C l^{-1} (MC 3 and 4)	4.71	5.41
Acetate 19 mg C l^{-1} (MC 5 and 6)	5.41	5.24
Acetate 11 mg C l^{-1} (MC 7 and 8)	0.75	1.22

Table 4.3: Probability values indicating Statistical Significance of differences in concentration of N denitrified between treatments during Trial II. Probabilities determined by Tukey pair-wise Comparisons

	Autotrophic control (MC 2)	Glucose 22 mg C l^{-1} (MC 3 and 4)	Acetate 19 mg C l^{-1} (MC 5 and 6)	Low acetate 11 mg C l^{-1} (MC 7 and 8)
Sterile control (MC 1)	0.99	**< 0.001**	**< 0.001**	**0.04**
Autotrophic control (MC 2)	-	**< 0.001**	**< 0.001**	**0.02**
Glucose 22 mg C l^{-1} (MC 3 and 4)	-	-	0.45	**< 0.001**
Acetate 19 mg C l^{-1} (MC 5 and 6)	-	-	-	**< 0.001**

4.3.6 Summary

- No denitrification activity was observed in the controls.
- Nitrate reduction activity was observed in all active treatments. With low acetate (11 mg C l^{-1}), NO_3^- was reduced to NO_2^- only and no overall denitrification was observed. In the glucose and acetate treatments (22 and 19 mg C l^{-1}), the effluents were characterised by variable NO_3^- and NO_2^- concentrations were the ratio of NO_3^- to NO_2^- fluctuated from high to low concomitantly. The magnitude of the ratio-fluctuations increased over time, so that effluents were composed of nearly all NO_3^- or nearly all NO_2^-. This dynamic was more pronounced in the glucose treatment compared to acetate.
- Denitrification (TON loss) was observed from the effluents of the acetate treatment from day 47 onwards, prior to influent DOC increase.
- Denitrification was not observed in the effluents of the glucose treatment until the second phase of the trial, when the influent OC was raised.
- A residual NO_2^- concentration remained in the effluents of both the glucose and acetate treatments when the influent OC increased to 34 and 37 mg C l^{-1}, respectively. The NO_2^- in the acetate effluents, however, was < 1.5 mg N l^{-1}, whereas glucose effluents contained NO_2^-N in the range 2.3-4.6 mg N l^{-1}.
- The mean denitrification efficiencies (d 8 to end of trial) were: 5 % in the low acetate treatment, 31.4 % and 36.1 % in the glucose and higher acetate treatments respectively.
- All treatment microcosms exhibited a residual effluent DOC of approximately 4 mg C l^{-1}.
- In the two acetate treatments, increased influent OC produced a greater proportion of influent DOC used in denitrification activity.
- Microcosms supplied with acetate treatment consumed a greater portion of influent OC in denitrification activity compared glucose (~ 10 % more).
- Statistically, the glucose and acetate treatments showed a significantly higher concentration of NO_3^- denitrified than the controls and low-acetate treatment, though they did not differ significantly from each other in this respect.
- Similarly, the glucose and acetate treatment showed significantly greater effluent NO_2^- concentrations than the low-acetate treatment, but did not differ significantly from each other.

4.4 TRIAL III

The objective of this trial was to assess the potential interaction between the OC amendment and alternative electron acceptors; Fe^{3+}, Mn^{4+} and SO_4^{2-}. The results

presented in this chapter describe denitrification activity only; Fe^{3+}, Mn^{4+} and SO_4^{2-} activity is considered in Chapter 6. The various treatment types in this trial were concerned with differing mineral content in the microcosm sandstone matrix; all microcosms were given the same influent feed solution containing sodium acetate at a concentration of 35 mg C l^{-1}. On day 37, however, the influent OC dose was increased to 50 mg C l^{-1} so that concentration of OC available for microcosm biomass would be in excess of the stoichiometric requirement for complete N denitrification. The nominal NO_3^- input concentration was 15 mg N l^{-1} in all microcosms. The results for trial III are presented in Figures 4.7 to 4.9, the microcosm identification codes were:

- **MC 1** Sterile ($HgCl_2$) control, Lower Greensand (LGS)
- **MC 2** Sterile ($HgCl_2$) control, Devonian sandstone
- **MC 3** } Devonian sandstone
- **MC 4** }
- **MC 5** } Lower Greensand
- **MC 6** }
- **MC 7** } Triassic sandstone
- **MC 8** }

4.4.1 Control Treatments (MC 1 and MC 2)

In the sterile control microcosms, MC 1, MC 2, the NO_3^- concentration remained at the influent value throughout the trial and NO_2^- was not detected in the microcosm effluents, indicating the absence of denitrification activity (Figure 4.7 a). The effluent DOC also remained at the influent concentration, and the increase in effluent DOC on d 37 of the trial reflected changes to influent DOC concentration.

4.4.2 Devonian Sandstone Microcosms (MC 3 and MC 4)

In the microcosms filled with Devonian sandstone material, the effluent NO_3^- concentration began to fall after day 4 in both replicates (Figure 4.7 b, c) and NO_2^- was detected in the effluent on day 9 in MC 3 and day 10 in MC 4. This suggested that NO_3^- removal occurred within the microcosms during the first 9-10 days of the trial, even though the diagnostic presence of NO_2^- was not observed. Indeed, the TON plots showed that, during the first 9 days, effluent TON decreased to < 10 mg N l^{-1} (Figure 4.8 a, b). Furthermore, NO_2^- is scarcely present throughout the trial and its contribution to the overall TON did not exceed that of the NO_3^-. Conversely, NO_3^- persisted in the effluent, albeit at concentrations < 4 mg N l^{-1} and complete denitrification did not occur until d 44 in MC 3 and 38 in MC 4, coinciding with the influent DOC increase to 50 mg C l^{-1}.

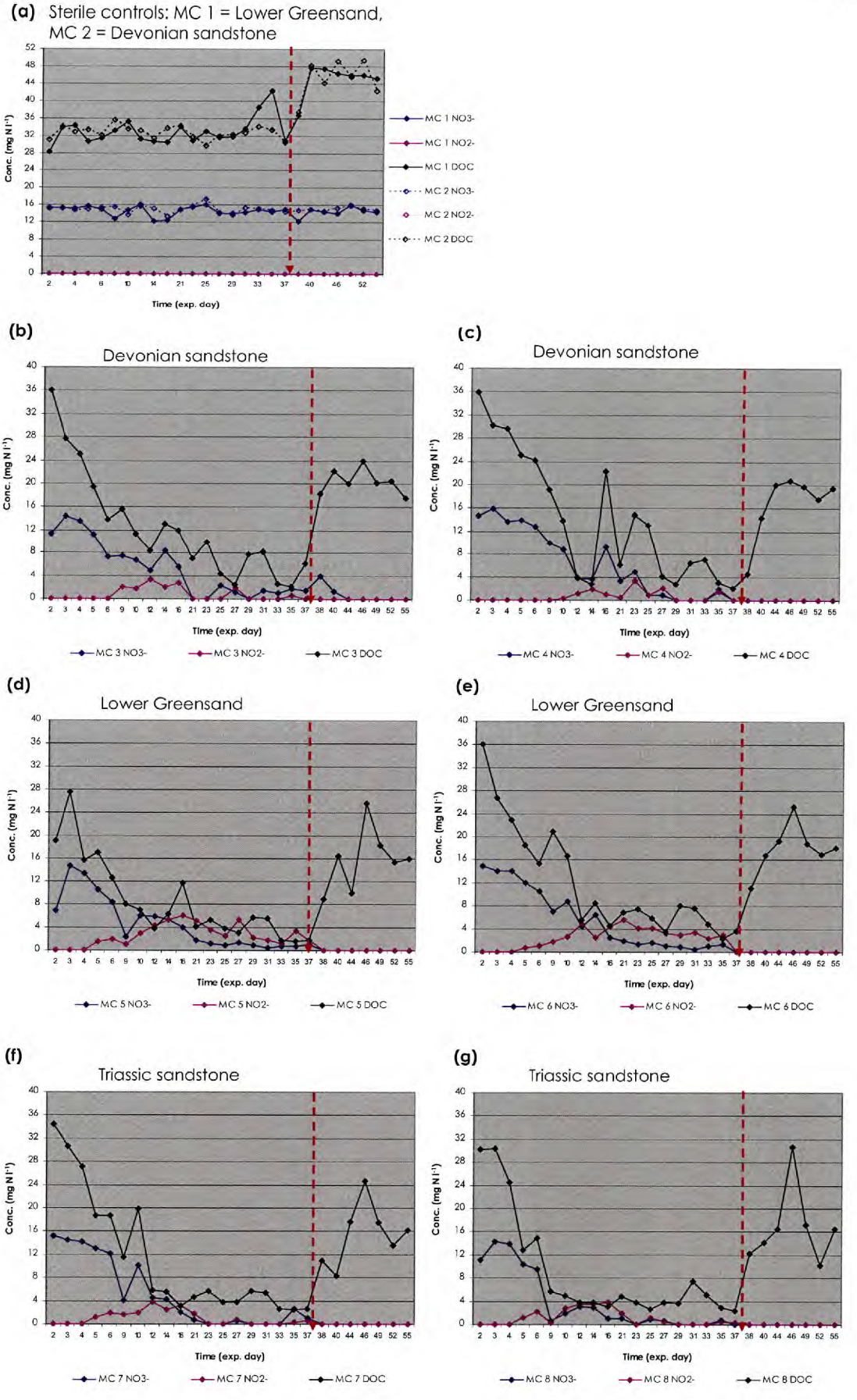


Figure 4.7 (a-g): Influence of acetate input on effluent concentrations of NO_3^-, NO_2^- and DOC during microcosm Trial III, (concentration scale for DOC is in units: mg C l^{-1}). Dashed red lines indicate 35 → 50 mg C l^{-1} influent OC increase

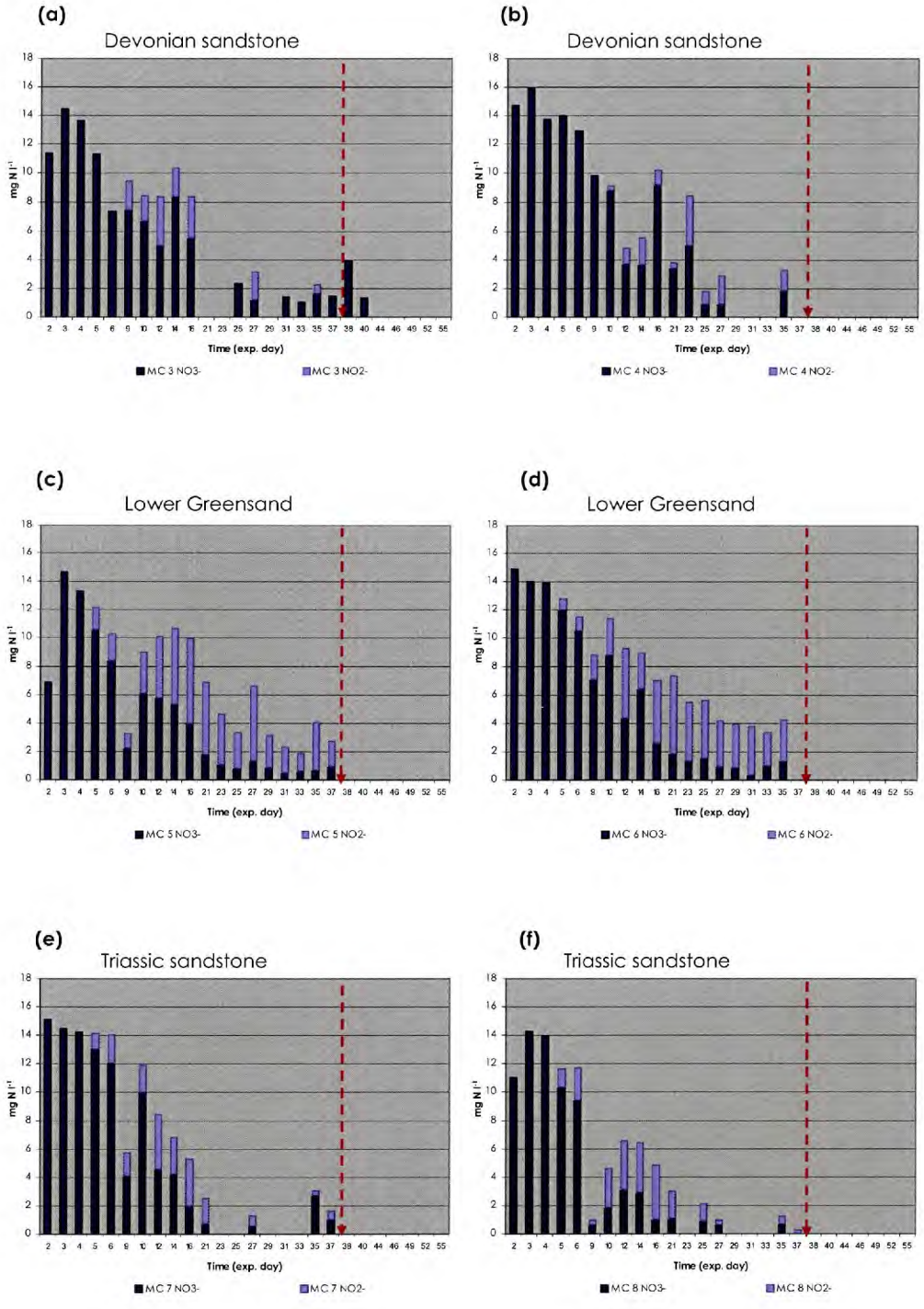


Figure 4.8 (a-f): Influence of acetate input on concentrations of NO_3^- and NO_2^- as portions of the effluent TON during microcosm Trial III, dashed red lines indicate 35 → 50 mg C l^{-1} influent OC increase

Effluent DOC declined over the first 12 days, falling by 28 mg C l^{-1} in MC 3 and 32 mg C l^{-1} in MC 4. Following this, effluent DOC continued to fall overall with increasing and decreasing fluctuations in both treatment replicates (Figure 4.7 b, c). On d 37, the influent OC was increased to 50 mg C l^{-1}, and this was reflected in the effluent DOC, which increased to approximately 20 mg C l^{-1} in both replicates for the remainder of the trial, demonstrating that the microcosms had an upper limit to the amount of OC that could be utilised in biologically mediated processes. Interestingly, the increase in influent OC appeared to correspond to the excess DOC in the effluent, suggesting that in this case, it was largely unutilised. This was also evident from the mass balance calculations, which showed minor differences in the quantity of DOC consumed for denitrification-related activity between the two OC input doses (5-6 % difference), compared to effluent DOC. Effluent DOC was notably higher following the increased input dose, increasing by 10 % in MC 4 and 19 % in MC 3 (Figure 4.9).

4.4.3 Lower Greensand Microcosms (MC 5 and MC 6)

In the microcosms filled with Lower Greensand material, effluent NO_3^- exhibited an initial period of decline during the first 21 days, after which, the concentration stabilized in the effluents from both replicates, at ± 1.0 mg N l^{-1} (Figure 4.7 d, e). This concentration persisted until day 37 in MC 5 and day 35 in MC 6, after which no NO_3^- was detected. Nitrite concentration peaked at 6.0 mg N l^{-1} in MC 5 on d 16 and 5.48 mg N l^{-1} in MC 6 on d 21; it subsequently declined in both microcosms and was not detected in the effluent after day 37. The results suggest that the denitrification was tending towards completion on d 37, at the lower influent OC dose (35 mg C l^{-1}), but the increased dose (50 mg C l^{-1}) on d 37, facilitated the complete reaction, and NO_x^- was not detected in the effluents under the higher dosage regime. This was clearly demonstrated from the TON plots for example (Figure 4.8 c, d).

Effluent DOC decreased during the first 12 days of the trial, followed by a period of fluctuation between 2 and 8 mg C l^{-1} (Figure. 4.7 d, e) until day 37, when the influent OC was increased. After this time, effluent DOC peaked at 25 mg C l^{-1} and persisted in the effluent, fluctuating around 20 mg N l^{-1}. Again, the influent OC amendment (15 mg C l^{-1}) approximated to the excess DOC measured in the effluent. This implied little additional utilisation of influent acetate in biological processes and that the majority of DOC in the effluent was acetate breakthough. This was reflected in the DOC mass balances, (Figure 4.9), where the proportion of acetate-C consumed in denitrification-related activity was varied by 3-4 % but the effluent DOC increased by 19 % in MC 5 and 16 % in MC 6.

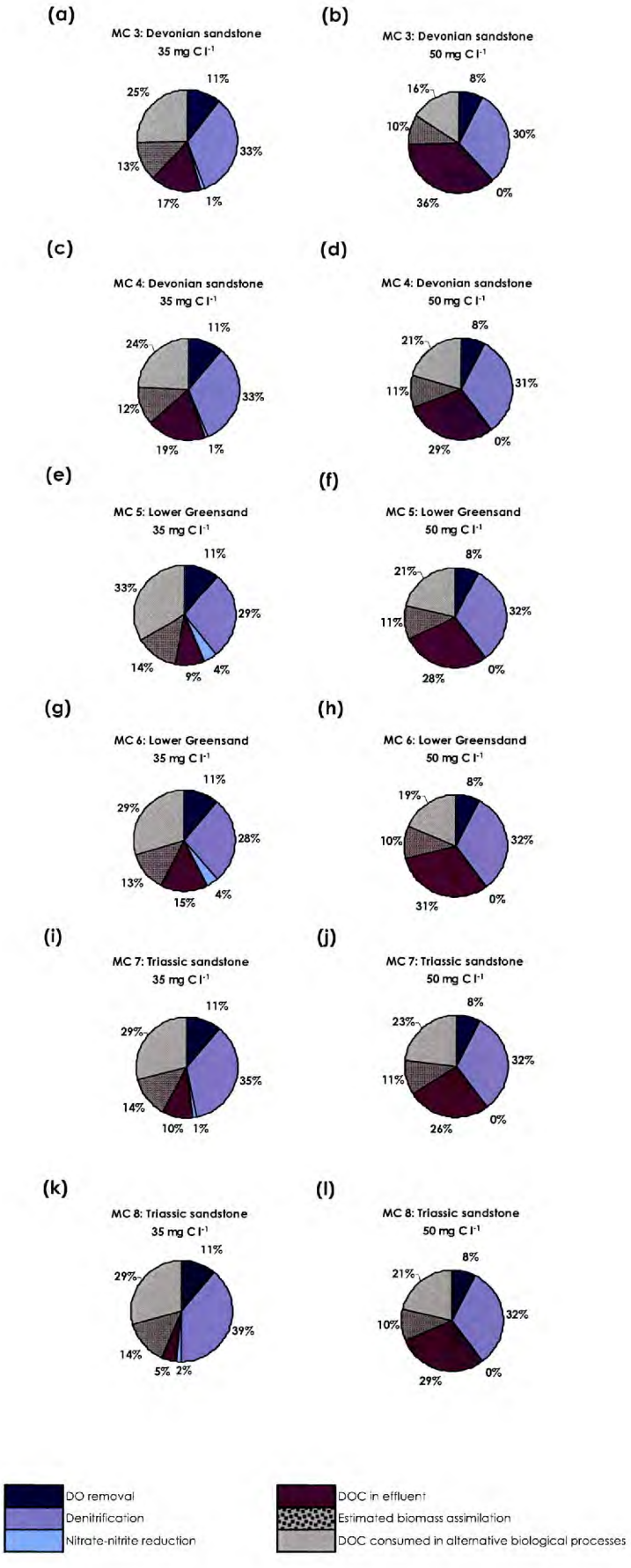


Figure 4.9 (a-l): Influence of acetate input on C mass balance and utilisation during microcosm Trial III

4.4.4 Triassic Sandstone Microcosms (MC 7 and MC 8)

In the Triassic sandstone microcosms, effluent NO_3^- concentrations decreased to ± 1 mg N l^{-1} during the first 23 days of the trial and were generally absent after this time (Figure 4.7 f, g). During the same period, NO_2^- was detected in the effluent, peaking at approximately 4 mg N l^{-1} and was generally absent from the effluent after d 23. Between days 16 and 23, NO_2^- constituted the majority of the TON observed in the effluent (Figure 4.8 e, f). In this treatment, denitrification activity was observed from day 5, but appreciable TON loss occurred from d 9 onwards. By d 23, complete denitrification was evident, though there were sporadic incidents of NO_3^- and NO_2^- residuals in the effluent. However, NO_x^- was not detected after d 37 under the increased in influent dose.

The effluent DOC showed a marked decline from the influent concentration to approximately 6 mg C l^{-1} during the first 10-12 days in MC 7 and 8, respectively. Subsequently, it fluctuated, approximating to 4 mg C l^{-1} until d 37, where, following the influent OC increase, the effluent DOC was also raised (Figure 4.7, f, g). The mean effluent DOC concentrations after the influent OC was increased to 50 mg C l^{-1} were 15.5 and 16.9 mg N l^{-1} in MC 7 and 8, respectively, indicating that the majority of the effluent DOC was substrate breakthrough. Increased availability of influent OC eliminated the occasional detection of traces of NO_x^- in the effluent. The DOC mass balance showed that the effluent DOC was greater following the increased input dose (Figure 4.9), increasing by 16 % in MC 7 and 24 % in MC 8, whereas the fraction of DOC consumed in denitrification related reactions varied by only 3-4 %.

4.4.5 General Linear Model Results

The statistical analysis by GLM showed that there was a highly significant difference in the concentration of N denitrified in microcosms of different aquifer materials ($P = <0.001$). Tukey pair-wise comparisons showed that the two sterile control microcosms (MC 1, MC 2) were significantly different from the biologically active treatments (MC 3 - 8), as anticipated (Table 4.5). Of the active microcosm treatments, the only significant difference in concentration of NO_3^- denitrified was detected between the Lower Greensand and the Triassic sandstone microcosms. In this case, the Triassic sandstone microcosms showed significantly more denitrification than the Lower Greensand, as can be seen from the mean denitrification values in Table 4.4. The concentration of N denitrified in the Devonian sandstone microcosms was intermediate between the Triassic sandstone and Lower Greensand microcosms and therefore was not significantly different to either type.

The GLM for effluent NO_2^- concentration showed that there was a highly significant difference amongst the active treatment groups, (MC 3 - 8) ($P < 0.001$). Lower Greensand produced a significantly larger mean NO_2^- concentration compared to the Devonian sandstone and the Triassic sandstone microcosms ($P = 0.001$), (refer to Table 4.4 and 4.5). Conversely, the difference in effluent NO_2^- concentration between the Devonian and Triassic sandstone microcosms was not significant, ($P = 0.68$).

Therefore, Lower Greensand was identified as having a lower concentration of NO_3^- denitrified and a greater concentration of NO_2^- in the effluent, implying that denitrification was less efficient in this aquifer material compared to Devonian and Triassic sandstone.

Table 4.4: Mean concentration values of N-denitrified and effluent NO_2^- for microcosms composed of different aquifer materials during Trial III

Treatment	Mean concentration of N denitrified during Trial (mg l^{-1})	Mean concentration of effluent NO_2^- during Trial (mg l^{-1})
Lower Greensand Control (MC 1)	0.63	0.00
Devonian sandstone Control (MC 2)	0.32	0.00
Devonian sandstone (MC 3 and 4)	12.10	0.64
Lower Greensand (MC 5 and 6)	11.15	2.17
Triassic sandstone (MC 7 and 8)	13.16	0.82

Table 4.5: Probability values indicating Statistical Significance of differences in concentration of N denitrified in microcosms composed of different aquifer materials during Trial III. Probabilities determined by Tukey pair-wise Comparisons

	Devonian sandstone Control (MC 2)	Devonian sandstone (MC 3 and 4)	Lower Greensand (MC 5 and 6)	Triassic sandstone (MC 7 and 8)
Lower Greensand control (MC 1)	0.99	**< 0.001**	**< 0.001**	**< 0.001**
Devonian sandstone Control (MC 2)	-	**< 0.001**	**< 0.001**	**< 0.001**
Devonian sandstone (MC 3 and 4)	-	-	0.24	0.16
Lower Greensand (MC 5 and 6)	-	-	-	**< 0.001**

4.4.6 Summary

- No denitrification activity was observed in the sterile control treatments.
- Denitrification was observed in all biologically active microcosms, however, the relative efficiency of denitrification activity differed between the aquifer materials tested.
- The differences in the concentration of N denitrified followed the order: Lower Greensand < Devonian sandstone < Triassic sandstone, however, the difference in N denitrified was only significant between the Triassic sandstone and Lower Greensand aquifer materials.
- The Lower Greensand microcosms also showed a significantly higher effluent NO_2^- concentration, compared to that measured in the Devonian and Triassic sandstone.
- Mean denitrification efficiencies across the whole trial (encompassing both input OC doses) were: 80.7 % in the Devonian sandstone microcosms; 74.3 % in the Lower Greensand microcosms; 87.7 in the Triassic sandstone microcosms.
- A residual amount of DOC was present in the microcosm effluents, equivalent to approximately 4 mg C l^{-1}, when denitrification was established.
- Effluent DOC increased in direct proportion with the increased influent OC dose (+15 mg C l^{-1}) on day 37 indicating substrate breakthrough. However, raising the input dose eliminated the trace TON residual (~ 1 mg N l^{-1}) for the remainder of the experiment.
- The proportion of influent DOC used in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] ranged from 38-45 % in the Devonian microcosms, 40-44 % in the Lower Greensand microcosms and 40-52 % in the Triassic sandstone microcosms. The concentration of input OC used in denitrification was similar under both the 35 mg C l^{-1} and 50 mg C l^{-1} dosage regimes, although when considered as a proportion of the total C mass balance, the amount consumed in denitrification was less under the higher OC dose.

4.5 TRIAL IV

This Trial examined denitrification activity under conditions representative of UK aquifers using sandstone and raw NO_2^- contaminated groundwater obtained from an operational public water supply facility (refer to Chapter 2 for details). Different concentrations of glucose, currently proposed as the OC substrate for the ISBD pilot Trial, were used to assess the influence of denitrification on water quality, particularly with regards to NO_2^- persistence. An alternative substrate; acetic acid, was also

tested as a potential OC substrate to support denitrification. The treatment regimes and microcosm identification codes for this trial were:

- **MC 1** } Autotrophic control
- **MC 2**
- **MC 3** } Glucose treatment; 35 mg C l^{-1}
- **MC 4**
- **MC 5** Glucose treatment; 28 mg C l^{-1}
- **MC 6** Glucose treatment; 44 mg C l^{-1}
- **MC 7** } Acetic acid treatment; 35 mg C l^{-1}
- **MC 8**

The nominal NO_3^- input concentration was 15 mg N l^{-1} in all microcosms. The results for Trial IV are presented in Figures 4.10 to 4.12.

4.5.1 Control Treatments: no OC (MC 1 and MC 2)

The autotrophic controls show that the NO_3^- remained at influent level throughout the trial and NO_2^- was not detected (Figure 4.10). The DOC of the microcosm effluent ranged from 1-6 mg C l^{-1}.

4.5.2 Glucose Treatments: 35 mg C l^{-1} (MC 3 and 4)

In MC 3, NO_3^- reduction commenced between d 1 and 4 (Figure 4.10) and subsequently, complete denitrification, was initiated by d 6 (Figure 4.11). Complete denitrification was achieved by d 15, but NO_x^- was detected occasionally in the effluent throughout the trial. In MC 4, TON loss, concurrent with the detection of effluent NO_2^- was observed on d 4, however, NO_2^- persisted in the effluent from this microcosm for the duration of the experiment, generally at concentrations $\geq$ 4 mg N l^{-1} and there was little further TON loss (Figure 4.11). Complete denitrification was not achieved in this treatment, except for an isolated occurrence on day 19. Between d 23 and 24, there was a 48 hour recess in operation, when the microcosms were not pumped. However, this recess did not appear to affect NO_x^- activity in this treatment.

The concentrations of DOC in the effluents decreased to approximately 4 mg C l^{-1} during the first week and remained at this concentration for the duration of the trial (Figure 4.10 b, c), although values as high as 8 mg C l^{-1} were occasionally measured in MC 3 effluent. The operational recess was probably responsible for the increased effluent DOC observed in both replicates on d 25.

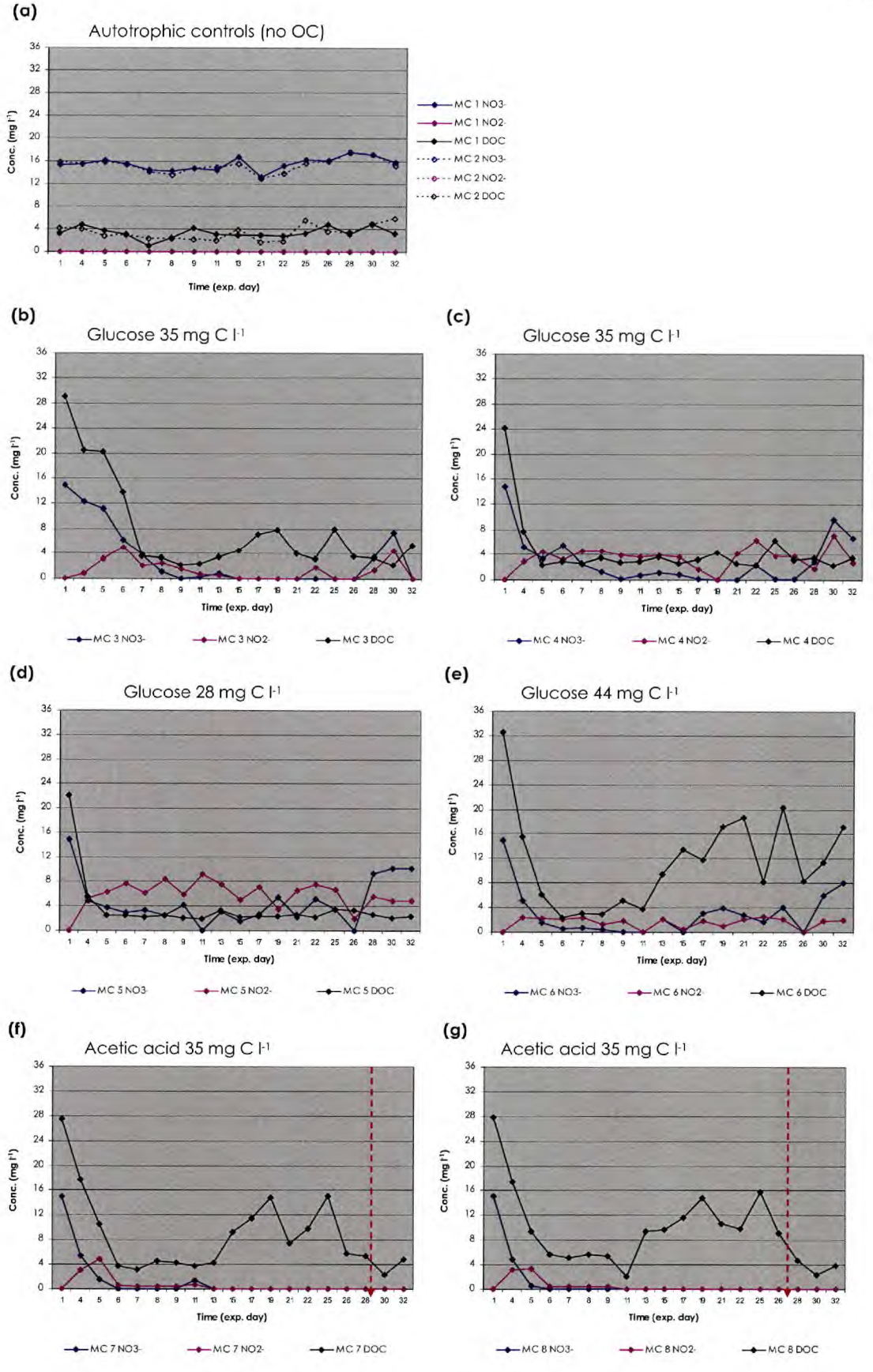


Figure 4.10 (a-g): Influence of OC input on effluent concentrations of NO_3^-, NO_2^- and DOC during microcosm Trial IV. Concentration scale for NO_x^- = mg N l^{-1}, concentration scale for DOC = mg C l^{-1}). Dashed red lines indicate decreased influent OC dose to 30 mg C l^{-1}

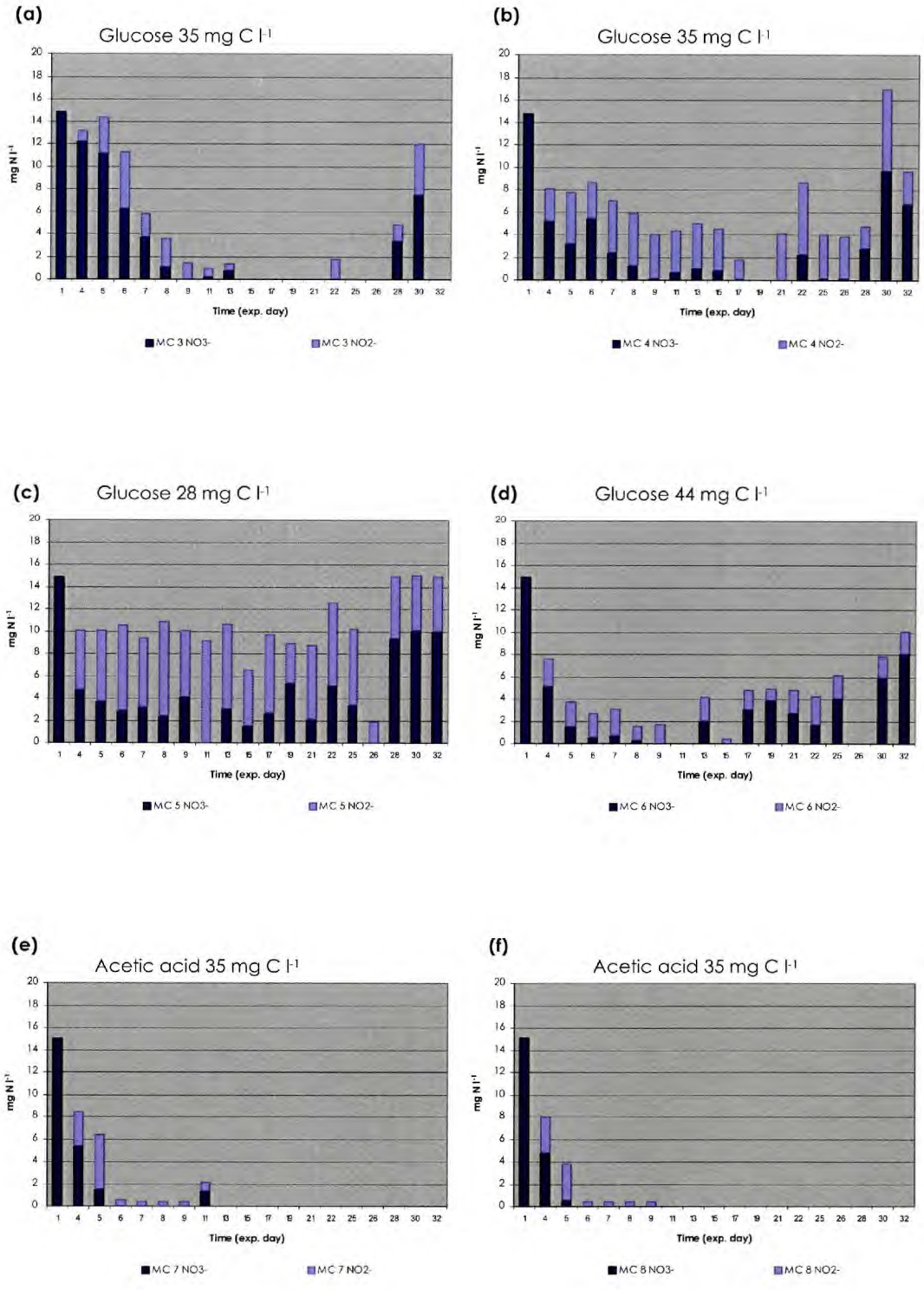


Figure 4.11 (a-f): Influence of acetate input on concentrations of NO_3^- and NO_2^- as portions of the effluent TON during microcosm Trial IV

The differences in denitrification between the two microcosms are reflected in the DOC utilisation profiles, (Figure. 4.12 a, b), which show that 47 % of the influent OC was directed towards denitrification activity [DO removal + denitrification + NO_3^-→ NO_2^- reduction] in MC 3, compared to 38 % in MC 4. Conversely, a higher proportion of influent OC (38 %) went towards alternative biological activity in MC 4, compared to 28 % in MC 3.

4.5.3 Low Glucose Treatment: Glucose 28 mg C l^{-1} (MC 5)

The microcosm that was fed glucose at a concentration of 28 mg C l^{-1} exhibited an initial decrease in effluent NO_3^-, which fell to 5 mg N l^{-1} by day 4. Subsequently, the NO_3^- concentration fluctuated between 0 and 5 mg N l^{-1}, increasing to approximately 10 mg N l^{-1} during the final 3 days of the experiment (Figure 4.10 d). Concurrently, effluent NO_2^- increased to 5 mg N l^{-1} and persisted between this value and 9 mg N l^{-1}. Effluent TON declined to approximately 10 mg N l^{-1}, but no further decreases were generally observed (Figure 4.11 c). Nitrite remained the principal component of the TON, until the final 3 days of the trial; therefore, it is apparent that, whilst some compete denitrification occurred, the NO_3^-→NO_2^- reduction reaction dominated in this treatment.

The concentration of DOC in the effluent was reduced to < 4 mg C l^{-1} by d 5 and remained consistently at this value (Figure 4.10). The DOC measured in the effluent constituted 2 % of the influent OC. Of the 98 % that was therefore consumed in biological activity, 41 % of this was involved in denitrification reactions.

The interruption to microcosm pumping did not influence either the NO_x^- or DOC concentrations in this treatment.

4.5.4 High Glucose Treatment: Glucose 44 mg C l^{-1} (MC 6)

In MC 6, fed glucose at a concentration of 44 mg C l^{-1}, denitrification activity was evident from d 4. The NO_3^- concentration in the effluent decreased to 5.17 mg N l^{-1} whilst NO_2^- was detected at a concentration of 2.39 mg N l^{-1} and the TON decreased from the influent value to 7.56 mg N l^{-1} (Figure 4.10 e; 4.11 d). Subsequently, both NO_3^- and NO_2^- typically remained between 0 and 4 mg N l^{-1}, (although NO_3^- did increase above this amount during the final two days of the experiment). Whilst effluent TON concentration decreased below the detection limit by d 11, it subsequently increased once more, measuring 10.04 mg N l^{-1} by the end of the experiment (Figure 4.11 d). Thus, denitrification activity in this microcosm was approaching 100 % efficiency over the first 16 days of the experiment, but this was not sustained, even

though the OC supply was maintained at a consistent input dose throughout. The operational recess period did not seem to impact on NO_x^- activity in this treatment.

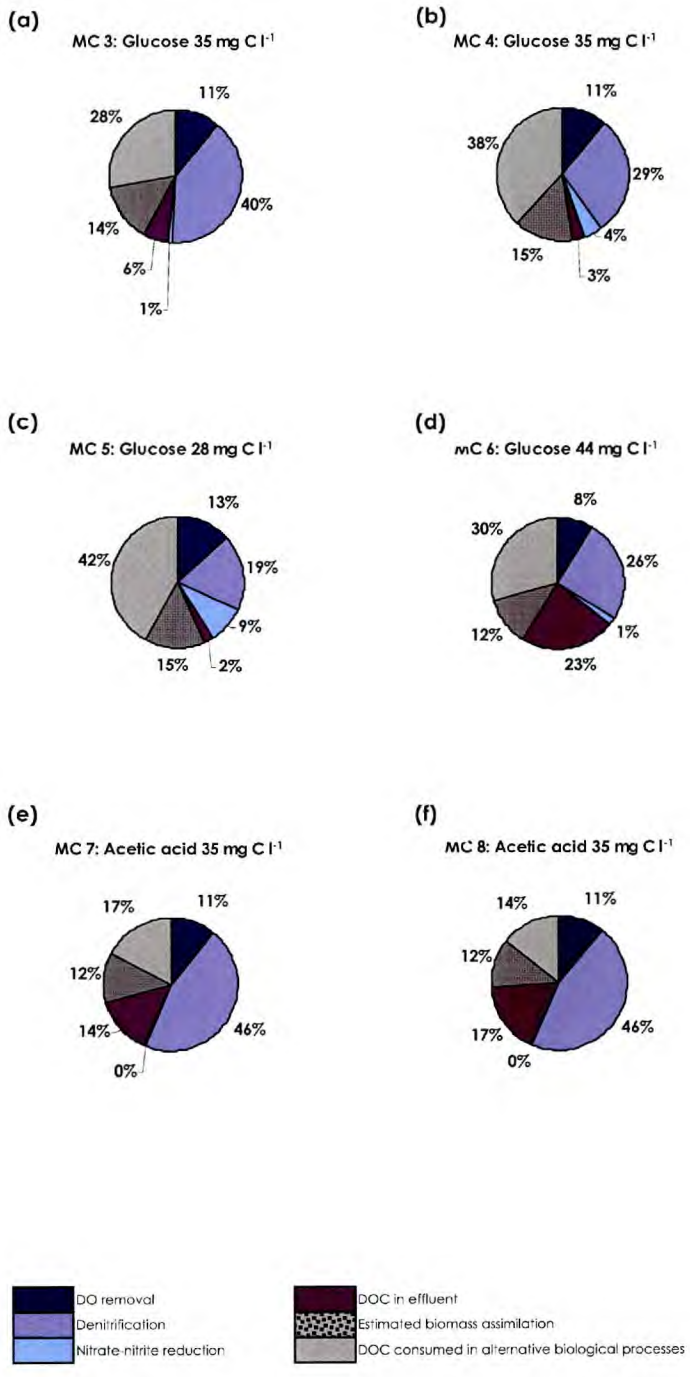


Figure 4.12 (a-f): Influence of OC input on C mass balance and utilisation during microcosm Trial IV

Effluent DOC decreased to < 4 mg C l^{-1} by d 6 of the trial and remained at this value until d 11, it became more variable (Figure 4.11 e). The arrest to microcosm pumping during d 23-24 was apparently responsible for the peak in effluent DOC on d 25. However, the reasons for the other variations in effluent DOC are uncertain but may relate to competitive inhibition of denitrifying bacteria by other microbial groups, as discussed in Section 4.6. The DOC mass balance for this treatment (Figure 4.12 d) suggested that effluent DOC comprised 23 % of the influent OC and of the 77 % used in biological activity, only 35 % was consumed for denitrification purposes.

4.5.5 Acetic Acid Treatment: 35 mg C l^{-1} (MC 7 and MC 8)

In the acetic acid treatments (35 mg C l^{-1}), denitrification activity was observed from d 4; NO_3^- concentration decreased from the influent value to approximately 5 mg N l^{-1} and NO_2^- was detected in the effluent at a concentration of 3 mg N l^{-1} in both replicate microcosms (Figure 4.10 f, g). Additionally, the TON dropped from the input concentration to approximately 8 mg N l^{-1} (Figure 4.11 e, f). By d 6, no NO_3^- was detected in the effluent and remained largely undetected in MC 7 and was completely removed below detectable limits in the effluent of MC 8. Nitrite persisted at low concentration, < 0.5 mg N l^{-1} in the effluents until d 13 in MC 7 and day 11 in MC 8. Thus, complete denitrification was observed during the second week of the trial and was maintained during the remaining experimental period. The imbalance in NO_3^- and NO_2^- reduction, which caused NO_2^- to persist for up to 5 days, diminished without additional amendment to the influent OC dose. Indeed, once complete denitrification was established, the effluent DOC increased, suggesting that less OC was required to sustain the microbial community within the microcosms. In this regard, influent OC was decreased by 5 mg C l^{-1} (to 30 mg C l^{-1}) during the final 4 days of the trial and this alteration to the OC supply regime did not affect the NO_x^- profile, which continued to attain complete denitrification.

The DOC in this treatment was consumed to approximately 4 mg C l^{-1} in MC 7 by d 6, and remained at this value until all TON had been removed, by d 13. After d 13, however, effluent DOC increased to concentrations of up to 15 mg C l^{-1}, varying between this amount and 5 mg C l^{-1} (Figure 4.10 f). Similarly, in MC 8, the DOC was consumed to ≤ 5 mg C l^{-1} during the first 11 days, and once all TON had been removed, the effluent DOC increased, peaking at 16 mg C l^{-1} on d 25. The effluent DOC concentration for days 30 and 32 had returned to the 4 mg C l^{-1} concentration previously observed in the effluents from this treatment. These results coincided with the 5 mg l^{-1} reduction in influent DOC; however, since the trend in effluent DOC was approaching this value prior to the change to influent OC, this was not necessarily indicative of a microbiological response.

The DOC mass balances showed that 83-86 % of the influent OC was consumed within the microcosms, and that 57 % was used for denitrification purposes [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] in both microcosms (Figure 4.12 e, f). Nitrate reduction to NO_2^-, as an independent reaction from denitrification, was not important in this treatment, since complete denitrification quickly established.

4.5.6 General Linear Model Results

Significant differences in the concentration of NO_3^- denitrified between the treatments groups were detected by the GLM (P = 0.01). As anticipated, Tukey pair-wise comparisons subsequently showed that all treatments supplied with OC had significantly higher denitrification than the autotrophic controls (refer to Tables 4.6 and 4.7). Furthermore, highly significant differences in denitrification were detected between the acetic acid treatment (35 mg C l^{-1}) and all glucose treatments (35, 28 and 44 mg C l^{-1}).

Of the three different glucose treatment regimes, the two higher does, (35 and 44 mg C l^{-1}) showed highly significant differences in denitrification when compared to the low glucose treatment (28 mg C l^{-1}). However, these two higher glucose treatments (35 and 44 mg C l^{-1}) did not show a significant difference in the concentration of N denitrified, compared to each other, (refer to Table 4.7 for Tukey P values).

The difference in mean effluent NO_2^- concentrations between treatment groups amended with OC was not found to be significant, though the mean values, listed in Table 4.6 indicates that there is some degree of variation.

Table 4.6: Mean concentration values of N-denitrified and effluent NO_2^- for treatment regimes in Trial IV

Treatment	Mean concentration of N denitrified during Trial (mg l^{-1})	Mean concentration of effluent NO_2^- during Trial (mg l^{-1})
Autotrophic controls (MC 1 and 2)	0.43	0.00
Glucose 35 mg C l^{-1} (MC 3 and 4)	11.19	2.36
Glucose 28 mg C l^{-1} (MC 5)	5.21	6.04
Glucose 44 mg C l^{-1} (MC 6)	11.19	1.5
Acetic acid 35 mg C l^{-1} (MC 7 and 8)	14.85	0.10

Table 4.7: Probability values indicating Statistical Significance of differences in concentration of N denitrified between treatments during Trial III. Probabilities determined by Tukey pair-wise Comparisons

	Glucose 35 mg C l^{-1} (MC 3 and 4)	Glucose 28 mg C l^{-1} (MC 5)	Glucose 44 mg C l^{-1} (MC 6)	Acetic acid 35 mg C l^{-1} (MC 7 and 8)
Autotrophic controls (MC 1 and 2)	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
Glucose 35 mg C l^{-1} (MC 3 and 4)	-	**< 0.001**	0.99	**< 0.001**
Glucose 28 mg C l^{-1} (MC 5)	-	-	**< 0.001**	**< 0.001**
Glucose 44 mg C l^{-1} (MC 6)	-	-	-	**< 0.001**

4.5.7 Summary

- No denitrification activity was observed in the autotrophic controls.
- Denitrification activity was observed in all active treatments, although in the low-glucose microcosms (28 mg C l^{-1}), $NO_3^- \rightarrow NO_2^-$ reduction prevailed.
- Complete denitrification was only sustained in the acetic acid treatment; conversely, NO_2^- persisted in all glucose-treatments.
- Mean denitrification efficiencies for day 8-32 were: 76.3 % in the mid-range glucose treatment; 31.4 % in the low-glucose treatment; 74 % in the high glucose treatment and 99.1 % in the acetic acid treatment.
- The concentration of NO_3^- denitrified in the low-glucose treatment was significantly lower than all treatments, there was no significant difference between mid- and high- glucose treatments and the acetic acid treatment denitrified significantly more NO_3^- than all other treatments.
- Conversely, there was no significant difference in concentration of effluent NO_2^- between microcosms supplied with OC.
- In the glucose treatments where influent OC was ≤ 35 mg C l^{-1}, effluent DOC was typically around 5 mg C l^{-1}. In the high glucose treatment and the acetic acid treatment, effluent DOC exhibited increases of 15 and 10 mg C l^{-1} above this value, respectively.
- The operational recess did not appear to affect NO_x^- dynamics in any treatment, but did cause effluent DOC to increase.
- Influent DOC used in denitrification activity [DO removal + denitrification + $NO_3^- \rightarrow NO_2^-$ reduction] was: 44-57 % in the mid-range glucose treatment; 41 % in the low-glucose treatment; 35 % in the high glucose treatment; 57 % in the acetic acid treatment.

4.6 DISCUSSION

4.6.1 Abiotic, Autotrophic and Endogenous Denitrification

As the results presented in this chapter demonstrate, NO_3^- reduction occurred in all biologically active treatments, whereas in control treatments, no significant NO_3^- reduction was observed. The abiotic (chemically sterilised) controls employed in the experiments showed that the denitrification detected was microbiologically mediated and since denitrification was not detected in the abiotic controls, it can be concluded that chemical NO_3^- reduction was not significant under the conditions and time scales of the experiments. In the control microcosms which were operated autotrophically, no denitrification activity was observed. This suggested that denitrification in the microcosms supplied with OC was supported by heterotrophic organisms and that autotrophic denitrification, for example, supported by an inorganic electron donor, was not significant in these experiements.

Furthermore, these results implied that endogenous denitrification was undetectable, in agreement with previous work. For example; Janda (1988) advocated the use of an exogenous substrate during denitrification of drinking water because the rate of endogenous respiration approximated to only 10 % of exogenous respiration, and Her and Huang (1995) measured endogenous denitrification efficiency as being 9.2-17.8 %.

4.6.2 Residual Organic Carbon in Microcosm Effluents

Many studies of biological denitrification identify a residual OC content in the denitrified water (Her and Huang, 1995; Mosheni-Bandpi *et al.*, 1999; Gomez *et al.*, 2000). It is often assumed that this carbon is simply a portion of the influent/media OC substrate that is unused, for example, owing to an excessive initial C/N ratio, and the precise nature of the carbon is not determined. As such, this 'carbon breakthrough' is typically dismissed as a residual which must be removed by post-denitrification treatments. In the denitrification trials presented here, an organic carbon residual was detected in all trials, at concentrations of ± 2-4 mg C l^{-1}. This was defined as the lowest concentration of effluent DOC which could consistently be detected for an individual microcosm, regardless of NO_x^- activity.

The carbon residual can be tentatively distinguished from influent carbon 'breakthrough,' where effluent DOC exceeds this residual concentration, and may be directly linked to influent OC passing through the column without being oxidised and hence being detected in the effluent. For instance, during Trial III (Section 4.4.4), the effluent of Triassic sandstone microcosms fed with sodium acetate at 35 mg C l^{-1} (MC 7 and 8, Figure 4.7) had a DOC concentration of ± 4 mg C l^{-1} between d 12 and

37. However, when the OC in the influent was increased at the end of d 37, a proportional increase in effluent DOC was observed, demonstrating breakthrough of the influent carbon, as described by Her and Huang (1995), for example, under excessive C/N ratio. It was assumed, however, that where effluent DOC was in the range ± 2-4 mg C l^{-1}, that this was not substrate breakthrough and was hence described as a residual OC concentration, which may have been caused by either physico-chemical or biological processes within the microcosm.

In heterotrophic denitrification processes, for example at WWTPs, OC residuals are typically produced as a result of microbiological activity, and categorised as soluble microbial products (SMPs). The definition 'SMP' may be used broadly to describe any soluble materials present in the effluent of a biological system that were not present in the influent; collectively referring to organic compounds produced from the microbial catabolism of substrates (usually accompanying biomass growth) and decay of biomass (Barker and Stuckey, 1999). A variety of compounds are thus encompassed in the SMP category, owing to ambiguities in absolute definitions and the difficulties in analytically characterising SMPs. However SMPs that have been identified include: polysaccharides, proteins, nucleic acids, amino acids organic acids, humic and fulvic acids, structural components of cells, exocellular enzymes and products of energy metabolism (Barker and Stuckey, 1999).

Similarly, Hamon and Fustec (1991) observed residual OC concentrations in the range of 1-4 mg C l^{-1} in heterotrophic denitrification treatments, and attributed this to the products of microbial autolysis. Therefore, it is reasonable to assume that in the microcosm supplied with OC, the residual was attributable to SMPs and the products of microbial biomass decay.

However, during the microcosm experiements, the effluents of the autotrophic control microcosms consistently contained an OC residual of similar concentration to that observed in the effluents of the biologically active microcosms. This is contradictory to the hypothesis that heterotrophic microbial activity was ultimately responsible for the effluent OC residual. Therefore, an alternative explanation for the OC residual might be the presence of physico-chemical phenomena, such as leaching. For example, the Triassic sandstone which was used in most of the microcosm Trials, reportedly has a solid-bound OC content of 0.147 % (Thornton *et al.*, 2000), which approximates to a concentration value of 1.47 mg g^{-1} sand. It may be possible that the treatment of the sandstone material during the microcosm trials, for example, crushing and sieving and subsequently, the exposure to an artificial microcosm feed solution of low anticipated ionic/dissolved solids content during

microcosm operation, caused the solid bound OC content to be slowly released into solution.

Without further analysis, however, the nature of the OC residual cannot be fully elucidated but its presence could have further implications for post-denitrification treatments (considered further in Chapter 8). The occurrence of a residual OC concentration in the autotrophic control microcosms, regardless of its nature, suggests that biological denitrification is unlikely to have a pronounced detrimental or additional impact on the OC content of the denitrified water compared to raw groundwater resources.

4.6.3 Formation of Ammonium via DNRA pathway

When NO_3^- reduction proceeds via the DNRA pathway, NH_4^+ is produced rather than N_2 gas. This may occur in carbon rich environments where the NO_3-N is the limiting factor; the formation of NH_4^+ allows microbes to utilize more electrons per N atom, as reviewed in Chapter 1. The formation of NH_4^+ poses a risk to ISBD plants, where the injection of OC may produce sites of relatively high C/N concentration, if optimal dispersion of the injected substrate is not achieved, as observed by Hamon and Fustec (1991), for example.

In these experiments, microcosm samples (effluent and feed solutions) were analysed for NH_4^+ as part of the N analysis suite and in all samples examined, NH_4^+ was below the lower detection limit of the analytical instrument limit (0.25 NH_4^+ as N mg l^{-1}).

4.6.4 Formation and Persistence of NO_2^-

Nitrite, the first intermediate product of denitrification activity, is commonly observed in the effluent of heterotrophic denitrification systems during the first few days of OC supply (e.g. Fourmentraux-Chevron *et al.*, 1999; Nutall *et al.*, 1999; Smith *et al.*, 2001). Nitrite was detected in these experiments during the first 4 days, indicating that NO_3^- reduction had initiated. The concentration of NO_2^- in the system typically increases during the next few days, corresponding to the lag period in which the microbial enzyme pathways necessary to reduce NO_2^- are produced (refer to Chapter 1). During this time, continued NO_3^- reduction is responsible for the increase in effluent NO_2^- concentrations, but once the NO_2^- reduction pathways become active, both NO_3^- and NO_2^- concentrations can decrease, indicating a net loss in TON from the system (i.e. denitrification), as depicted in Figure 4.13.

Efficient denitrification systems should exhibit similar NO_x^- concentration profiles to those depicted in Figure 4.13, where NO_2^- accumulation is transient; although the

concentration of NO_2^- produced in the effluent may be temporarily in excess of regulatory requirements (e.g. EU MAC = 0.5 mg $NO_2^- l^{-1}$).

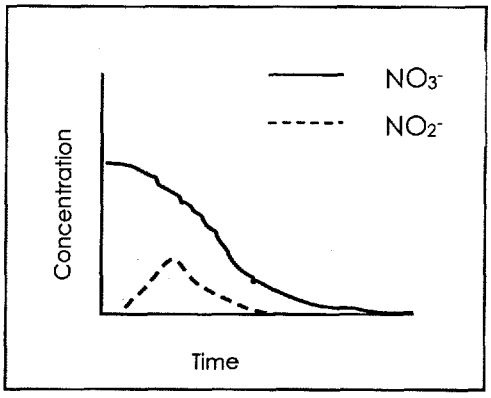


Figure 4.13: Schematic representation of the Concentration Profiles of NO_3^- and NO_2^- in flow-through denitrification systems

Several examples of ISBD or microcosm denitrification describe a transient presence of NO_2^- in the denitrified water (Hamon and Fustec, 1991; Bates and Spalding 1998; Abdelouas *et al.*, 1999; Nutall *et al.*, 1999), but the scarcity of NO_2^- data reported in published literature has been noted (Bates and Spalding, 1998). Conversely, results from the microcosm experiments presented here reveal a more persistent presence of NO_2^- in the effluents of active microcosms, likely to impinge on the quality of denitrified water.

In microcosms that were fed with an OC dose below the stoichiometrically calculated optimum dose (including biomass assimilation factor), for example, in Trials I and II; microcosm effluents contained NO_3^- and NO_2^-, but no overall TON was lost, as depicted in Figure 4.14 (a) and (b). This suggested that a small portion of the NO_3^- was reduced to NO_2^-, but no further reduction occurred and NO_3^- remained the dominant N species in the effluent. In some cases, the NO_3^- reduction activity also fluctuated between periods of $NO_3^- \rightarrow NO_2^-$ reduction and no reduction activity at all, hence a series of maxima and minima in the NO_3^- concentrations were observed. These concentration fluxes were oppositely reflected in the NO_2^- profile, as depicted in Figure 4.14 (b). Under these OC-limited conditions, denitrification activity was electron limited and the majority of the electron resource was consumed by DO leaving only sufficient for NO_3^- reduction only (refer to Chapter 1).

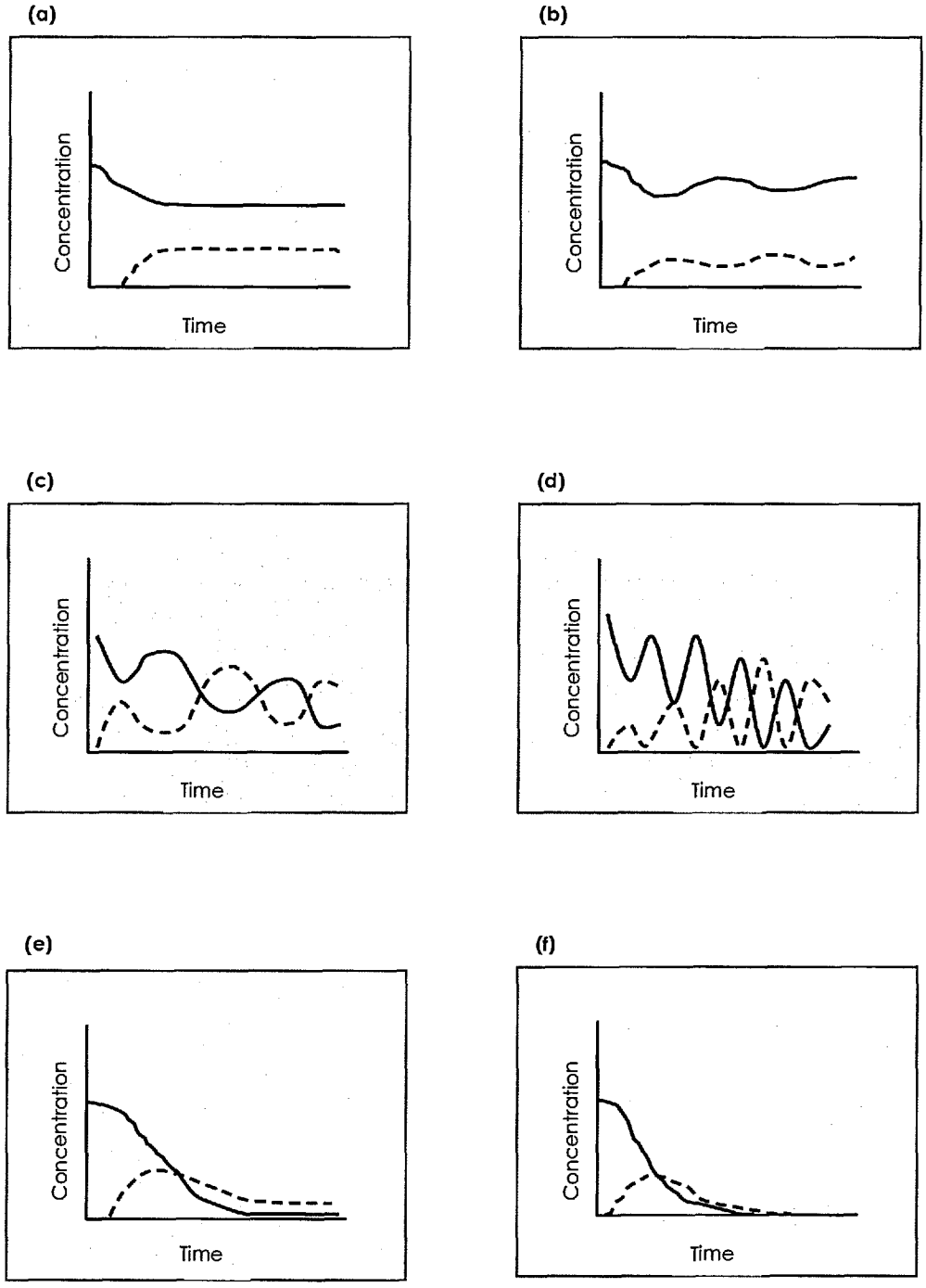


Figure 4.14 (a - f): Schematic representation of the various NO_3^- (——) and NO_2^- (- - -) concentration profiles observed during microcosm experiments

Microcosm treatments receiving OC doses calculated to be optimal with regards to the stoichiometric denitrification and biomass growth requirements, for example the acetate (19 mg C l^{-1}) and glucose (22 mg C l^{-1}) treatments in Trial II, exhibited NO_x^- profiles similar to those depicted in Figure 4.14 (c) and (d) respectively. In these microcosms, NO_3^- was reduced initially to NO_2^- only, but once NO_2^- reduction activity

had initiated, the effluent NO_3^- concentration decreased over time, with a corresponding increase in NO_2^-. Nitrite became the dominant N-species in the effluents and complete denitrification was observed on sporadic occasions suggesting that, although full denitrification was occurring, there was an imbalance between NO_3^- and NO_2^- reduction activity, with NO_3^- reduction consuming the majority of available OC.

These NO_3^- and NO_2^- dynamics were complicated further by an alternating cycle of NO_3^- and NO_2^- dominance that became progressively more exaggerated over time. This cyclic pattern was particularly erratic in the glucose treatment (Figure 4.14 d), compared to the acetate treatment (Figure 4.14 c). From these simplified NO_3^- and NO_2^- profiles it can be seen that NO_2^- (produced from NO_3^- reduction) exhibited a sequence of maxima and minima, which increased in magnitude over the duration of the experiment. A similarly fluctuating NO_3^- response was observed by Dahab (1993), although NO_2^- concentrations were not measured in this instance.

The progression of NO_3^- reduction in this succession of arrested cycles is defined by periods of increasing NO_3^- reduction activity, punctuated by inactive phases, during which time NO_2^- is washed out of the microcosm system and the NO_3^- fed to the microcosms simply passed through and into the effluent. The reason for the series of apparently inactive periods remains unclear.

It may be possible that NO_3^- reduction was inhibited by the presence of rising NO_2^- concentrations owing to the toxicity of the ion. In this case, increases in NO_2^- would inhibit NO_3^- reduction but this effect would be reversed when the concentration of NO_2^- in the system decreases, under the flow through conditions in the microcosm. However, this remains speculative, particularly given the relatively low concentrations of NO_2^- compared to published data which document inhibitory effects at concentrations in the range: 30-250 mg NO_2-N l^{-1} at pH 6-7 (Glass *et al.*, 1997). Furthermore, the shape of the NO_x^- profiles would have perhaps shown more abrupt changes if NO_2^- toxicity had been the mechanism of denitrification inhibition.

An alternative explanation for the unstable denitrification observed in this experiment is that the OC dosage applied, although calculated to be stoichiometrically balanced for complete denitrification, was insufficient in practice, due to the presence of other sinks for the OC, not considered in the stoichiometric calculation of OC demand. For example, in the case of these experiments, the electron shortage was potentially exacerbated by DO intrusion into the system possibly through the tubing delivering feed solution to the microcosm. Similarly, the erratic effluent NO_3^-

reduction observed by Dahab (1993) was attributed to inconsistent microbial growth and insufficient OC, aggravated by DO contamination of the experimental system. These factors probably account for the dominance of the $NO_3^- \rightarrow NO_2^-$ reaction.

If the fluctuations observed in the NO_3^- reduction activity were indeed caused by an artefact of the experimental system, it demonstrates the sensitivity of the microbial community to fluctuating environmental conditions and the importance of changes in OC dose, even of the magnitude of only a few mg l^{-1}. For example, the glucose microcosms exhibiting the effluent NO_x^- fluctuations described by Figure 4.14 (d) had an influent OC dose of 22 mg C l^{-1}; contrastingly, in Trial IV, a microcosm fed glucose at an influent dose of 28 mg C l^{-1} gave an NO_x^- profile similar to that depicted in Figure 4.14 (e), in spite of the changes to microcosm tubing.

The microbial community did appear to gradually increase its NO_3^- reduction capacity, however, during successive periods of NO_3^- reduction. This was probably due to an increase in biomass volume and possibly because of adaptation to the environmental and OC supply conditions. The results suggest that cyclic patterns in denitrification activity between NO_3^- and NO_2^- concentration, where NO_3^- reduction to NO_2^- initially became established as the dominant reaction, only stabilised to produce complete denitrification and TON loss with increased OC dose. Nitrite, however, continued to persist in the effluent at ± 4 mg C l^{-1}, suggesting that more OC was required, although the acetate treatment was more effective at stimulating complete denitrification and NO_2^- removal compared to glucose.

Indeed, in Trial III, where microcosms were fed an OC dose of 35 mg C l^{-1}, complete denitrification was achieved by day 23, but NO_2^- was occasionally detected in the effluent. However, no NO_2^- was detectable during the final 15 days of the trial when the OC dose was increased to 50 mg C l^{-1}.

Conversely, during Trial IV, a range of glucose doses between 28-44 mg C l^{-1} did not produce marked differences in the NO_x^- concentration profiles, which resembled those depicted in Figure 4.14 e. Statistically, there were no significant differences in NO_2^- production between the three glucose doses of 28, 35 and 44 mg C l^{-1}, and no significant differences in the amount of N denitrified in the top two dose were apparent, suggesting that the maximum denitrification efficiency under glucose dose had been reached, but NO_2^- was able to persist in microcosms treated with this OC source. This was in contrast to the acetic acid microcosms also studied, where complete denitrification was achieved in < 14 days and was sustained throughout the trial even when the OC dose was reduced from 35 mg C l^{-1} to 30 mg C l^{-1}. The NO_x^-

profiles in this treatment are depicted in Figure 4.14 (f), and closely resemble an idealized denitrification system. The 14 day 'start up' period required to achieve complete denitrification is consistent with reported start up times of 15-21 days before consistent denitrification is achieved, (Hamon and Fustec, 1991); Fourmentraux-Chevron *et al.*, 1998; Devlin *et al.*, 2002).

Therefore, it appears that in the majority of the microcosm experiements presented here, OC limitation is the principal cause of NO_2^- accumulation and persistence in the effluents. This could be explained by competitive inhibition and to a lesser extent, could be due to the selection of those organisms only capable of NO_3^- reduction (e.g. Glass *et al.*, 1998).

4.6.5 C/N Ratio

Throughout the microcosm trials presented here, a range of C/N ratios were used, as detailed in Table 4.8. The differing ratio of C/N administered to the microcosms in the influent solution corresponds to a range of 15 mg C l^{-1} in the influents for all the glucose and acetate treatments in Trial II and III and 16 mg C l^{-1} for the glucose treatment in Trial IV ('low' concentration treatments not included). The C/N ratios, ranging from 1.46 – 3.33 in the microcosm experiments were within the range of C/N ratios reported in literature, summarised in Table 4.9. The C/N doses in the microcosm experiments were generally in excess of the stoichiometric quantity, but are relatively conservative compared to the C/N values reported in the literature. Many of the microcosm experiments experienced C limitation, which resulted in incomplete denitrification, where NO_3^- reduction to NO_2^- became the dominant reaction. Where this occurred, an increase in influent OC concentration stimulated denitrification activity and generally resulted in the complete biological reduction of NO_3^- and NO_2^-.

During these experiments denitrification efficiency increased with increasing C/N ratio in the influent dose until maximum denitrification was achieved (depicted in Figure 4.15), in agreement with the observations of Her and Huang (1995). In the case of the acetate treatments, this relationship was approximately linear, ($R = 0.99$), but the relationship between denitrification efficiency and glucose C/N input was described by a second order polynomial relationship ($R = 0.88$).

Figure 4.15 illustrates that the highest denitrification observed in the microcosms supplied with glucose was 84 %, at an influent C/N of 2.33; the next incremental dose (C/N = 2.46) resulted in a decrease in denitrification efficiency to 70 ±1 %. Therefore, from these experiments, it appeared difficult to achieve and moreover, to sustain denitrification with glucose, compared, for example, to the other substrates used in these experiements: acetate and acetic acid.

Table 4.8: Summary of Influent C/N ratios used in microcosm treatments, denitrification efficiency and influent OC consumption, (calculated from empirical N and C consumption data, Trial I not included due to insufficient data; 'Low' OC treatments from Trial II and II not included). Treatments in which complete denitrification was achieved are shaded

Trial / MC ID	Carbon substrate	Influent conc. (mg C l^{-1})	Time (day no.)	Influent C/N ratio	Denitrification efficiency (%)	Influent OC consumed (%)
II / MC 3 & 4	Glucose	22.0	8-60	1.46	21.3	79.9
		27.0	64-70	1.80	18.0	62.3
		32.0	72-78	2.13	58.0	83.2
		37.0	79-84	2.46	71.0	87.0
II / MC 5 & 6	Acetate	19.0	8-60	1.26	19.7	82.4
		24.0	64-70	1.60	44.2	89.4
		29.0	72-78	1.93	65. 0	84.6
		34.0	79-84	2.26	91.2	82.0
III / MC 3 & 4	Acetate	35.0	23-37	2.33	88.3	82.7
		50.0	38-55	3.33	97.5	63.1
III / MC 5 & 6	Acetate	35.0	23-37	2.33	75.3	87.2
		50.0	38-55	3.33	100.0	66.2
III / MC 7 & 8	Acetate	35.0	23-37	2.33	95.6	88.6
		50.0	38-55	3.33	100.0	67.7
IV / MC 3 & 4	Glucose	35.0	19-32	2.33	72.3	88.3
IV / MC 5	Glucose	28.0	19-32	1.86	27.4	90.5
IV / MC 6	Glucose	44.0	19-32	2.93	63.9	63.5
IV / MC 7 & 8	Acetic Acid	35.0	19-28	2.33	100.0	70.6
IV / MC 7 & 8	Acetic Acid	30.0	29-32	2.00	100.0	88.4

Table 4.9: Details of selected C/N ratios used in denitrification experiments relevant to ISBD or reactors for groundwater denitrification treatment

Reference	OC substrate	C/N ratio in influent dose for maximum denitrification efficiency (e.g. > 90 %)
Mercado *et al.* (1988)	Sucrose	3.57
Hamon and Fustec (1991)	Ethanol	1.25
Dahab (1993)	Ethanol	1.0-1.5
Her and Huang (1995)	Methanol	0.6-10.0
	Acetic acid	> 1.9
	Glucose	> 2.0
	Benzoic acid	3.0-3.6
Mosheni-Bandpi *et al.* (1999)	Acetic acid	4.3
Also citing: Croll *et al.* (1985)	Acetic acid	4.7
Dahab and Lee (1988)	Acetic acid	4.05
Bockle *et al.* (1986)	Acetic acid	3.9
Nutall *et al.* (1999)	Acetate	1.42-2.0
Devlin *et al.* (2000)	Acetate	1.6
Gomez *et al.* (2000)	Methanol	1.1
	Ethanol	1.08
	Sucrose	2.5

A hypothetical C/N requirement was extrapolated from the equation of the polynomial curve fitted to the data ($y = 58.4x^2 - 163x + 131.3$), and the optimum C/N for 100 % denitrification was estimated as 2.58. This value is consistent with the results of Her and Huang (1995) which showed that C/N ratios in excess of 2.0 were necessary for glucose denitrifying sludge bioreactors; and similarly with the findings of Gomez *et al.*, (2000) which showed a sucrose C/N requirement of 2.5 in groundwater treatment filters. Conversely, the apparent decrease in denitrification efficiency once the C/N ratio of 2.33 was exceeded conflicts with the findings of Her and Huang (1995). They did not report loss of denitrification efficiency with increased glucose C/N ratio, although it was observed with alternative OC substrates: methanol and benzoic acid. In this case, it was assumed that denitrifying sludge reactors had been poisoned, as complete inhibition of denitrification resulted (with increasing C/N ratio) and this coincided with carbon breakthrough.

Bates and Spalding (1998) also observed a decrease in the rate of denitrification when influent ethanol dose was doubled from 50 → 100 mg l^{-1}, hypothesising that the decrease in activity was either due to a toxic effect exerted by the ethanol, or increased competition for the electron resource facilitated by a drop in redox potential under the higher ethanol dose. Alternatively, Martienssen and Schops proposed that the unstable denitrification conditions observed under high C/N ratio

(>2.5) in methanol reactors was due to a shift in the microbial community in favour of NO_2^- sensitive strains, which apparently were only capable of NO_3^- reduction. The influence of microbial community structure is considered further in Chapter 7.

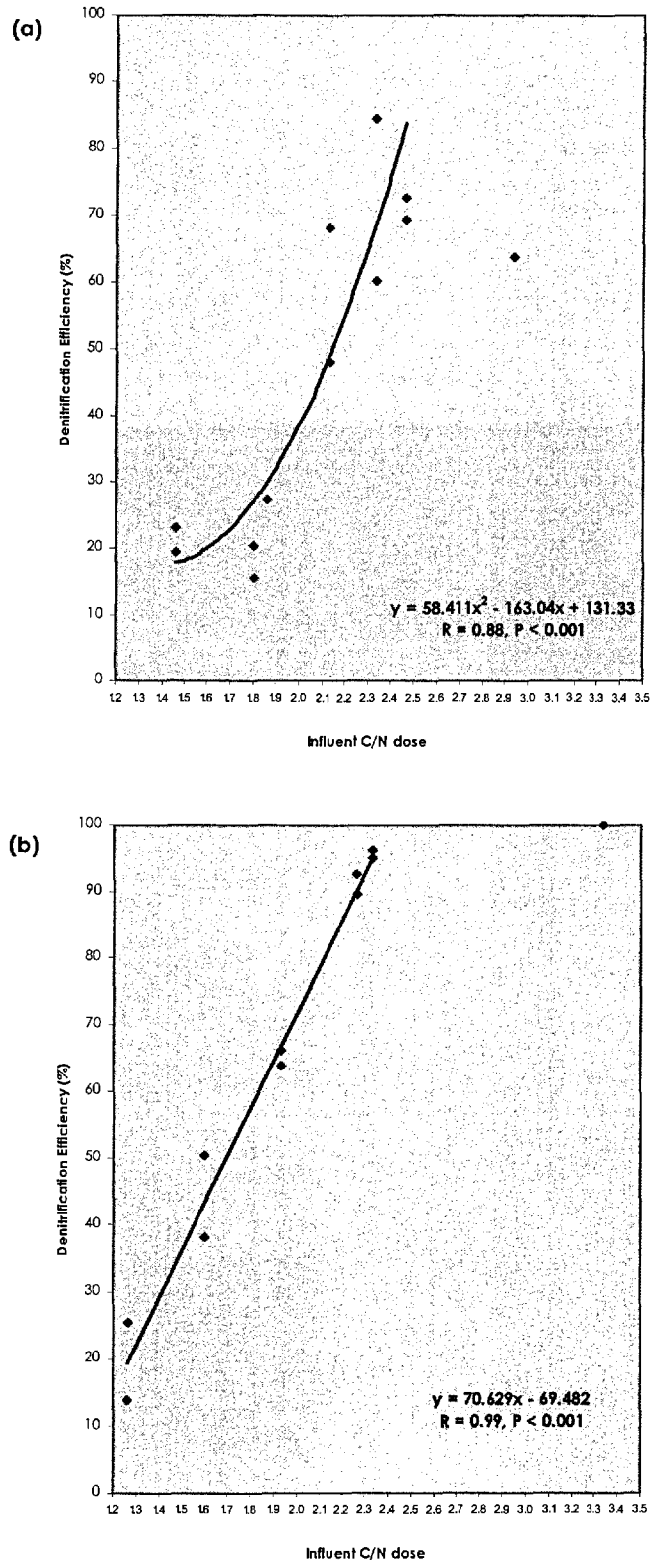


Figure 4.15: Denitrification efficiency (%) compared to influent C/N dose for **(a)** glucose and **(b)** acetate, (including regression details)

The reason for the decrease in denitrification activity of glucose microcosms with high C/N (> 2.33) remains unclear. At an influent C/N of 2.46, OC consumption (%) remained high in accordance with the OC consumption at lower C/N ratios, implying that some changes in the structure of the microbial community may have been responsible for the comparative decrease in denitrification efficiency. However, under the highest C/N ratio of 2.93, OC consumption was also less (where carbon breakthrough occurred), which might alternatively indicate that the denitrifying fraction of the biomass was inhibited.

In contrast, microcosms fed with acetate achieved complete denitrification activity under excessive influent C/N ratios (where carbon breakthrough was observed). For example, from the data presented in Figure 4.15 b, it appears that 100 % denitrification efficiency could have been realised at a lower influent ratio than the plotted C/N ratio of 3.3. Using the equation from the fitted linear regression line (y = 70.6x - 69.5), an optimum C/N ratio for complete denitrification was calculated as 2.4. This value is greater than the C/N ratios described by Nutall *et al.* (1999) and Devlin *et al.* (2000): 1.4-2.0 and 1.6 respectively, however, the DO concentration in the experiments of Nutall *et al.* (1999) is not described and Devlin *et al.* (2000) conducted their batch experiments anaerobically, and thus the C/N ratio is lower as it does not include a component for DO consumption.

The acetic acid treatment in Trial IV was initiated with an influent C/N dose of 2.33 and complete denitrification was observed within 13 days, after which time carbon breakthough in the microcosm effluent was observed. In response to this, the OC dose in the influent was reduced, giving a C/N ratio of 2.0 with no detrimental effect on denitrification efficiency. This second acetic acid dosage regime reflected the results of Her and Huang (1995) which achieved 97-99 % denitrification using an acetic acid treatment with a C/N ratio of 1.9. Conversely, these values were low compared with the reports of Mosheni-Bandpi *et al.* (1999) and results cited therein (Table 4.9) which advocated C/N ratios > 3.9 when using acetic acid.

There was insufficient experimental data to examine the effects of C/N ratio on denitrification over a range of acetic acid concentrations, therefore, it remains unclear whether the lower OC dose would have been sufficient to induce complete denitrification from the outset, or whether the slightly higher (5 mg C l^{-1}) aided the establishment of a fully denitrifying microbial community, which could then adapt to a reduced OC dose without loss to denitrification function. The manner in which the higher initial influent OC is completely consumed (to the residual level) and

subsequently exhibits breakthough once complete denitrification has been attained, would suggest that the latter hypothesis was more likely.

4.6.6 Organic Carbon Losses

In all the microcosm experiments, it was apparent that a portion of the influent OC was unaccounted for, according to the OC mass balances and summarised in Table 4.10. From the data summarised in Table 4.10, it is difficult to determine a relationship between the influent C/N ratio and the proportion of unidentified OC consumption. However, it appears that in glucose and acetate treatments of similar C/N ratio, the proportion of unidentified OC consumption is greater in the glucose treatments. Furthermore, the acetic acid treatment, which was the most efficient denitrification experiment in these trials, exhibited a markedly lower proportion of OC lost to unidentified activities.

Table 4.10: Summary of OC consumed in alternative biological microcosm processes other than denitrification–related activities (as a proportion of total OC consumed)

Trial / MC ID	Carbon substrate	Time (day no.)	Influent C/N	OC consumed in alternative biological processes (%)
I / MC 3 & 4	Glucose	4-21	1.46	33.0
I / MC 5 & 6	Glucose	4-21	0.73	29.0
I / MC 7 & 8	Glucose	4-21	2.6	41.5
II / MC 3 & 4	Glucose	8-84	1.46 → 2.46	30.0
II / MC 5 & 6	Acetate	8-84	1.26 → 2.26	21.75
II / MC 7 & 8	Acetate	8-51	0.73	27.0
III / MC 3 & 4	Acetate	9-55	2.33 → 3.33	21.5
III / MC 5 & 6	Acetate	9-55	2.33 → 3.33	25.5
III / MC 7 & 8	Acetate	9-55	2.33 → 3.33	25.5
IV / MC 3 & 4	Glucose	8-32	2.33	33.0
IV / MC 5	Glucose	8-32	1.86	42.0
IV / MC 6	Glucose	8-32	2.93	30.0
IV / MC 7 & 8	Acetic acid	8-32	2.33 → 2.0	15.5

It is assumed that the OC 'lost' from denitrification-related activities was either assimilated as biomass, in excess of the estimated quantity that was accounted for (15 % of the influent dose), or consumed in alternative microbial energy-generating processes.

Interestingly, the acetic acid microcosms clearly demonstrated higher denitrification efficiencies than the glucose and acetate treatments, with the smallest amount of influent OC used in alternative electron-consuming processes. The influent C/N ratio in this treatment was relatively conservative compared with those used in the glucose

and acetate experiments and also compared to those reported in the literature (Table 4.9) demonstrating the differing suitability of various OC substrates for ISBD.

4.6.7 Effect of OC Substrate Type

Many studies have been carried out to find the most suitable substrate for engineered biological denitrification (refer to chapter 1.5.2); substrates that have been assessed with regards to ISBD of drinking water resources include: sucrose, glucose, sodium acetate, methanol, ethanol and acetic acid (e.g. Mateju *et al.*, 1992).

In terms of denitrification activity and OC consumption, acetic acid was the most favourable substrate, showing the highest denitrification efficiency, in the shortest time period with the least amount of NO_2^- persistence. The acetic acid treatments were also able to sustain denitrification under the most conservative C/N ratio (2.0). The microcosms receiving acetate could also sustain complete denitrification, but required a higher C/N ratio and a longer start up time, whereas the glucose treatments were incapable of sustaining complete denitrification, in the context of these experiments. However, hypothetical optimum C/N ratios, for acetate and glucose were similar, calculated as 2.40 and 2.35 respectively, in contrast to speculation that the non-fermentable acetate might have been markedly more efficient than the fermentable glucose. This may be because the microcosm experiments were conducted on the threshold of C-limitation, which subdued differences between acetate and glucose availability to fermentative organisms. In regard to overall denitrification performance, however, acetate was a superior substrate to glucose, giving less erratic and more reproducible results with less NO_2^- persistence. Interestingly, acetic acid and acetate treatments showed a greater degree of difference in denitrification activity, although acetate is essentially a salt of acetic acid.

For example, the differences observed in optimum C/N ratios and denitrification activity with differing organic substrates has previously been liked to molecular weight (Her and Huang, 1995), and degradability (Mosheni-Bandpi *et al.*, 1999; Stief, 2001). However, Her and Huang (1995) noted little difference between acetic acid (C/N = >1.9) and glucose, (C/N = 2.0), which have molecular weights (MW) of 60 and 180 respectively, in spite of their observation that increased C/N was related to increased MW. The results from the microcosm experiments do not support this concept, except in the case of glucose compared to acetic acid, although acetic acid is more readily metabolised than glucose (Mosheni-Bandpi *et al.*, 1999) so this may be responsible for the observed result.

Generally, comparisons between OC substrates for ISBD purposes are not made in terms of chemical degradability, or bioavailability, but instead are compared in terms of the diversity of the microbial community they support. After C/N limitation, microbial community is the next factor to be considered when defining denitrification kinetics, particularly with regards to substrate utilisation. The denitrification results presented here are reviewed in terms of microbial community in Chapter 7.

4.6.8 Effects of Operational Interruption

During the various experiments, microcosm operation was interrupted on a daily basis to allow essential maintenance and sampling to be carried out. These interruptions did not exceed 5 hours and during this period, the microcosm inlets were clamped to prevent drainage of the interstitial fluid from the microcosms and to minimise contamination with O_2. However, to determine the effect of a longer idle period on denitrification activity, the experiments incorporated period of 24 and 48 hour recesses, during which time, the microcosm inlets were sealed and pumping was terminated.

In the course of microcosm Trial II, three recesses of 24 hours indicated that there was no discernible effect on NO_x^- activity, although the NO_3^- and NO_2^- profiles for glucose and acetate treatments in this experiment were erratic, regardless of pumping activity. However, the DOC concentration showed a greater response to the recesses, and following an idle period, the concentration of DOC in the microcosm effluent increased temporarily during the 2 days following the recess, before returning to background concentration. This response was more pronounced in the glucose treatment, where effluent DOC rose after all three recesses, compared to the response of the acetate treatment, where effluent DOC only exhibited an increase on one out of three recess occasions. The microcosms receiving acetate at a concentration less than the stoichiometric optimum did not show a response in effluent DOC, probably owing to the lower OC availability and the ability of the microbial biomass to consume all the OC supplied.

In Trial IV, there was a longer recess period of 48 hours, which occurred once the microcosms had reached a steady state with regards to denitrification activity. In this instance, no effects on NO_x^- concentrations were observed in either the glucose or acetic acid treatments. Conversely, the DOC profiles did respond to the interruption; in the glucose treatments, increased effluent DOC was observed in both 35 and 44 mg C l^{-1} treatments, with the response of the high glucose treatment being more pronounced. This response was also observed in the acetic acid treatment, although

in this treatment plus the high glucose treatment, there was already evidence of OC breakthrough in the effluent so the results were not as marked overall. Similarly to the findings from Trial II, the effluent DOC returned to background values after two days.

The apparent increase in effluent DOC following a 24-48 h break in microcosm pumping could possibly be because the rate of OC uptake by the microbial biomass temporarily decreased following the shut down period. In this case, a decreased rate of OC uptake by the microcosm biomass would have resulted in a small amount of substrate breakthrough into the effluent. It follows that one of the OC consumptive processes other than denitrification, such as assimilation, must have been affected, since denitrification activity was sustained at similar pre- and post- shut down levels. An alternative explanation for the increase in effluent DOC following the shut down could be an increased concentration of organic products from bacterial cell lyses, previously proposed by Hamon and Fustec (1991) for example, who observed similar increases in effluent DOC following an unplanned interruption to pumping.

Regardless of the DOC effects, the results from the NO_x^- concentration profiles indicated that once established, denitrifying communities retained their functionality following a period without OC and NO_3^- supply, of up to 48 hours. Indeed, Smith *et al.* (2001) also postulated that denitrifying communities remained acclimatised to the injection of extraneous substrates during ISBD field trials over a period of 15 months. These findings are pertinent with regards to operational designs favouring intermittent OC injection and are relevant to Water Utilities, indicating that episodic events such as equipment failure and shut down during maintenance procedures will not impair the effectiveness of ISBD systems.

CHAPTER 5

IMPACTS OF STIMULATED MICROBIAL ACTIVITY ON AQUIFER BIOFOULING

This chapter examines the effects of denitrification activity on the porosity and flow characteristics of the aquifer matrix.

5.1 OVERVIEW

5.2 RESULTS

5.2.1 Hydraulic Conductivity

5.2.2 Dye Experiments

5.2.3 Microcosm Sand Biofilm Content

5.2.3.1 Extracellular Polymeric Substances

5.2.3.2 ATP

5.3 DISCUSSION

5.1 OVERVIEW

In all aquifer bioremediation schemes, there is an inherent risk of biofouling; where the stimulated biomass potentially proliferates to the extent that it impedes operation and water flow. Biofouling may be prohibitive to bioremediation for potable water resource treatment unless carefully managed, (as reviewed in Chapter 1). However, where incidents of biofouling have been reported, quantitative assessment of the various potential contributing factors are typically lacking, and most authors simply concede that biofouling requires further investigation (e.g. Hamon and Fustec, 1991; Dahab, 1993; ITRC, 2002).

Laboratory microcosm experiments were used to assess biofouling under conditions representative of UK groundwater conditions. The following parameters were determined to gain a comprehensive body of data;

- Hydraulic conductivity across the sand columns;
- Formation of preferential flow-channels;
- Biomass concentration;
- Extracellular polymer (EPS) production.

5.2 RESULTS

5.2.1 Hydraulic Conductivity

Hydraulic conductivity is widely used for monitoring biofouling in porous materials (Chapter 2, Section 2.3.3). Hydraulic conductivity (K), may be calculated from 'real time' *in situ* pressure measurements (refer to Chapters 2 and 3 for details). The raw data from pressure transducers, given in Appendix 3, which were used to calculate head loss, or pressure differential, within the microcosm, is characteristic of data obtained from constant head permeameters (refer to Chapter 3). The pressure at the top of the sand column remained approximately constant throughout the experiments, reflecting the hydrostatic pressure. The pressure measured at the base of the sand column fluctuated throughout the experiments, influenced by the hydrostatic pressure plus the water pressure within the sand column. Where the pressure measured at the base of the microcosms increased, the head loss also increased, corresponding to a decrease in hydraulic conductivity.

The specific pressure measured in individual microcosms was influenced by unique factors such as sand-packing during assembly and formation of flow channels etc.

during operation, so to facilitate comparison between separate microcosms, the ratio of K/K_0, (where $K_0 = K$ on exp. day 1) was calculated rather than using actual values of K. Therefore, all microcosm treatments had an initial hydraulic conductivity ratio = 1 (Vandevivere and Baveye, 1992 a), as presented in Figure 5.1. The range of values of K/K_0 measured for the microcosms were consistent with the reports of Vandevivere and Baveye (1992 a). Specifically, values of K_0 ranged from: 0.8-1.13 m d^{-1} in microcosms packed with Triassic sandstone and 0.7-0.86 m d^{-1} in microcosms filled with Greensand. Changes in the microcosm hydraulic conductivity over time described the dynamics of the physical resistance of the microcosm sand to influent flow, caused for example, by variations in bulk pore space and frictional properties of the sand (e.g. Baveye *et al.*, 1998).

Generally, the results suggested that there was little or no change in hydraulic conductivity during the microcosm experiments. Control microcosms exhibited an approximately constant K/K_0 ratio throughout experimental periods, with small fluctuations, demonstrating the dynamic nature of the experimental system and the sensitivity of the pressure transducers. Variations in K/K_0 in the control microcosms could be attributed to physicochemical phenomena such as: gradual flushing of a fraction of the fine sand particles; precipitation of minerals in the oxidising environment of the microcosms treated with $HgCl_2$ (sterile controls) and filtration/flushing of suspended solids in the autotrophic control in microcosm Trial IV, (where natural groundwater was used). Generally, however, no net change in hydraulic conductivity was observed in the control microcosms.

Throughout microcosm Trial I, the microcosms receiving glucose at 22 mg C l^{-1} (MC 4) and 39 mg C l^{-1} (MC 8) both exhibited suppressed K/K_0 values, compared to the sterile control (MC 1). However, no overall trends were observed in this relatively short experiment. In microcosm Trial II, the glucose microcosm at 22 mg C l^{-1} (MC 3) showed a reduction in hydraulic conductivity of 0.2 orders during the middle phase of the experiment (d 20- d 37), however, this recovered by 50 % to give a hydraulic conductivity decrease of 0.1 orders from d 70 onwards. A similar pattern was observed during microcosm Trial IV, where the glucose treatment at 35 mg C l^{-1} (MC 4) exhibited a maximum decrease in K/K_0 approaching 0.3 orders, on d 8-9, but appeared to stabilise at a net K-loss of 0.1 - 0.15 towards the end of the experiment. The glucose treatment at 44 mg C l^{-1} (Trial IV, MC 6) exhibited a more consistent decrease, approaching a K-loss of 0.3 orders after d 20. However, the greatest reduction in hydraulic conductivity was observed in the acetic acid microcosm, Trial IV, though the OC concentration was only 35 mg C l^{-1} and reduced to 28 mg C l^{-1} during the final three days of the experiment. In this microcosm (MC 8), K/K_0

decreased by up to 0.35 orders, before stabilising at a K/K_0 decreased by approximately 0.25 orders. The reduction in influent acetic acid concentration (35→30 mg C l^{-1}) during the last 3 days of the experimental period had little overall effect on hydraulic conductivity.

The reduction in hydraulic conductivity observed in these experiments compared to the controls was attributed to biological activity and additionally to the filtration action of the microcosm sand on suspended particles present in the groundwater, in the case of microcosm Trial IV. This filtration phenomenon has been previously documented (Baveye *et al.*, 1998; Rinck-Pfeiffer *et al.*, 2000) and can be inferred from these experiments owing to the relatively greater degree of K-loss in microcosm Trial IV, where natural, raw groundwater was supplied to the microcosms, compared to Trials I and II, where influent solutions were prepared from purified laboratory water.

However, biological activity did not necessarily lead to overall decreases in K/K_0. For example, in Trial II, the acetate and glucose treatments had similar influent OC concentrations (19 and 22 mg C l^{-1} respectively) and exhibited comparable denitrification activities, but a relative decrease in hydraulic conductivity was only observed in the glucose treatment, not in the acetate treatment. This suggested that variations in observed K-loss were influenced by substrate type. For example, the results from the acetic acid microcosm showed a greater degree of K-loss compared to the glucose microcosm treated with the same influent OC concentration of 35 mg C l^{-1}. These observations are given further consideration in Section 5.3.

From a qualitative perspective, the K/K_0 profiles measured for glucose were more dynamic than for either acetate or acetic acid. Fluctuations in hydraulic conductivity have been previously attributed to localised clogging of pore lumen within the bulk sample, followed by spontaneous unclogging, caused by biomass detachment under increased pressure and shear stress, for example (Baveye *et al.*, 1998). Alternatively, such fluctuations may be due to the production and subsequent release of gas bubbles from the sand column (e.g. Balks *et al.*, 1997; Baveye *et al.*, 1998), particularly where the fluctuations are relatively large.

It is difficult to put the results from the microcosm experiments into a practical engineering context, given the lack of published quantitative data from ISBD clogging incidents. However, compared to data from specific studies on sand clogging, the degree of reduction in hydraulic conductivity observed here was insignificant and would not impact the operation of ISBD systems. For example, Vandevivere and Baveye (1992, a, b) and Rinck-Pfeiffer *et al.* (2000) report decreases

in K over 1-4 orders of magnitude in sand column experiments, in comparison to a maximum K decrease of 0.35 in these experiments. These findings are discussed further in Section 5.3.

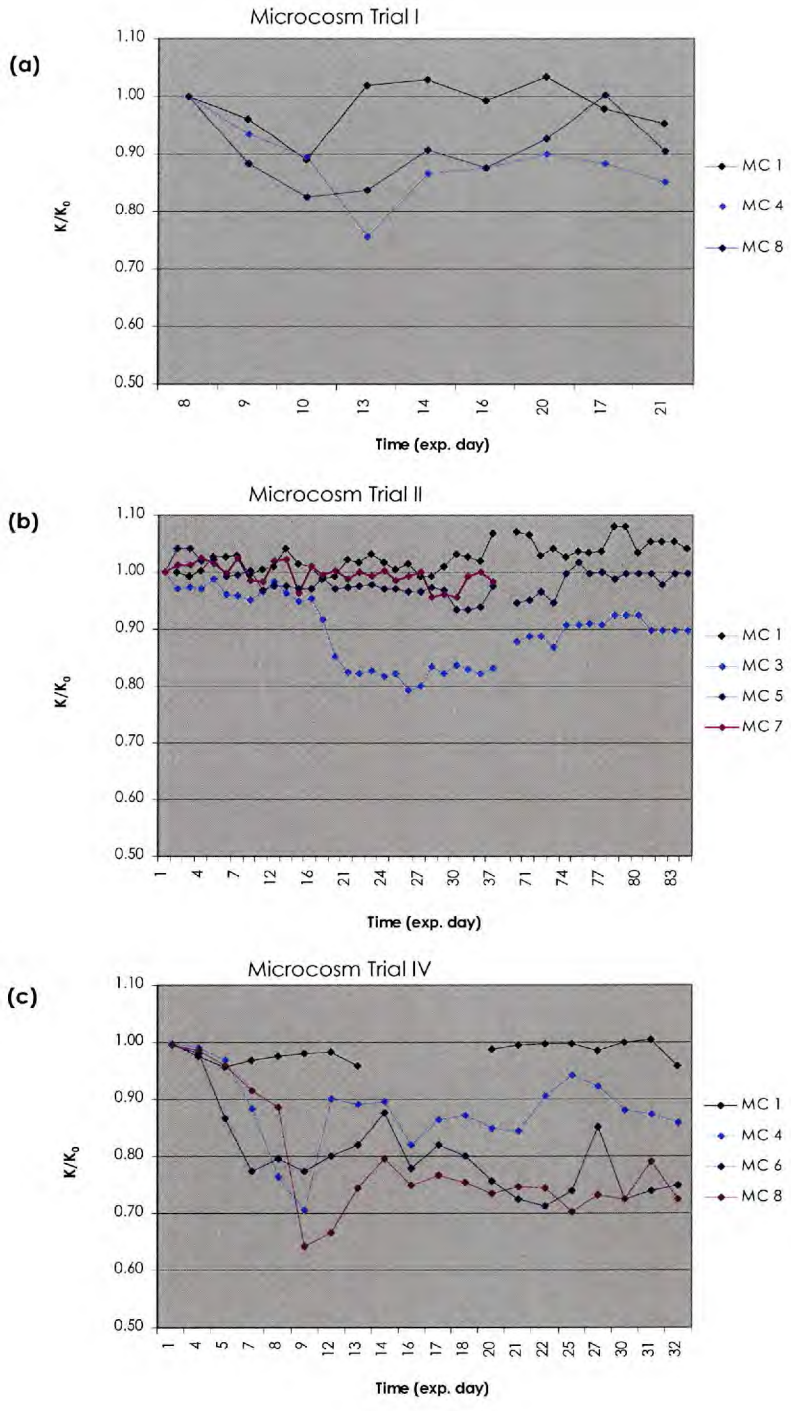


Figure 5.1: Changes in hydraulic conductivity (K/K_0); **(a)** MC 1 = control, MC 4 = 23 mg glucose-C l^{-1}, MC 8 = 39 glucose-C mg l^{-1}; **(b)** MC 1 = control, MC 3 = 22 mg glucose-C l^{-1}, MC 5 = 19 mg acetate-C l^{-1}, MC 7 = 11 mg acetate-C l^{-1} (discontinued early); **(c)** MC 1 = control (absent data represents period where control microcosms were not operated), MC 4 = 35 glucose mg-C l^{-1}, MC 6 = 44 mg glucose-C l^{-1}, MC 8 = at 35 mg acetic acid-C l^{-1}

5.2.2 Dye Experiments

Inert chemicals such as salts (e.g. bromide) or dyes (e.g. sodium fluorescein) are typically used as tracers in hydrogeological surveys, to monitor groundwater movement and dilution rates (e.g. Aldous and Fawell, 1986; ITRC, 2002). Similar compounds are used in laboratory experiments to assess the flow parameters of sand/soil columns, where the breakthrough curve of the tracer (BTC) is typically used to analyse flow patterns, and/or ensure that the arrangement of the packed material is homogenous (Sharp *et al.*, 1999; Rinck-Pfeiffer *et al.*, 2000; Huang, *et al.*, 2002). For example, during the design of the microcosm apparatus used in these experiments, a dye tracer was used in conjunction with a simplified solute transport model (Oakes and Edworthy, 1976) to demonstrate that preferential flow channels within clean (before experimentation) packed microcosms were insignificant (Cartmell, 1997).

To assess the relationship between microbial activity and microcosm flow characteristics, BTC curves for biologically active sand microcosms and control treatments were compared in this laboratory-based programme of biodenitrification. Daily visual observations of the operation of the microcosms revealed a considerable amount of disruption to the sand column in the microcosms caused by the release of gases during microbiological activity, which did not occur in control microcosms (Figure 5.2).

Small, spherical gas bubbles ascended to the column surface and were released. However, where bubbles formed irregular shapes or aggregated together to form larger bubbles, they became less mobile, forming pockets of desaturated sand, as shown in Figure 5.3. Continued aggregation of gases could lead to more significant desaturation, forming bubbles that extended in a plane across the cross sectional area of the microcosm, eventually pushing the overlying sand upwards within the column (Figure 5.3 b). Where bubbles aggregated in this fashion, they were released by gentle agitation of the microcosm, with the consequence of disruption to the sand column, but avoiding the more considerable impact of severe desaturation.

The movement of bubbles through the sand encouraged the finer particulate fractions of sand to be moved to the top of the sand column and become suspended as gas bubbles were released. This had the cumulative effect of decreasing the height of the sand column within the microcosm over time and concomitantly increased the volume of solution overlying the sand at the top of the column, since the effluent outflow tube was at a fixed height (Figure 5.3 d). Furthermore, the ascending bubbles left visible channels of up to 1.5 mm in width in

the sand column, where flow appeared to be at an accelerated velocity (although this was not quantified) (Figure 5.3 c).

The combination of these artefacts meant that quantitative comparisons of BTCs between control (clean) and biologically active microcosms were problematic, since preferential flow channels were visibly obvious, and the gas production responsible for the disturbances was not quantified. From a qualitative perspective however, the BTCs measured from biologically active microcosms (Figure 5.4) generally showed decreased time to initial dye breakthrough, compared to the control microcosms. In some cases, the concentration of the dye rose sharply following the initial breakthrough, for example as depicted in Figure 5.4c, MC 6. This was due to relatively undiluted concentrations of dye being transported through the sand at comparatively accelerated pore velocities. Both these observations have been described as characteristics of primary porosity, or preferential flow channels with advective flow (Sharp *et al.*, 1999; Kamra and Lennartz, 2004). In some of the active microcosms, conversely, the attainment of 100% dye breakthrough was slower than that observed in the clean sand control microcosms, in spite of the presence of preferential flow channels. This indicated that some of the dye was dispersed within the sand column, where its transport was comparatively slower, suggesting that dispersive/diffusive flow processes were also significant. Similar flow characteristics were described by Sharp *et al.* (1999) documenting development of both primary (advective) and secondary (diffusive) flow processes in non-mucoid biofilms grown on flat-plate reactors.

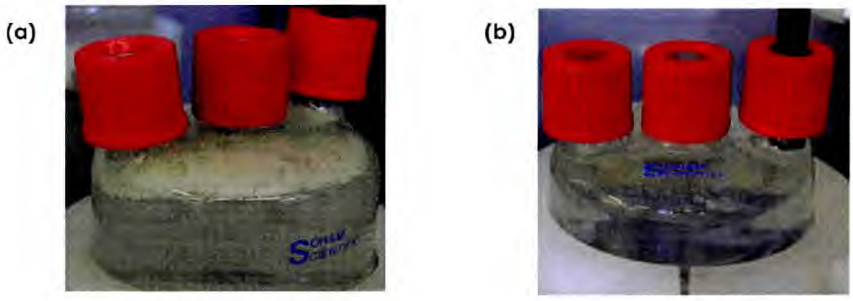


Figure 5.2: **(a)** gas bubbles accumulating at top of biologically active microcosms compared to **(b)**, control microcosm with no bubbles

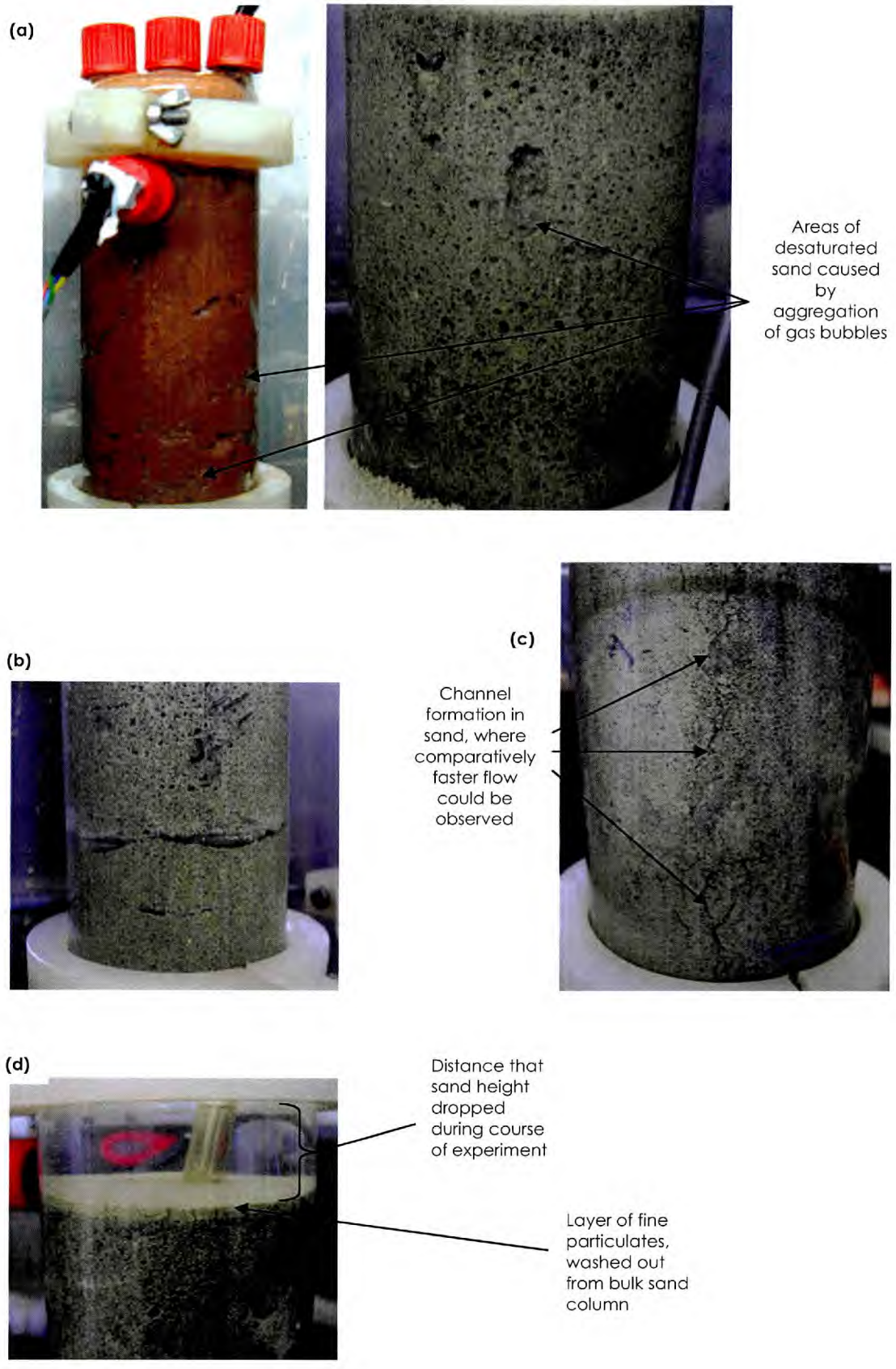


Figure 5.3: **(a)** Gas bubbles aggregated in sand column; **(b)** bubbles forming large pockets; **(c)** preferential flow channel caused by smaller bubbles ascending through sand column, along interface with glass; **(d)** fine particulate fraction of sandstone overlying the larger grain sized fraction which comprised the bulk of microcosm sand.

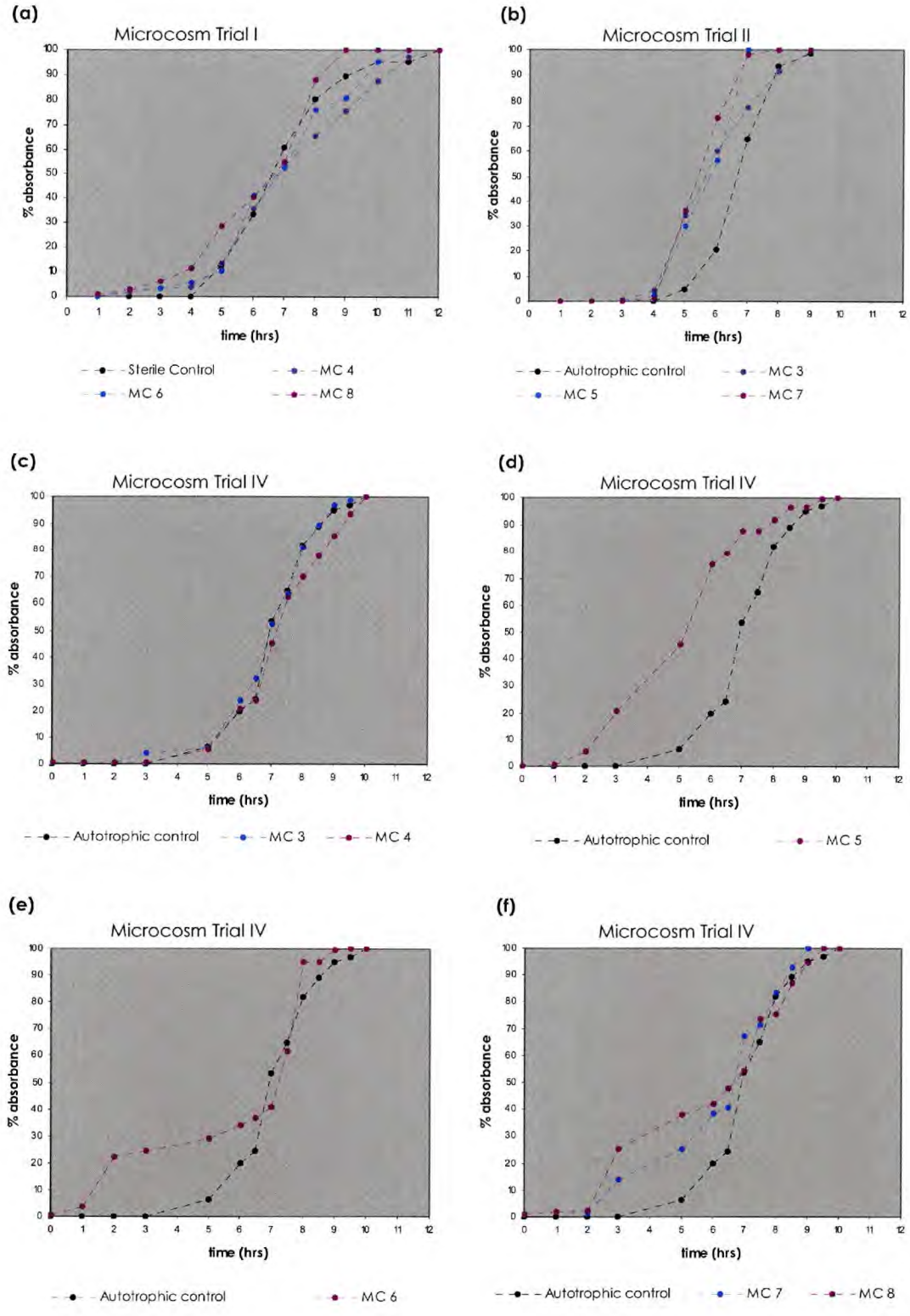


Figure 5.4: Dye breakthrough curves: **(a)** MC 4 = 22 mg glucose-C l^{-1}, MC 6 = 11glucose-C l^{-1}, MC 8 = 39 glucose-C l^{-1}; **(b)** MC 3 = 22 mg glucose-C l^{-1}, MC 5 = 19 mg acetate-C l^{-1} and MC 7 = 11 mg acetate-C l^{-1}: **(c)** MC 3 & 4 = 35 mg glucose-C l^{-1}; **(d)** MC 5 = 28 mg glucose-C l^{-1}; **(e)** MC 6 = 44 mg glucose-C l^{-1}; **(f)** MC 7 & 8 = 35 mg acetic acid-C l^{-1}

In the case of these experiments, changes in flow characteristics of the sand columns were attributed to gas production rather than biofilm development. The implications of aquifer clogging from gas evolution are discussed in Section 5.3.

5.2.3 Microcosm Sand Biomass Content

The development of a viable microbial community within the microcosms is necessary for denitrification activity, however, clogging of the aquifer material due to excessive biomass proliferation has frequently been observed in ISBD experiments, as discussed in Chapter 1. To study the relationship between decreased permeability in the aquifer material and biomass proliferation, two elements of the biovolume: cell density (using ATP measurement) and extracellular polymers were measured using sandstone from microcosm trials II and IV, as described in Chapters 2 and 3.

5.2.3.1 ATP Measurement

During the course of the laboratory measurements, attempts were repeatedly made to quantify the ATP content of the microcosm sand, as described in Chapters 2 and 3. A variety of adaptations of the protocol described by Cartmell (1997) were tested, including:

- Different light-capture instrumentation;
- Different concentration combinations of the Lumac reagents;
- Exclusion of Lumac NRB reagent, which caused turbidity in the samples.

During these experiments, however, no cellular ATP could be detected in the sand extracts, in spite of attempts to improve the sensitivity and precision of the methods. The biochemical activity within the microcosms clearly inferred that a microbial community had established, but it was not possible to quantify this using a standard ATP method.

Previously, Cartmell (1997) successfully quantified the amount of ATP from microcosm sand samples using similar extraction and instrumentation protocols with an estimated percentage recovery of 60 %. Indeed, Cartmell (1997) documented microcosm ATP concentrations of up to 100 ng ATP g^{-1} sand (dry wt) (adjusted for percentage recovery). The assessment of % recovery of sandstone ATP in the experiments of Cartmell (1997) was based on the addition of a known concentration ATP spike, prepared from a freeze-dried salt (Celsis Lumac, UK), to sterilised sand. Losses of ATP during extraction/quantification have previously been attributed to de-phosphorylation of ATP by cellular enzymes and adsorption onto positively charged soil/sand particles (e.g. De Nobli *et al.*, 1996), for example. This should be minimised

by the use of trichloroacetic acid and phosphate buffer in the extractant, respectively (De Nobli *et al.*, 1996). However, as the % recovery reported by Cartmell (1997) demonstrates, in sterilised sand where the activity of microbial enzymes should be negligible, 40 % of the spiked ATP could not be recovered. Indeed, the extreme sensitivity of the extraction procedure has been described as a major disadvantage of the technique (Lazarova and Manem, 1995). If the concentration of ATP in the sample under investigation is initially low, the losses experienced during the extraction/quantification procedure may be sufficient to consume all of the cellular ATP, so that none may be detected in the sample extract. Indeed, when the same extraction/measurement procedure was used on an aggregate sample of biofilm, collected from the microcosm inlet tubing of the glucose treatments over the duration of the experiment; the ATP content was quantified as 31.30 mg ATP g^{-1} (fresh wt).

An alternative explanation for the inability to detect ATP in the microcosm sand samples could be that the physical disruption process (ultrasonication) used to lyse the bacterial cells was insufficient. The original recovery method was developed for the disruption of soils, but sand was difficult to disperse in the extractant by ultrasonication using a water bath; other researchers have used bead-beating techniques to disrupt biomass in sand, for example (Calvo-Bado *et al.*, 2003). The use of a DNA indicator test could help investigate this further in future works.

In the case of these experiments, it was concluded that the original concentration of ATP in the samples was too low to be detected. This may have been exacerbated during the sampling procedure itself, where microcosm sand was sectioned into volumes of approximately 98 cm^3 and homogenised. If microbial growth within the sand had been very heterogeneous, in relatively isolated aggregates, as some authors suggest (e.g. Vandevivere and Baveye, 1992 a; Rittman, 1993), the process of mixing bulk volumes of sand may have the effect of further diluting the ATP. Although Cartmell (1997) was able to recover ATP from microcosm sand samples using similar methods to those employed here, it should be noted that the actual microcosm experiments were incubated at a considerably higher temperature (25 °C) and the aquifer material was pre-conditioned with microbial nutrients prior to denitrification experiments. In this instance, conventional methods of quantifying biomass, such as dry wt measurements or culturing, although comparatively crude, would have been useful to compliment the ATP determinations.

It is feasible that future biomass determinations in ISBD applications may involve more sophisticated enumeration techniques such as fluorescent *in situ* hybridisation (FISH,

which is currently being developed for use in wastewater activated sludge (e.g. Patureau, *et al.*, 2001; Lee, *et al.*, 2002. The initial processes of amplifying and recovering bacterial DNA from environmental sandstone samples has been successfully demonstrated as part of these experiments (refer to Chapter 7).

5.2.3.2 Extracellular Polymeric Substances

Samples collected from microcosms used in Trial III were not found to contain any EPS-p, as the colorimetric reaction between the sample extracts and protein reagents gave absorbance readings below than the lowest protein standard (50 μg ml^{-1} bovine serum albumin) and similar to the reagent blank. Alternatively, samples for the EPS-c assay became turbid during preparation, which compromised the colorimetric assay (for example, see Figure 5.5).

No meaningful results were therefore obtained from the analyses of microcosm samples collected during Trial III, but the analytical procedures were subsequently reviewed prior to microcosm Trial IV. In the case of the EPS-c determinations, the cause of reagent contamination was identified as a contaminated batch of formaldehyde and subsequently removed. For the EPS-p assays, a more sensitive extraction method (Zhang *et al.*, 1999) and an alternative assay protocol were selected (as described in Chapter 3).

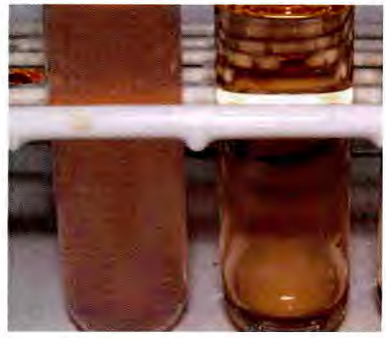


Figure 5.5: Carbohydrate assay showing a contaminated sample on the left, compared to a non-contaminated sample on the right

The results from sandstone samples collected during microcosm Trial IV are presented in Figure 5.7, it was not possible to estimate the percentage recovery from the extraction processes, but a positive control was included and demonstrated that both protein and polysaccharide protocols were successful. Protein and carbohydrate were detected in all microcosms, including the autotrophic control microcosms, which received raw groundwater amended with NO_3^- but no OC. As

would be expected, the greatest quantities of EPS were found in the bottom 5 cm of the sand column, from the inlet chamber upward, a smaller amount was detected in the 5-10 cm section of the non-control microcosms. In sections further than 10 cm away from the inlet point, no more EPS-p or EPS-c was recovered. In some microcosms, a white 'slime' developed, coating the sand lying next to the glass wall of the microcosm adjacent to the inlet, as depicted in Figure 5.6. This slime-coated sand was held together as aggregated portions, presumably bound by the slime content, assumed to be EPS.

The EPS-p recovered from the 0-5 cm section ranged from 4 - 28 ± 2 μg g^{-1} sand (dry wt) whilst the EPS-c ranged from 8-14 ± 2 μg g^{-1} sand (dry wt). In the sand sampled from this bottom section, there was a marked increase in the EPS-p content of the glucose microcosms, compared to the control and acetic acid treatments. Conversely, the EPS-c content was similar in all microcosms, as shown in Figure 5.7.

In the sand sampled from the 5-10 cm sections, no EPS were detected in the control microcosms and no EPS-c was found in the lowest concentration glucose treatment (MC 5). Of the remaining glucose and acetic acid microcosms, EPS-c concentrations were similar to those observed in the bottom 0-5 cm of microcosm sand, but the EPS-p were reduced in the 5 – 10 cm layer of the sandstone (Figure 5.7). In both sections sampled for EPS determination (0-5, 5-10 cm), the glucose treatments contained more EPS-p, whereas acetic acid treatments had larger amounts of EPS-c, as quantified by the ratios of EPS-p/EPS-c presented in Table 5.1.

As these results demonstrate, more EPS were found in the vicinity of the nutrient injection point, decreasing with increased distance away from the nutrient input source. This pattern of EPS deposition has been previously quantified in sand columns (Vandevivere and Baveye, 1992 b; Rinck-Pfeiffer *et al.*, 2000) and observed at clogged ISBD field experiments (e.g. Mercado *et al.*, 1988; Hamon and Fustec, 1991; Abdelouas, 1999).

Whilst biofilm thickness is generally attributed to physical factors such as the shear stress acting on the biofilm, the composition of the biofilm is related to nutrient availability, specifically, C, N and P concentrations (Alves *et al.*, 2002). Also, for a given content of P in groundwater, the ratio of EPS-p/EPS-c has been found to increase with increased C/N ratio (Cuervo-Lopez, *et al.*, 1999; Alves *et al.*, 2002). However, this was not apparent from the microcosm results presented here, where C/N ratio had no discernible effect over the relatively small concentration range tested.

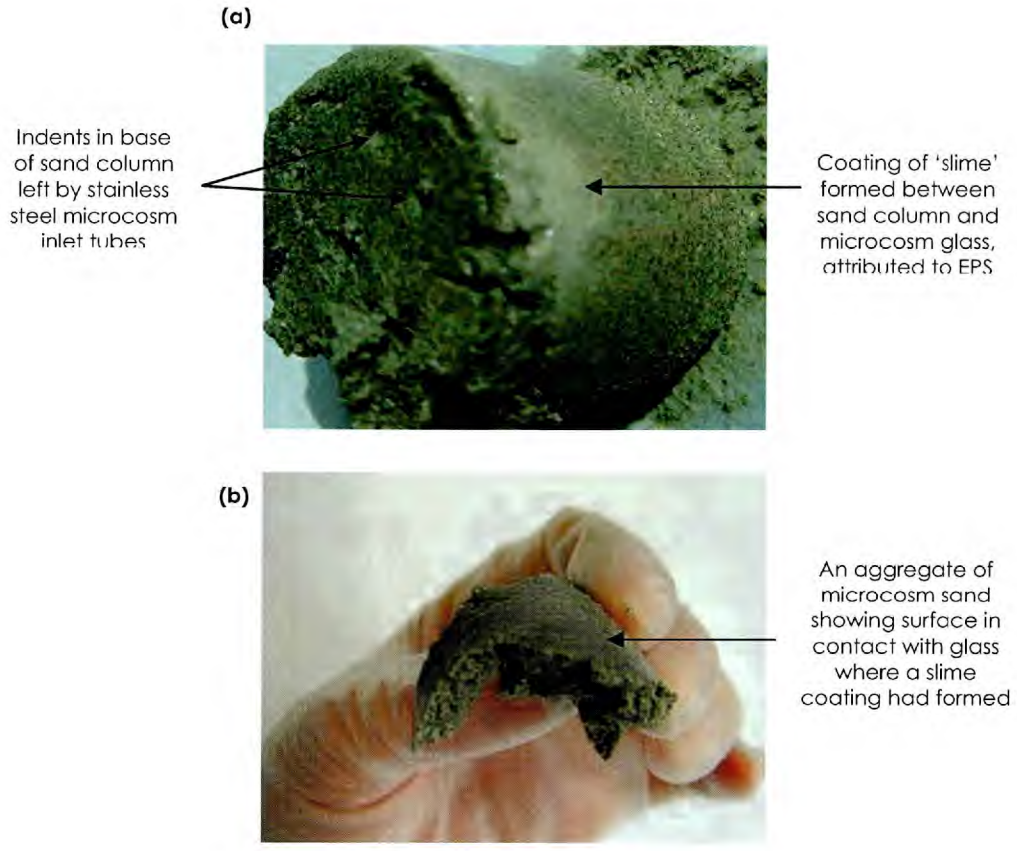


Figure 5.6: Effects of EPS production on sandstone from microcosm 7 (acetic acid treatment) at termination of Trial IV. **(a)** Slime deposits on sandstone removed from base of microcosm, (showing base of sand column uppermost); **(b)** sandstone from base of microcosm 7, aggregated by slime development at peripheral edge in contact with microcosm glass

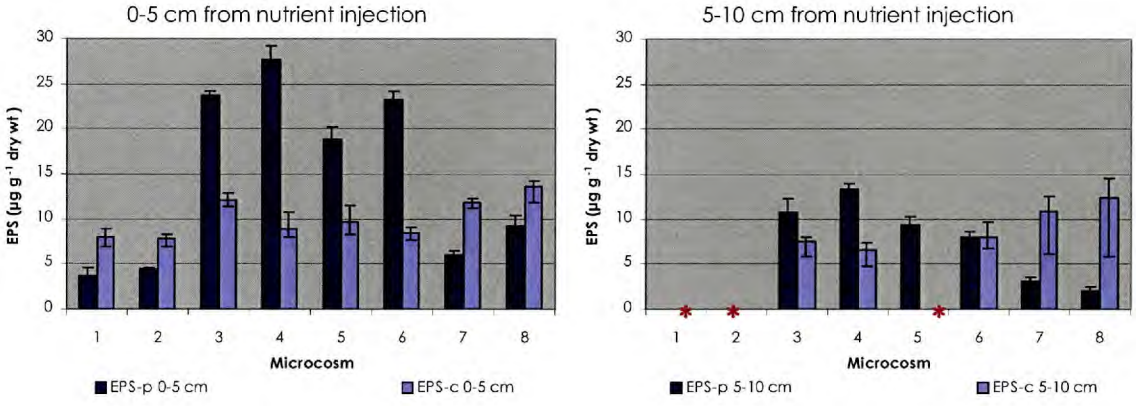


Figure 5.7: Mean EPS-p and EPS-c concentration of samples extracted from sandstone collected at the termination of microcosm Trial IV. (*) = < lower detection limit. MC 1 and MC 2 = controls (no OC), MC 3 and MC 4 = 35 mg C l^{-1} glucose, MC 5 = 28 mg C l^{-1} glucose, MC 6 = 44 mg C l^{-1} glucose, MC 7 and 8 = 35 mg C l^{-1} acetic acid. Y-error bars indicate min. and max. values

The type of OC substrate did apparently influence the composition of the EPS, and glucose treatments contained the larger amount of protein, as described in Table 5.1. Such variation according to substrate type has been documented by Sponza (2002) and Cuervo-Lopez *et al.* (1999), where acetate gave a lower EPS-p/EPS-c ratio compared to glucose, but had a higher specific denitrification rate. Similarly, the acetic acid treatments in these experiments were more efficient in terms of denitrification activity, compared to the glucose treatments, as discussed in Chapters 4 and 5. However, the relationship between EPS-p, EPS-c and denitrification efficiency apparently varies, with some reports correlating improved efficiency with higher EPS-p/EPS-c ratios (Lazarova *et al.*, 1994); whereas others associate lower EPS-p/EPS-c with increased denitrification rate (Cuervo-Lopez *et al.*, 1999). Therefore, the link between quantity and type of EPS produced and denitrification activity is uncertain.

Table 5.1: Ratios of EPS-p/EPS-c in microcosm sand from Trial IV

Microcosm	Treatment	EPS-p/EPS-c (µg/µg) 0-5 cm	EPS-p/EPS-c (µg/µg) 5-10 cm
1	Control (no OC)	0.46	-
2	Control (no OC)	0.56	-
3	35 mg C l^{-1} glucose	1.96	1.42
4	35 mg C l^{-1} glucose	3.13	2.02
5	28 mg C l^{-1} glucose	1.94	-
6	44 mg C l^{-1} glucose	2.75	1.00
7	35 mg C l^{-1} acetic acid	0.51	0.28
8	35 mg C l^{-1} acetic acid	0.68	0.16

In the context of these experiments, the variations in EPS formed under different substrate regimes may be a manifestation of the different microbial communities established; for instance, various authors have described differential EPS production under biofilms of different dominant bacterial species (e.g. Lazarova *et al.*, 1994). This could be explained by something as straight forward as the varying capability of different strains of bacteria to produce EPS (Vandevivere and Baveye, 1992 b).

Alternatively, the EPS composition may be an indirect indication of biomass concentration, where high EPS-p/EPS-c content has previously been linked with increased biofilm cell content (Alves *et al.*, 2002). From the microcosm results, the pronounced decrease in EPS-p concentration compared to EPS-c concentration at increased distance from the nutrient injection point would support this hypothesis, in light of the general view that biomass concentration also decreases in this plane

(Vandevivere and Baveye, 1992 b; Rinck-Pfieffer *et al*, 2000). Although a plausible explanation, biomass concentration data would be requires to verify whether this was actually the case.

The lack of quantitative studies of EPS in ISBD-comparative applications makes it difficult to put these results into a broader context. Similar laboratory experiments (Rinck-Pfeiffer *et al.*, 2000), carried out using secondary treated wastewater intended for aquifer recharge, at 20 °C, described EPS polysaccharides concentrations in the range of 0.1-1.0 mg g^{-1} dry wt, compared to a range of 0.0-13.6 µg g^{-1} dry wt measured here. The implications of the microcosm EPS determinations are discussed in the general context of biofouling in the following Section.

5.3 DISCUSSION

The clogging of aquifer material during both laboratory denitrification and ISBD applications is commonly reported qualitatively, but comparatively little quantitative information is available regarding the mechanisms and causes of clogging (e.g. Mercado *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993; Abdelouas *et al.*, 1999). Clogging is usually diagnosed from manifested effects, such as:

- progressive resistance and eventual termination of flow (Hamon and Fustec, 1991),
- increased head loss and leakage from the influent point (Dahab, 1993),
- visual clogging of equipment/wells (e.g. Mercado *et al.*, 1988),

Under the experimental conditions reported here, 'clogging' of the sandstone matrix was not observed in any of the microcosms stimulated with OC, in contrast to much of the ISBD reports from the literature. This further highlighted the need to quantify the progressive processes of clogging, to establish why clogging may occur in some scenarios, but not in others.

As discussed in Chapter 1, biofouling, or clogging, is a complex interaction of physical, chemical and biological phenomena. In these experiments, hydraulic conductivity was used as a general measure of loss in permeability of the porous aquifer material. The principal aim was to study biological causes of clogging, though incorporation of control data also highlighted clogging from physical and chemical means. The results indicated that physical and chemical influences on hydraulic conductivity were minimal, but in some cases, biological activity coincided

with reduced hydraulic conductivity. It was not possible to further define the factors responsible for this behaviour, since the methods used to quantify microcosm biomass lacked sufficient sensitivity to detect the very small biomass apparently supported in the aquifer matrix.

The evidence from these microcosms indicated that biomass (ATP and EPS) content in the sand, produced during the period of OC amendment, was small; the EPS that was observed visually was mainly present at the periphery between sandstone and microcosm glass, and not within the porous matrix. The distribution of biomass within the microcosms was therefore thought to generally be of a patchy, non-continuous nature, forming clusters of cell aggregates in the pore lumen, as previously described for low substrate loading conditions (Vanevivere and Baveye, 1992 a; Rittmann, 1993).

Under these conditions, it seemed likely that the observed decreases in hydraulic conductivity, which were comparatively minimal, were caused by the evolution of gases such as N_2, from denitrification activity. Indeed, Vandevivere and Baveye (1992 a) showed that in sand columns with biomass densities < 4.0 mg wet wt g^{-1} sand, hydraulic conductivity was unaffected. Furthermore, they observed that in columns where glucose supply was growth-limiting, as in the majority of microcosm experiments in this study, EPS were apparently absent. In other experiments, Vandevivere and Baveye (1992 b) demonstrated that hydraulic conductivity was not necessarily affected by EPS production, and that the characteristics of EPS extruded from bacterial cells had a critical influence. For instance, EPS that encapsulated the cells had less impact than EPS that was extruded in long strands.

The apparent differences in EPS production by different bacterial species and strains could be influenced by the type of OC substrate. For instance, microcosms treated with glucose and acetic acid exhibited differences in EPS-p/EPS-c composition (Table 5.1). The characterisation of EPS production under different OC substrates and doses could also be relevant to remedial steps to ameliorate clogging at ISBD installations and particularly in biological denitrification reactors involving filters. Potentially, measures such as intermittent operation, or push-pull operation (where direction of flow is reversed) could cause the sloughing of biomass resulting in perturbed water quality in terms of cell counts and also organic content.

The influence of gas bubbles on hydraulic conductivity depends on the volume of gas within the bulk volume of the sand; anecdotally, the volume of gas produced was greater in the more active microcosms, for example as depicted in Figure 5.8.

The rheological changes observed within the sand columns were exacerbated by the agglomeration of bubbles into larger, irregular bubbles or 'air pockets.' Where such gas bubbles became trapped in the pore network, their presence reduced hydraulic conductivity by reducing the size of the water-conducting pores (Baveye *et al.*, 1998). Alternatively, their ascendance through the sand could produce continuous channels, which remained in tact as preferential pathways, as shown in Figure 5.9, for example. The entrapment of gas bubbles in a porous medium may be aggravated by the presence of EPS, and the effect of the reduction on hydraulic conductivity may be exacerbated in media of mixed grain size (Baveye *et al.*, 1998).

However, although gas clogging has been observed in laboratory columns, there is a paucity of evidence demonstrating this behaviour in the field (Baveye *et al.*, 1998). Indeed, release of gaseous products has not been cited as a cause of clogging in the ISBD literature reviewed for this thesis (e.g. Mercado *et al.*, 1988; Janda *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993). In many ISBD field trials, however, it is possible that gases are simply vented to the atmosphere at observation boreholes, or in the injection/abstraction wells (e.g. Braester *et al.*, 1988). Alternatively, under the increased pressure anticipated in the subsurface, the gaseous products of denitrification may be dissolved in solution (Dahab, 1993), until the pressure decreases to ambient levels, for example at the abstraction well, where products may be released in the gas phase. Therefore, in operational practice, gas generation does not appear to be a significant factor in ISBD field systems, although it is widely observed in, and affects the flow characteristics of laboratory microcosms.

Although biological clogging was not observed in the laboratory microcosm experiments reported here, it is exclusively reported to be the cause of aquifer plugging observed in ISBD field trials. Dahab (1993) noted that clogging was particularly poorly reported in laboratory microcosm studies, however, this may be due to empirical differences between microcosm simulations and actual *in situ* experiments, which contribute to the frequency of biofouling incidents in field trials, compared to laboratory microcosm experiments. Whilst the microcosm approach to the study of subsurface and aquifer environments is accepted and is moreover a necessity, some authors caution the extrapolation of controlled laboratory column experiments to field situations (Baveye *et al.*, 1998).

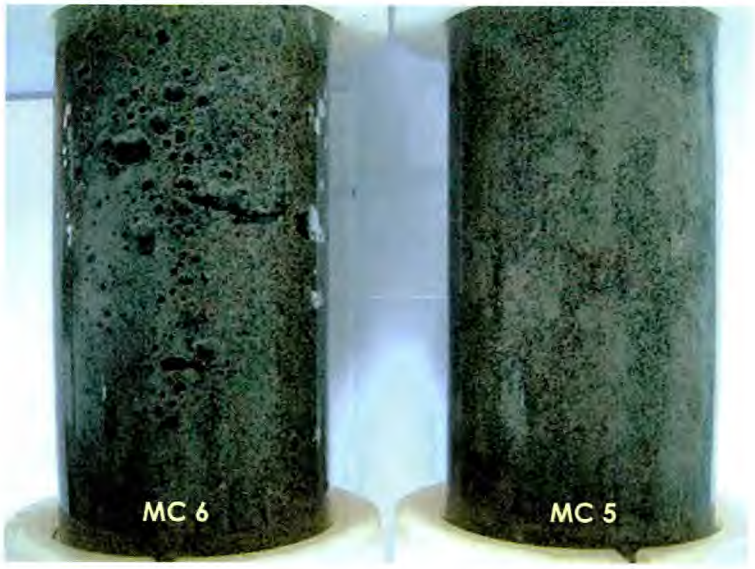


Figure 5.8: Comparison of gas pockets in upper sections of microcosms of high (MC 6) and low (MC 5) relative denitrification activity during Trial IV. MC 6 received influent glucose dose of 44 mg C l^{-1} compared to 28 mg C l^{-1} in MC 5

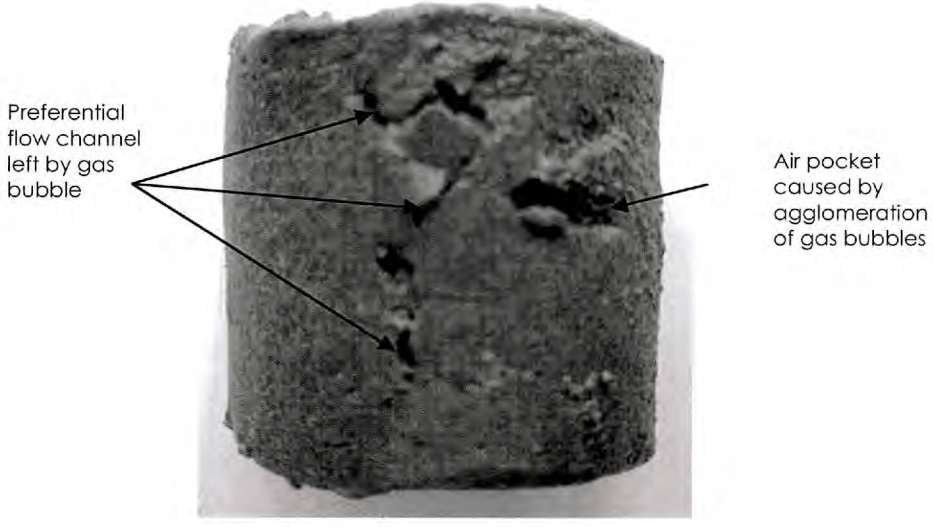


Figure 5.9: Section of sand removed from MC 7, Trial IV at the end of the experiment, showing preferential flow channels caused by ascending gas bubbles

In terms of field studies, individual aquifer characteristics may contribute to the varying observations of biofouling. For example, aquifers with relatively high DO or NO_3^- contents would require a higher concentration of OC amendment, and

therefore, a greater degree of biomass proliferation would be anticipated. Certainly, Rittmann (1993) documented clear differences in biofilm development under high and low substrate loadings. Alternatively, increased groundwater temperature could increase the risk of aquifer biofouling in ISBD applications, since it should increase the rate of microbial metabolism, and therefore can indirectly be responsible for increased biomass growth. The degree of reduction in soil column permeability has been shown to be increased as a function of temperature (Balks *et al.*, 1997).

The type of OC substrate used, however, emerges as one of the critical factors in potential biofouling of ISBD applications, owing to its influence over the enhanced microbial biomass, in terms of species diversity and biofilm development, (particularly its influence on EPS production). Although biofouling was not observed in the microcosm sand here, it is worth noting that during routine cleaning/disinfection of the microcosm inlet tubing, considerable quantities of biomass were removed from the tubing of the glucose-fed microcosms, every 3-4 days. Conversely, biofilm was not visually evident in either the acetate or the acetic acid tubes, on any cleaning occasion.

Interestingly, although the application of various substrates has been thoroughly researched at the laboratory scale, field scale ISBD applications have been surprisingly limited in the range of substrates used, for example, as detailed in Table 1.4 (Chapter1); only ethanol has been applied with any frequency. It appears, therefore, that this substrate has repeatedly been selected on the basis of its denitrification yield, with less consideration for potential biofouling.

Various operational approaches have been adopted to ameliorate aquifer biofouling, principally, intermittent injection of the OC substrate (e.g. Hamon and Fustec, 1991) and the recycling of a portion of the abstracted groundwater into the injection borehole in conjunction with the OC amendment (e.g. Janda *et al.*, 1988). The adoption of such strategies has met with limited success and currently, biofouling continues to be an issue for investigation, with regards to *in situ* bioremediation not only of NO_3^- contaminated groundwater, but for other aquifer 'clean up' methodologies (e.g. Nutall *et al.*, 1999; Baveye *et al.*, 1998). These results show that a major management factor in controlling the extent of biofouling in operational ISBD systems will be the selection of an appropriate OC source.

CHAPTER 6

POTENTIAL FOR ALTERNATIVE ELECTRON ACCEPTING PROCESSES IN GROUNDWATER ISBD SYSTEMS

6.1 OVERVIEW

6.2 RESULTS

6.2.1 Microcosm Trial III

6.2.2 Microcosm trial IV

6.3 DISCUSSION

6.3.1 Iron and Sulphate activities

6.3.2 Manganese and Sulphate activities

6.3.3 Summary and Conclusions

6.1 OVERVIEW

This Chapter reviews the potential for the alternative electron acceptors, Fe^{3+}, Mn^{4+} and SO_4^{2-} to become active under the enhanced anaerobic environment caused by ISBD. To achieve complete denitrification, experimental evidence advocates that the quantity of OC injected into the aquifer must be in excess of the stoichiometric requirement for DO and N consumption, to meet the needs of biomass assimilation. However, it has been demonstrated that microbial groups other than those involved in denitrification (including DO consumption) may additionally become established and compete for the OC electron resource. Commonly, fermentative bacteria are thought to be responsible for the increased competition for electrons, although under the anaerobic conditions that develop, it is also feasible that microbial groups capable of utilising alternative TEAs (terminal electron acceptors) might become active, particularly if a non-fermentable substrate, such as acetate is used as the OC source (refer to Chapter 1 for further details). Under idealised ISBD conditions, the OC injection should be optimised to balance the OC supply with the demand for denitrification activity, and prevent further reducing conditions from developing. However, if the OC injection is not optimally dispersed and mixed with the influent groundwater, OC concentrations significantly in excess of the denitrification environment may accumulate within localised pore networks. Under such conditions, TEAPs (terminal electron accepting processes) other than denitrification may initiate.

The assessment of alternative TEA activity during denitrification was the principal focus of microcosm Trial III, where denitrifying microcosms were packed with sandstones of varying Fe and Mn mineral content, and SO_4^{2-} was dissolved in the influent feed solution. The concentration of total Fe in the sandstones was highest in the Devonian sandstone, followed by the Lower Greensand and lastly the Triassic sandstone, whereas the concentration of total Mn was similar in the Triassic sandstone and Lower Greensand but higher in the Devonian sandstone (refer to Chapter 2 for details of sandstone Fe and Mn concentrations and Chapter 3 for descriptions of microcosm preparation). The NO_3^- and OC amendments supplied to the microcosms were similar for all sandstone types (15 mg NO_3-N l^{-1} and 35-50 mg acetate C l^{-1}). During the experimental period, dissolved Fe and Mn (filtered through 0.45 µm) sampled from the microcosm interstitial solution and SO_4^{2-} in microcosm effluents were quantified, in conjunction with denitrification activity (details of denitrification activity discussed in Chapter 4). The results from the experimental assessments of Fe^{2+}, Mn^{2+} and SO_4^{2-} activity during microcosm Trial III are presented in Section 6.2.1, with supplemental results from microcosm Trial IV in Section 6.2.2. It should be noted, however, that Fe and Mn ions were not actually speciated, and therefore, Fe^{2+} and

Mn^{2+} are inferred from total dissolved Fe and Mn concentrations, following filtering through 0.45 µm and immediate acidification, as described in Chapter 3.

6.2 RESULTS

6.2.1 Microcosm Trial III

Throughout the experiment, the ORP (mV) measured in the active microcosms decreased over time, compared to control microcosms which tended to remain at a consistent redox value (redox values presented in Chapter 8). This was indicative of the development of anoxic conditions and coincided with increased denitrification activity. Whilst the ORP probes could not be used for absolute measurements of oxidation/reduction capacity within the microcosms, they did show that the ORP status in the Devonian sandstone microcosm was notably lower than that in the Lower Greensand and the Triassic sandstone microcosms, though the influent DO, NO_3^- and OC were similar in all three microcosm types as microcosms were fed from the same batch of feed solution.

The SO_4^{2-} supplied to the microcosms during weeks 4 and 7 was accounted for in the microcosm effluents, and thus it was assumed that SO_4^{2-} did not react within the sand columns, regardless of sandstone type. Indeed, in some cases, there was a small increase in SO_4^{2-} apparent in effluent samples, which suggested that quantities of SO_4^{2-} were dissolving from the microcosm sandstone material (not reported in the context of these experiments).

The concentrations of dissolved Fe and Mn measured in the biologically active microcosms throughout the experiment are presented in Figure 6.1. The control microcosms were not analysed for $Fe_{(aq)}$ and $Mn_{(aq)}$ in this experiment, since the use of $HgCl_2$ as a sterilising agent to distinguish biological denitrification activity from chemical denitrification, meant that a comparison could not be made between non-sterile and sterile microcosms, since the chemistry of the feed solutions was different.

The analysis incorporated quality checks such as blank correction and samples spikes and coefficient of variation was < 20 %. The GFAAS instrument was calibrated using a standard range of 10-50 µg l^{-1} for both Fe and Mn. Samples containing Fe/Mn in excess of this range were diluted appropriately and re-analysed; the instrument's software extrapolates measurements of < 10 µg l^{-1}. These extrapolated values have been included, since the instrument's calibration curves are automatically forced through zero and have correlation coefficient of > 0.99. Microcosm feed solutions were prepared from PURITE RO water and contained < 1.0 µg l^{-1} $Fe_{(aq)}$ or $Mn_{(aq)}$.

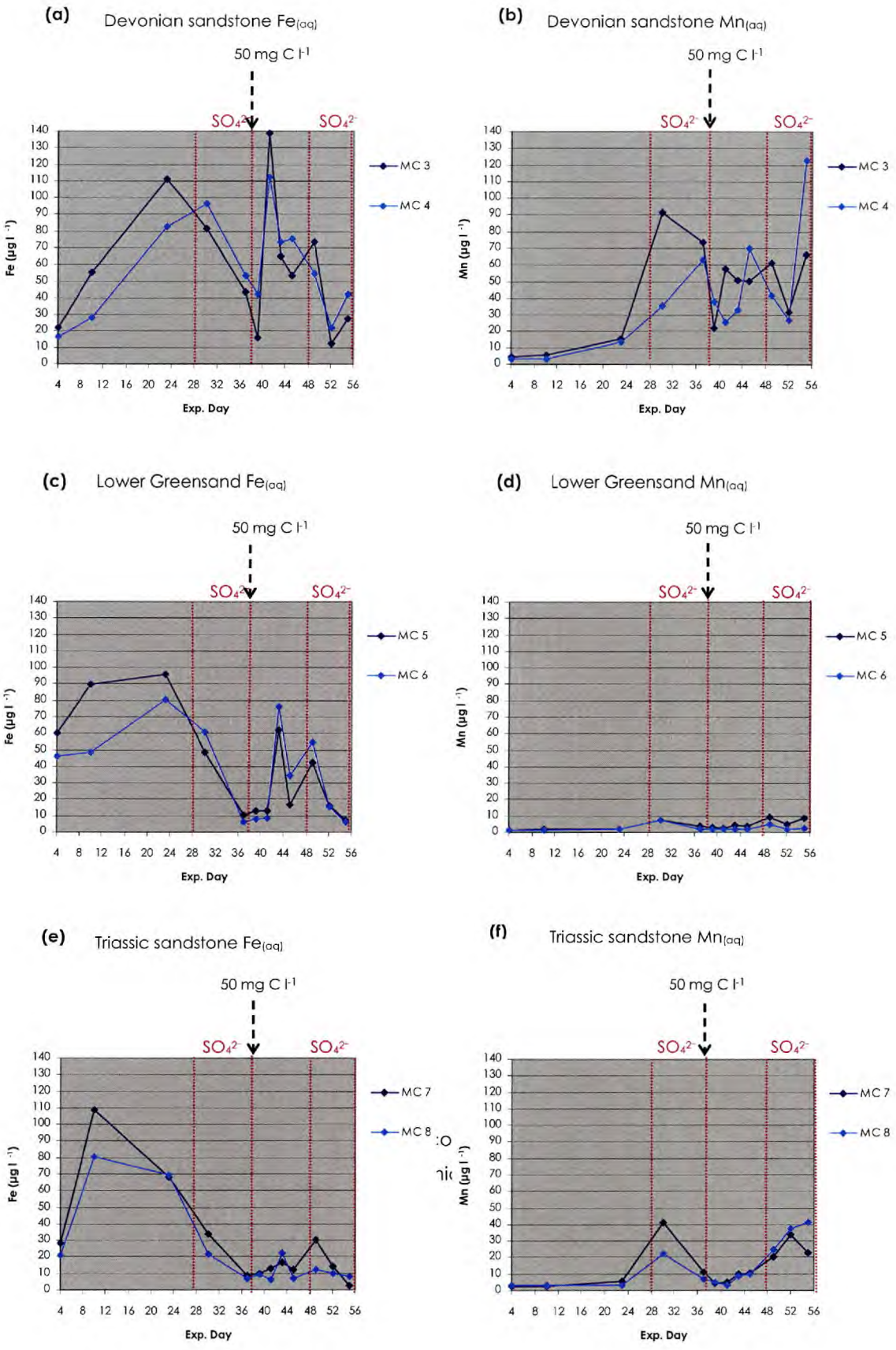


Figure 6.1: Dissolved (filterable) Fe and Mn concentrations in interstitial microcosm solution sampled during experimental Trial III, indicating periods of SO_4^{2-} addition to microcosm feed solution

The interstitial $Fe_{(aq)}$ concentrations were generally larger than the observed $Mn_{(aq)}$ concentrations, with the exception of the Devonian sandstone microcosm, where similar concentrations of each were observed. Initially, the profiles of $Fe_{(aq)}$ concentration in the interstitial solution over time were comparable in all three sandstone types, characterised by a peak in concentration between d 4 and d 30. Thereafter, the concentration fluctuated, with the magnitude of the fluctuations greatest in the Devonian sandstone, followed by the Lower Greensand and finally the Triassic sandstone. The mean concentrations of $Fe_{(aq)}$ and $Mn_{(aq)}$ measured over the duration of the experiment were comparable in the Lower Greensand and Triassic sandstone microcosms but considerably larger in the Devonian sandstone microcosms. The two periods of SO_4^{2-} addition coincided with decreases in interstitial $Fe_{(aq)}$ in the Devonian and Lower Greensand microcosms. This was also observed, but to a lesser extent in the Triassic sandstone microcosms. In all three sandstone types, increased fluctuation in $Fe_{(aq)}$ was observed following the increase in influent OC, on d 37.

The interstitial Mn concentrations in the Devonian sandstone microcosms exhibited the most dynamic profile over time. However, though Mn concentrations varied, there was an underlying trend of increasing Mn concentration apparent over the duration of the experiment. The overall mean concentration of Mn in the interstitial solution measured for Devonian sandstone was higher than the Lower Greensand and Triassic sandstone microcosms.

A GLM ANOVA and Tukey test of the concentrations of $Fe_{(aq)}$ and $Mn_{(aq)}$ respectively, (using the methodology described in Chapter 4, Section 4.1.2) showed that the overall mean concentrations of $Fe_{(aq)}$ and $Mn_{(aq)}$ were significantly higher ($P < 0.05$) in the interstitial solution from the Devonian sandstone microcosms (ANOVA tables presented in Appendix 4).

The Mn concentration measured for the Lower Greensand microcosms was < 10 $\mu g\ l^{-1}$ throughout the experimental period, and was apparently unaffected by either the SO_4^{2-} amendments or the increase in influent OC. Contrastingly, $Mn_{(aq)}$ measured for the Triassic sandstone microcosm exhibited an increasing trend in $Mn_{(aq)}$ following the increase in influent OC. Relatively minor variations in Mn concentrations were observed during the SO_4^{2-} phases, but no clear perturbations were apparent.

6.2.2 Microcosm Trial IV

During microcosm Trial IV, where Upper Greensand was used to fill the microcosms (refer to Chapters 2 and 3), samples of raw groundwater and interstitial microcosm solution were sent for $Fe_{(aq)}$ and $Mn_{(aq)}$ analysis at an external laboratory on five occasions, d 1, 7, 13, 20 and d 25, the results are presented in Table 6.1.

The concentrations of Fe and Mn in the representative samples of raw groundwater collected from the Fovant Public Supply borehole at the field site under investigation were less than the analytical limits of detection (16 µg l^{-1} Fe, 4.2 µg l^{-1} Mn). This was consistent with historical water chemistry of the public supply borehole (unreferenced information provided by Wessex Water), and from samples collected during the hydrogeological investigation of the site (refer to Appendix 1).

The interstitial microcosm solutions were found to contain $Fe_{(aq)}$ on only one sampling occasion, d 20. Conversely, $Mn_{(aq)}$ was observed in increasing concentration over the final two consecutive sampling occasions.

Table 6.1: Concentrations of $Fe_{(aq)}$ and $Mn_{(aq)}$ measured in interstitial solutions sampled of Upper Greensand microcosms, during Trial IV

	Exp. Day	Microcosm Treatment				
		Autotrophic Control (MC 2)	Glucose 35 mg C l^{-1} (MC 3)	Glucose 28 mg C l^{-1} (MC 5)	Glucose 44 mg C l^{-1} (MC 6)	Acetic acid 35 mg C l^{-1} (MC 7)
Fe (µg l^{-1})	1	< 16.0	< 16.0	< 16.0	< 16.0	< 16.0
	7	< 16.0	< 16.0	< 16.0	< 16.0	< 16.0
	13	< 16.0	< 16.0	< 16.0	< 16.0	< 16.0
	20	19.0	32.0	80.0	80.0	35.0
	25	0.0	0.0	0.0	0.0	0.0
Mn (µg l^{-1})	1	< 4.2	< 4.2	< 4.2	4.6	5.6
	7	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2
	13	< 4.2	< 4.2	< 4.2	< 4.2	< 4.2
	20	< 4.2	4.4	4.8	5.4	6.0
	25	< 4.2	25.9	22.2	24.6	22.5

The concentrations of Fe and Mn were smaller compared to those observed in Trial III. With relevance to the proposed ISBD pilot plant at the Fovant site (refer to Chapter 2), the results therefore indicated that *in situ* denitrification could potentially give rise to Mn dissolution, and thus warranted further monitoring.

6.3 DISCUSSION

In situ bioremediation schemes enhance the naturally occurring TEAPs within the subsurface to ameliorate polluted sites and contaminant plumes. *In situ* biological denitrification of groundwater aims to develop denitrification as the dominant TEAP, by supplementing the aquifer with sufficient OC for DO to be completely consumed as an electron acceptor, thereby permitting denitrification, as discussed in Chapter 1. However, it is imperative that the OC enhancement does not facilitate the further reduction of alternative electron acceptors: Fe^{3+}, Mn^{4+} and SO_4^{2-}, since the reduced forms of these species would impair groundwater quality and could cause fouling of the groundwater abstraction facility and water distribution networks.

In UK aquifers, $SO_4^{2-}{}_{(aq)}$ is present in pristine or relatively oxic groundwater, dissolved from SO_4^{2-} bearing minerals such as gypsum (Kimblin, 1995), the oxidised forms of Fe^{3+} and Mn^{4+}, conversely, are present as insoluble minerals such as oxides, hydroxides and carbonates (e.g. Chapelle, 2001). These aquifer mineral species may become involved in both abiotic and microbially mediated oxidation-reduction reactions, as electron acceptors, if the concentration of oxidisable substrate becomes sufficient (Chapelle, 2001).

The occurrence and distribution of these TEAPs is not simply determined according to microbial respiration, as a thermodynamic hierarchy (described in Chapter 1), but is also a function of physico-chemical parameters such as: Eh/pH, chemical saturation equilibriums and geochemistry; including availability of electron acceptors bound to the solid phase (e.g. Atta *et al.*, 1996; Ludvigsen *et al.*, 1998; Amirbahman *et al.*, 2003). Ultimately, however, microbial respiration is thought to drive the abiotic mechanisms, termed 'non-enzymatic' processes (e.g. Lovely, 1991; Gounot, 1994; Frederickson *et al.*, 1998; Broholm *et al.*, 2000). The complex interactions between abiotic and microbiological activities, which give rise to TEAPs are therefore difficult to appraise and comprehensive quantification of such biogeochemical phenomena requires a complex suite of experimentation, involving analysis of:

- Groundwater chemistry,
- Eh/pH,
- Aquifer geochemistry, including speciation of those ions concerned,
- Microbial community dynamics.

The experiments reported in this chapter therefore provide initial insight into the potential activity of the electron acceptors: Fe^{3+}, Mn^{4+} and SO_4^{2-} in conditions

representative of proposed ISBD operations in the UK. The data on Mn and Fe dissolution at drinking water-based ISBD experiments is scarce, but Hamon and Fustec (1991), reported concentrations of $Mn^{2+}_{(aq)}$ in the range 2-4 µg l^{-1}, but did not detect $Fe^{2+}_{(aq)}$; whilst higher concentrations were observed by Abdelouas *et al.* (1999): 180 µg Mn l^{-1} and 140 µg Fe l^{-1}. The latter results are more consistent with those reported here. The majority of published data describing groundwater Fe and Mn concentrations, however, is largely concerned with contaminant plumes and leachates (e.g. Ludvigsen *et al.*, 1998; Tucillo *et al.*, 1999; Thornton *et al.*, 2000; Kennedy and Everette, 2002; Broholm *et al.*, 2002).

The dissolved Fe and Mn recovered during this experiment were assumed to be the reduced ion species: Fe^{2+} and Mn^{2+} respectively, and therefore, the results suggested that microcosm operation had an effect on the mineral characteristics of the various sandstones examined. Furthermore, it was apparent that the degree of dissolved Fe and Mn in the interstitial microcosm solution related to the quantity of Fe and Mn (total) in the sandstone, as listed in Chapter 2 (page 55). Devonian sandstone microcosms exhibited statistically greater dissolved Fe and Mn concentrations in the interstitial water than the Lower Greensand and Triassic sandstone microcosms, for example. The apparent conservation of SO_4^{2-} from the microcosm influent solution in the microcosm effluent led to the conclusion that no sulphate reduction activity had occurred, at least at the scale of mg l^{-1}.

It was unclear from these experiments, however, whether $Fe^{2+}_{(aq)}$ and $Mn^{2+}_{(aq)}$ resulted from the reduction of oxidised mineral forms within the microcosms, or whether the reduced ions were present in the original sandstone, bound in the solid phase, and subsequently released owing to physico-chemical phenomena such as dissolution. Quantification of such physico-chemical parameters requires further investigation of the sandstone mineralogy, as considered in Chapter 10. Since the speciation of Fe and Mn minerals/ions was not determined here, a mass balance was not completed for the reactions. Mass balances for biogeochemical reactions are reportedly difficult to define, primarily owing to the large difference in the units of measurements for electron donor utilisation, compared to mineral forms of electron acceptor (Broholm, *et al.*, 2002; Amirbahman *et al.*, 2003).

Nevertheless, the results indicated that physico-chemical processes influenced Fe^{2+} concentrations in the interstitial solution whilst the Mn^{2+} concentration profile may have additionally been modulated by biotic factors.

6.3.1 Iron and Sulphate Activities

The concentration of dissolved Fe^{2+} measured during the first 23 days of experimental Trial III (refer to Figure 6.1), could be considered as 'wash out' processes, under the flow through regime. It is proposed that this was caused by equilibrium diffusion processes between the microcosm influent solution, which had trace concentrations of Fe and Mn, and the solid-bound reduced phases of Fe^{2+} and Mn^{2+}. It is unlikely that the initial rise in interstitial Fe^{2+} was the result of reductive dissolution of Fe^{3+}, since denitrification activity was not fully established within the microcosms, and therefore it is assumed competitive inhibition of dissimilatory iron-reducing bacteria (DIRB) by denitrifiers would have occurred, given the limitation in electron resource. Moreover, no Mn^{2+} was detected in the interstitial microcosm solution during this period. This further emphasised the unlikelihood of biological Fe reduction in the microcosms since Mn^{4+} is theoretically reduced in preference to Fe^{3+}, (where bioavailable forms of Mn^{4+} are present), (Chidthaisong and Conrad, 2000).

Conversely, where Fe^{2+} is present in the solid phase, for example, precipitated as authigenic minerals: siderite ($FeCO_3$), magnetite [$Fe^{2+}(Fe_3O_4)$, or Fe-sulphides; or is adsorbed onto the surfaces of crystalline Fe^{3+} oxides, precipitation and dissolution mechanisms occur to achieve a saturation equilibrium between the solid and aqueous phase (e.g. Broholm *et al.*, 2000; Amirbahman *et al.*, 2003). Broholm *et al.* (2000) observed that Triassic sandstone sampled from a site at a similar location (Mansfield, UK; NGR not given) to the source of Triassic sandstone used in these experiments, i.e. Sherwood Sandstone Group, was equally rich in Fe^{2+} and Fe^{3+}. Therefore, it is possible that the flushing of microcosms with an influent solution unsaturated with regards to Fe, during operation, could have caused the dissolution of Fe^{2+}-bearing minerals, according to the saturation conditions, as observed in similar laboratory experiements (Broholm *et al.*, 2000; Amirbahman *et al.*, 2003).

The concentrations of $Fe^{2+}_{(aq)}$ observed during Trial IV, which additionally incorporated control microcosms, would support this hypothesis, though the interstitial microcosm solution was only sampled on five occasions. As shown in Table 6.1, $Fe^{2+}_{(aq)}$ could not be detected in the samples, except on d 20, when up to 80 µg Fe l^{-1} was observed. This isolated incident of Fe^{2+}, detected in all samples, even those from the autotrophic control microcosm, coincided with the use of a microcosm feed solution that was prepared in Purite laboratory water (RO), during the previous 24 h. At all other times (during Trial IV), the microcosm feed solutions were prepared using raw groundwater taken from the site where the aquifer material was collected. Therefore, it is proposed that the natural groundwater was approximately at equilibrium with the solid aquifer phase, in terms of mineral saturation, and

alternatively, the feed solution was prepared in pure water (with no nutrient additions), was under-saturated with respect to Fe-bearing minerals, causing the dissolution of solid-bound Fe^{2+}. Conversely, the microcosm feed solutions used during Trial III were prepared in Purite water throughout the experiment, hence the persistent presence of $Fe^{2+}_{(aq)}$ over the duration.

The apparent release of Fe^{2+} from the solid phase during microcosm Trial III varied to some extent between the sandstone types tested. For example, Triassic sandstone, which had the lowest Fe content, showed a relatively large increase in Fe concentration on d 10. This could be explained by a rapid initial dissolution of solid-bound Fe^{2+} to achieve saturation equilibrium with the influent microcosm solution (prepared in RO water). The $Fe^{2+}_{(aq)}$ subsequently declined as solution flowed through the microcosms, continually diluting the soluble $Fe^{2+}_{(aq)}$ fraction and maintaining the equilibrium reaction in the dissolution phase. Presumably, a threshold was reached where the equilibrium remained constant, as the Fe^{2+} content of the solid phase became depleted, or until other geochemical sensitive parameters such as redox and pH, changed within the microcosm.

During Trial III, the Devonian sandstone, which had the highest total Fe content (refer to page 55), reached a maximum Fe concentration of similar magnitude to the Triassic sandstone (approximately 110 $\mu g\ l^{-1}$) which had the lowest total Fe content. This suggested that the fraction of soluble Fe^{2+} was similar in both types of sandstone. However, maximum release of Fe was slower the time in the Devonian sandstone microcosms (23 d), compared to the Triassic sandstone (10 d). Assuming that similar processes of equilibration were occurring in both sandstone types, this suggested that the rate of dissolution of Fe^{2+} from the solid phase was slower in the Devonian sandstone. This might be explained by differences in the reactivity of the Fe minerals between the sandstone, regardless of concentration (discussed subsequently). The Lower Greensand behaviour was intermediate between the Devonian and Triassic sandstone types in terms of Fe dissolution.

Following the maximum concentration of Fe^{2+} measured in the interstitial solutions during Trial III, the concentrations subsequently declined until d 37, in all microcosms (Figure 6.1). An alternative explanation to the shifting dissolution equilibrium constant for the decay in interstitial $Fe^{2+}_{(aq)}$ could be the re-precipitation of dissolved Fe^{2+} as Fe^{3+} (hydr)oxides, for example as a chemical oxidation process under the increased pH value in the denitrifying microcosms (> pH 8.6; refer to pH results presented in Chapter 8)(e.g. Braester and Martinell, 1988).

This decrease in $Fe^{2+}_{(aq)}$ was sustained in microcosms of all sandstone types until day 37, which coincided with an increase in influent OC from 35 → 50 mg C l^{-1}. Following the increased OC amendment, the interstitial $Fe^{2+}_{(aq)}$ became relatively dynamic, exhibiting concentration increases and decreases of varying magnitude. Since this period coincided with complete denitrification and carbon breakthrough, i.e. an excess of OC was available, it is hypothesised that the $Fe^{2+}_{(aq)}$ measured during this phase of the experiment could have been biogenic. For example, with excess electrons, microbially mediated Fe^{3+} reduction may have occurred; certainly, a wide range of bacterial species are capable of dissimilatory Fe^{3+} reduction (e.g. Lovely, 1991).

Conversely, Fe^{3+} reduction may also be attributed to non-enzymatic reactions, such as chemical reduction by organic acids of inorganic products of bacterial respiration, such as NO_2^-, for example (Lovely, 1991; Broholm *et al.*, 2000). Additionally, decreasing Eh can cause thermodynamic equilibrium to shift in favour of the reduced Fe^{2+} species (Lovely, 1991). However, such abiotic or 'non-enzymatic' reactions are considered to be of minor significance in natural sediments and anaerobic subsurface environments, where electron donors for microbial respiration are typically available and the circumneutral pH prevents the activity of many chemical reductants, which require more acidic conditions (Lovely, 1991). In the microcosm experiments, it is assumed that the basic pH that developed over time would have minimised abiotic reduction activity, and that microbial respiration would be the principal mechanism of Fe^{3+} reduction, either through heterotrophic or autotrophic metabolism.

The degree of Fe^{3+} reduction in microcosm Trial III, inferred from the $Fe^{2+}_{(aq)}$ concentration after day 37, seemed to vary in accordance with the total sandstone Fe concentration i.e.: Devonian sandstone > Lower Greensand > Triassic sandstone (refer to page 55). This may be either a reflection of the overall quantity of available Fe^{3+}, or may be due to the different types of Fe^{3+} minerals present and indeed Fe^{2+}-binding species present in each sandstone type. For example, amorphous Fe^{3+} minerals are reportedly more reactive than crystalline solids, (e.g. Amirbahman *et al.*, 2003), and furthermore, increased reactivity is correlated with increased surface area (Fredrickseon *et al.*, 1998). However, these interpretations cannot be substantiated further, without additional geochemical speciation of the aquifer mineral types. For example, the fluctuations in $Fe^{2+}_{(aq)}$ may not necessarily be due to changes in Fe^{3+} reduction kinetics, but could instead be caused by variation of the degree of Fe^{2+} that is in the aqueous phase, compared to that which subsequently reacts to form solid authigenic minerals such as siderite ($FeCO_3$).

The peaks observed in $Fe^{2+}_{(aq)}$ following the increased OC dose, on days 41-43, particularly in the Devonian sandstone and Lower Greensand microcosms, could be explained as an initial response to the increased electron availability, following the complete consumption of DO and NO_x^-. In this case, the relatively high $Fe^{2+}_{(aq)}$ values may correspond to a maximum Fe^{3+} reduction activity, which subsequently declines in magnitude over the following 5 days, until d 48. This could indicate that Fe^{2+} undergoes secondary reactions and is sequestered in the solid phase, as previously suggested. Alternatively, this could reflect the decreasing capacity of the DIRB to reduce Fe^{3+}, for instance as the Fe^{3+} minerals and bacterial cell surfaces become saturated with adsorbed Fe^{2+}, essentially preventing contact between the cell and the TEA.

Such physical phenomena have been proposed, for instance, by Zachara *et al.* (2001), who observed that the rate of dissimilatory Fe^{3+} reduction decreased as the surfaces of crystalline Fe^{3+} oxides became saturated with Fe^{2+}(s). More than 50 % of the biogenic Fe^{2+} remained in the solid phase, associated with the residual Fe^{3+} oxides. This process may be reversible, however, as removal of the sorbed Fe^{2+}, for example by reaction with an anion such as PO_4^- or CO_3^-, can allow bacterial reduction to continue (Fredrickson *et al.*, 1998). In this regard, the kinetics of dissimilatory Fe^{3+} reduction may be influenced by the secondary activity of the biogenic Fe^{2+}, however, this is largely speculative at present, since the microbial reduction of Fe^{3+} oxides is not well researched (Fredrickson *et al.*, 1998).

Two phases of SO_4^{2-} addition were incorporated during Trial III, coinciding with the different influent OC doses, 35 and 50 mg C l^{-1}, respectively. In all three sandstone types, the SO_4^{2-} additions coincided with decreases in $Fe^{2+}_{(aq)}$ (Figure 6.1). During the first SO_4^{2-} addition phase (d 28-37), it is presumed that the ion remained in the oxidised state, since residual NO_x^- was also present in the microcosms, and SO_4^{2-} reduction is unlikely to occur in the presence iof NO_x^- (e.g. Broholm *et al.*, 2000). Therefore, in the absence of reduced S^{2-} species, it is proposed that the observed decrease in $Fe^{2+}_{(aq)}$ was linked to a continuation of the established trend resulting from Fe^{2+}(s) dissolution, described previously. Contrastingly, during the second period of SO_4^{2-} addition, which coincided with a phase of complete NO_x^- removal and potential Fe^{3+} reduction to Fe^{2+} under the higher influent OC dose; it is possible that, a small degree of SO_4^{2-} reduction potentially occurred. This could not be quantified analytically, however, since the instrumentation was only capable of detecting concentrations in the mg l^{-1} range. Where electron donors are in adequate supply and TEAs are present, it may be possible for more than one microbial TEAP to exist (Ludvigsen *et al.*,

1998; Broholm *et al.*, 2000), in this case, Fe^{3+} reduction and SO_4^{2-} reduction. If this occurred, S^{2-} produced in the microcosms may undergo secondary reactions with the Fe^{2+} generated from Fe^{3+} reduction to form iron-sulphide precipitates such as mackinawite (FeS) or pyrite (FeS_2). These concurrent reactions have been observed in landfill leachates (Broholm *et al.*, 2000) and could be responsible for the decreased Fe^{2+} concentration in the aqueous phase, demonstrated in these experiements. Mineral speciation measurements are required, however, to further elucidate these processes and mechanisms.

6.3.2 Manganese and Sulphate Activities

The ranges of Mn concentrations measured in the solid phase of sandstones used in Trial III (Chapter 2, page 55), and subsequently observed in the aqueous phase, were much lower than the Fe concentrations, as is typical in the natural environment (e.g. Braester and Martinell, 1988). In contrast to the Fe, dissolved (filterable) Mn (assumed to be Mn^{2+}), was present in very low concentrations in the interstitial microcosm solution during the first 23 d of the experiment (Trial III): $\leq$ 5 µg Mn l^{-1} in the Triassic sandstone and Lower Greensand microcosms, and $\leq$ 15 µg Mn l^{-1} in the Devonian sandstone microcosms (Figure 6.1). Thus, there was no evidence for similar dissolution processes as observed for $Fe^{2+}_{(aq)}$ and therefore, the presence of reduced Mn^{2+} ions within the aquifer matrix was initially low.

Indeed, the concentration of interstitial $Mn^{2+}_{(aq)}$ in the Lower Greensand microcosms remained consistently small throughout the experimental period, suggesting either that there was little Mn^{4+} reduction, or that the reduced Mn^{2+} species remained in the solid phase, perhaps adsorbed to residual Mn- or Fe-oxides, or alternatively as the authigenic mineral, rhodochrosite ($MnCO_3$) or Mn_3O_4 (e.g. Gounot, 1994). Contrastingly, the Triassic sandstone, which had a similar concentration of total Mn in the raw aquifer material (80 mg kg^{-1} dry wt), exhibited some evidence of Mn^{2+} in the aqueous phase; $Mn^{2+}_{(aq)}$ increased to a maximum values on d 30, which could not be explained, followed by a general increasing trend in $Mn^{2+}_{(aq)}$ after d 39. This pattern in Mn^{2+} concentration coincided with the increased influent OC dose, and the complete removal of NO_x^- from the microcosms. It is assumed, therefore, that bacterial dissimilatory Mn^{4+} reduction had commenced, since the basic pH in these microcosms was approaching pH 9.5 (pH data presented in Chapter 8) and therefore is likely to have favoured the oxidised form: Mn^{4+} (e.g. Gounot, 1994).

The $Mn^{2+}_{(aq)}$ profiles of the Devonian sandstone microcosms were relatively erratic, and the concentration range of the aqueous phase was approximately equal to that of the $Fe^{2+}_{(aq)}$ profile for this sandstone type. However, a generally increasing trend in

$Mn^{2+}_{(aq)}$ was observed throughout the experiment, suggesting potentially increased Mn^{4+} reduction activity. The reduction of Mn^{4+} due to abiotic processes, such as redox-related reduction, is unlikely to have been significant, given the high pH of the microcosm effluent throughout (pH 8.5 - 9.5) (e.g. Gounot, 1994), therefore, Mn^{4+} reduction was attributed to microbial reduction.

The results obtained from microcosm Trial IV also exhibited an increasing trend in $Mn^{2+}_{(aq)}$, though only five samples were analysed. Since $Mn^{2+}_{(aq)}$ was not detectable in the autotrophic control microcosm, the presence of dissolved Mn was attributed to microbial Mn^{4+} reduction, similarly to the results from microcosm Trial III.

The effects of SO_4^{2-} on Mn reduction, and indeed dissolution of Mn^{2+}, were not clear from these experiements, where both increases and decreases in Mn^{2+} concentrations were observed. Indeed, some authors have described an increase in $Mn^{2+}_{(aq)}$ when S^{2-} from SO_4^{2-} was present, suggesting that S^{2-} can reduce Mn^{4+} (Gounot, 1994). Conversely, declining Mn^{2+} concentration in the aqueous phase has also been attributed to the presence of SO_4^{2-}, due to the adsorption of Mn^{2+} onto iron sulphides, precipitated during the reduction of Fe^{3+} and SO_4^{2-} (Amirbahman *et al.*, 2003).

To date, it seems that the geochemistry of Mn, particularly the oxides, is less well defined than the geochemistry of Fe; this may be partly due to the difficulties in distinguishing between enzymatic and non-enzymatic reduction reactions, since the non-enzymatic reactions typically involve products of microbial respiration such as NO_2^-, S^{2-} as well as Fe^{2+} (e.g. Gounot, 1994).

Similarly to the aqueous Fe^{2+} dynamics, the release of Mn^{2+} into the aqueous phase is likely to be controlled by the saturation of the aquifer solids with reduced Mn minerals, such as rhodochrosite (Amirbahman *et al.*, 2003). The secondary precipitation of reduced Mn^{2+} minerals and the adsorption of Mn^{2+} onto Fe and Mn oxides, within the aquifer material is thought to sequester a significant portion of the reduced species, commonly resulting in the overestimation of Mn^{4+} in the solid phase (Gounot, 1994).

6.3.3 Summary and Conclusions

In experimental Trial III, the concentrations of dissolved (filterable) Fe and Mn species in interstitial microcosm solutions fluctuated throughout the experiment, at concentrations approaching, and in some cases exceeding, the UK standards for these elements in drinking water, (200 and 50 µg l^{-1} for Fe and Mn respectively; SI

3184). The presence of dissolved species, assumed to be Fe^{2+} and Mn^{2+}, following increased influent OC dose, were assumed to be the products of microbial reduction. This was not conclusive, however, since autotrophic controls were not included in this Trial. Nonetheless, commencement of anaerobic biological activity, the absence of NO_x^- from the solution, and the availability of an electron resource, confirmed that the conditions for reductive dissolution of Fe^{3+} and Mn^{4+} had been established and this coincided with detection of soluble Fe^{2+} and Mn^{2+} in the interstitial solution. The results from Trial IV, where Mn^{2+} was detected in the interstitial solutions of biologically active microcosms, but not in the autotrophic control microcosm, supported the hypothesis that the reduction was microbially mediated.

Considering the results from microcosm Trials III and IV, it appeared that Mn reduction was the more prominent reaction, with Mn^{2+} concentrations exhibiting a consistent increasing trend. This would concur with thermodynamic theory and observations from ISBD field trials, where soluble Mn was detected in the denitrified groundwater at concentrations in excess of the background (Hamon and Fustec, 1991; Abdelouas *et al.*, 1999).

The Devonian sandstone which had the highest concentrations of Fe and Mn in the sandstone (Trial III), exhibited the highest concentrations in the aqueous phase, followed by Lower Greensand and finally the Triassic sandstone (with the exception of Mn, which was found in approximately equal quantities in the Lower Greensand and the Triassic sandstone). This may have been due either to the availability of Fe^{3+} and Mn^{4+} in the original aquifer material, or may be explained by the potential for the reduced species to precipitate as authigenic minerals or adsorb to the solid phase. In both cases, the reduced species may effectively be sequestered from the aqueous phase, obscuring the true extent of biochemical reduction taking place. For example, the Lower Greensand and Triassic sandstone had similar concentrations of Mn measured in the solid phase, but the Triassic sandstone exhibited a markedly larger $Mn^{2+}_{(aq)}$ during the experiment (Trial III). Conversely, the Lower Greensand had a significantly larger total Fe content in the aquifer material, compared to the Triassic, therefore potentially increasing Mn^{2+} adsorption.

From a practical application perspective, it would be desirable to be able to quickly forecast the potential for Fe and Mn dissolution under proposed ISBD treatment schemes, as this may have potentially deleterious impacts on the treated water quality, or compromise the operation of the abstraction facility through fouling events. However, the results from these experiments indicate that assessment of potential Fe and Mn reduction must be site specific and furthermore, accurate

quantification requires not only a chemical assessment of the groundwater, but additionally, a quantitative speciation of the aquifer mineralogy. This could be achieved by laboratory column studies, so long as the water used in the simulation is from the same source as the aquifer material, at the proposed ISBD site, for example, as described in Trial IV.

CHAPTER 7

ECOLOGICAL DYNAMICS OF DENITRIFYING BACTERIAL POPULATIONS IN THE MICROCOSM ENVIRONMENT

7.1 BRIEF INTRODUCTION TO DGGE

7.2 RESULTS OF COMMUNITY COMPARISONS USING DGGE GEL BANDING DATA

7.2.1 Analysis of Sample DGGE banding patterns using ANOVA

7.2.2 Analysis of Sample DGGE banding patterns using Raup and Crick Index

7.2.2.1 Description of Raup and Crick Method

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7.3 RESULTS OF ANALYSIS OF SEQUENCE DATA OBTAINED FROM DGGE GELS

7.3.1 Similarity of microcosm sample-Sequences to Known organisms

7.3.2 Summary of Sequence Data

7.4 DISCUSSION

7.1 BRIEF INTRODUCTION TO DGGE

Denaturant gradient gel electrophoresis is a molecular fingerprinting technique that is commonly used to elucidate ecological patterns in microbial community diversity (Liu and Stahl, 2002). It can provide a rapid phylogenetic assessment of bacterial populations in environmental samples, and is considered a superior method to more conventional techniques, such as those based on cultivation. Cultivation of bacteria from environmental samples is expected to recover only a small fraction of the tota; species present (Muyzer *et al.*, 1993; Schleifer and Wagner, 1997; Yoshie *et al.*, 2001), for example estimated as 1-15 % of the total bacteria present in activated sludge (Boon *et al.*, 2002).

Denaturing gradient gel electrophoresis is used to produce a 'fingerprint' of the microbial community structure by revealing the presence, and to some extent, abundance, of the constituent species, based on the total extracted DNA from the original environmental sample. The DNA extracted from the sample is amplified using polymerase chain reaction (PCR), commonly targeted towards fragments of the genome which code for 16S rRNA. The 16S rRNA gene is used as it is highly conserved between bacterial species, but contains smaller variable regions. The phylogenetic character of the organism may be resolved with regards to its relationship with other bacteria (from the conserved region), and subsequently, more subtle variation in nucleotide sequence may be used to discriminate between different organisms at the genus/species level (Liu and Stahl, 2002). Indeed, all types of rRNA and DNA genes coding for rRNA can be used in DGGE, but the 16S is the most frequently used molecule as it is of sufficient sequence length (approximately 1,500 base pairs) to accurately infer phylogenetic type, but is smaller than the largest rRNA molecule (23S rRNA: 3,000 base pairs), hence is easier to use experimentally (Liu and Stahl, 2002). The PCR procedure is a necessary step to increase the number of DNA molecules present in the sample, so that the information may be quantified. It also facilitates the selection of a particular region of the genomic DNA for exploration, e.g. the 16S rRNA gene, and permits the incorporation of a 'GC-clamp' into the amplified DNA (amplicons) which improves the resolution limits of the DGGE, allowing nearly all variations in nucleotide sequences to be detected (Muyzer *et al.*, 1993) (refer to Chapter 3, for details of methods).

During DGGE, electrophoresis is used to separate the amplicons of the 16S rRNA gene, which are of similar nucleotide length, but vary with regards to sequence composition. This technique was initially proposed by Muyzer *et al.* (1993). The variations in nucleotide sequence, which reflect phylogenetic type, impart different

physical qualities to the DNA molecule, specifically regarding the temperature at which the DNA melts (T_m) (Gelsomino *et al.*, 1999). When the DNA melts, the helical structure separates and its electrophoretic mobility is retarded. Melting can also be mediated by chemical denaturants such as formamide or urea, using an electrophoresis gel prepared with a linear gradient of low denaturant concentration at the top of the gel to high concentration at the base of the gel (on a vertical axis, where samples of DNA are loaded at the top). During DGGE, the DNA fragments move through the gel under a constant temperature and electric current until the concentration of denaturant is sufficient for the DNA to melt. At this point, the helical structure disassociates and the migration of the DNA molecule in the gel is arrested. The degree of migration through the gel is determined by the melting point of the DNA, which is ultimately a function of the specific nucleotide sequence of that particular DNA molecule (or 16S rRNA gene) (Muyzer *et al.*, 1993). A sample of the total DNA extracted from a microbial community gives a series of bands, visible on the DGGE gel, where each band represents a particular phylogenetic type (Yoshie *et al.*, 2001). The resulting banding patterns from different samples are frequently used to explore spatial/temporal variations in microbial community structure in response to environmental factors or perturbations (e.g. Yoshie *et al.*, 2001; Boon *et al.*, 2002).

The banding patterns generally reflect the composition of only the most dominant species within the community, although this may include those species un-culturable using standard cultivation techniques (Boon *et al.*, 2002; Evans *et al.*, 2005 *article in press*). For a more comprehensive picture of the community, a nested PCR protocol involving sequential repetitions of PCR using more group specific primers may be necessary (Gelsomino *et al.*, 1999). However, there are experimental artefacts that may potentially lead to misinterpretation of the ecological implications from banding patterns, including (Gelsomino *et al.*, 1999; Liu and Stahl, 2002):

- Incomplete recovery of the total microbial DNA from an environmental sample, which may be exacerbated by the shearing or loss of DNA fragments during the cell disruption procedure;
- Production of chimeras during the PCR phase leading to false banding information (where bands do not represent a true bacterial type);
- PCR-bias producing artificially stronger appearance of some bands;
- The production of more than one band from a single bacterial type which possesses, for example, several copies of the 16S rRNA gene with slightly different sequences; conversely two separate bacterial types may have similar 16S rRNA genes that migrate at similar positions in the gel giving the appearance of only one bacterial type.

The likelihood of these processes introducing experimental errors is uncertain and may be incidental (Gelsomino *et al.*, 1999). Molecular methods for exploration of microbial ecology are under continual development, but PCR-DGGE remains one of the most commonly applied protocols and robust techniques for elucidating diversity information on microbial communities from environmental samples (Gelsomino *et al.*, 2002; Yoshie *et al.*, 2001). Moreover, the bands on the DGGE gels may be excised and purified to resolve sequence information, therefore, permitting the determination of the relationships between the sampled organisms and known bacterial species (i.e. phylogenetic characterisation). A combination of the PCR-DGGE and subsequent sequence resolution techniques was used in this project to characterise the microbial communities present in the denitrifying microcosms (methods presented in Chapter 3). The DGGE gels produced were used to compare inferred communities between samples and subsequently, to produce bands for sequencing.

7.2 RESULTS OF COMMUNITY COMPARISONS USING DGGE GEL BANDING DATA

Following the DGGE procedure, the gels produced using the VrVf primer pair were stained and photographed as described in Chapter 3. The banding pattern on the DGGE gel (Figure 7.1) was used to assess the similarity between different samples, using a combination of statistical methods based on a matrix of 'presence/absence' data compiled using the Quantity One® software package (BIO-RAD Laboratories, CA, US). The presence/absence data essentially describes whether a particular band is present in the sample or not, and scores it accordingly as either positive (+) or negative (-). This data was compared using two approaches:

(i). conventional statistical comparison of the difference in similarity data using ANOVA;
(ii). Comparison of similarity between samples using the Raup and Crick test, a similarity index calculated from a simulated probability distribution.

7.2.1 Analysis of sample DGGE banding patterns using ANOVA

Since the ANOVA requires samples of numeric values, the raw presence/absence data were transformed to scores of similarity between pairs of samples, according to the Dice equation:

$$\mathbf{D} = 2j / (a+b)$$ (e.g. Clegg *et al.*, 2003)

Where: $\mathbf{D}$ = Dice Coefficient

j = Number of shared bands

a = Number of bands in first sample in pair

b = Number of bands in second

The Dice coefficient values for microcosm Trials II and III are presented in Table 7.1 and Table 7.2, respectively.

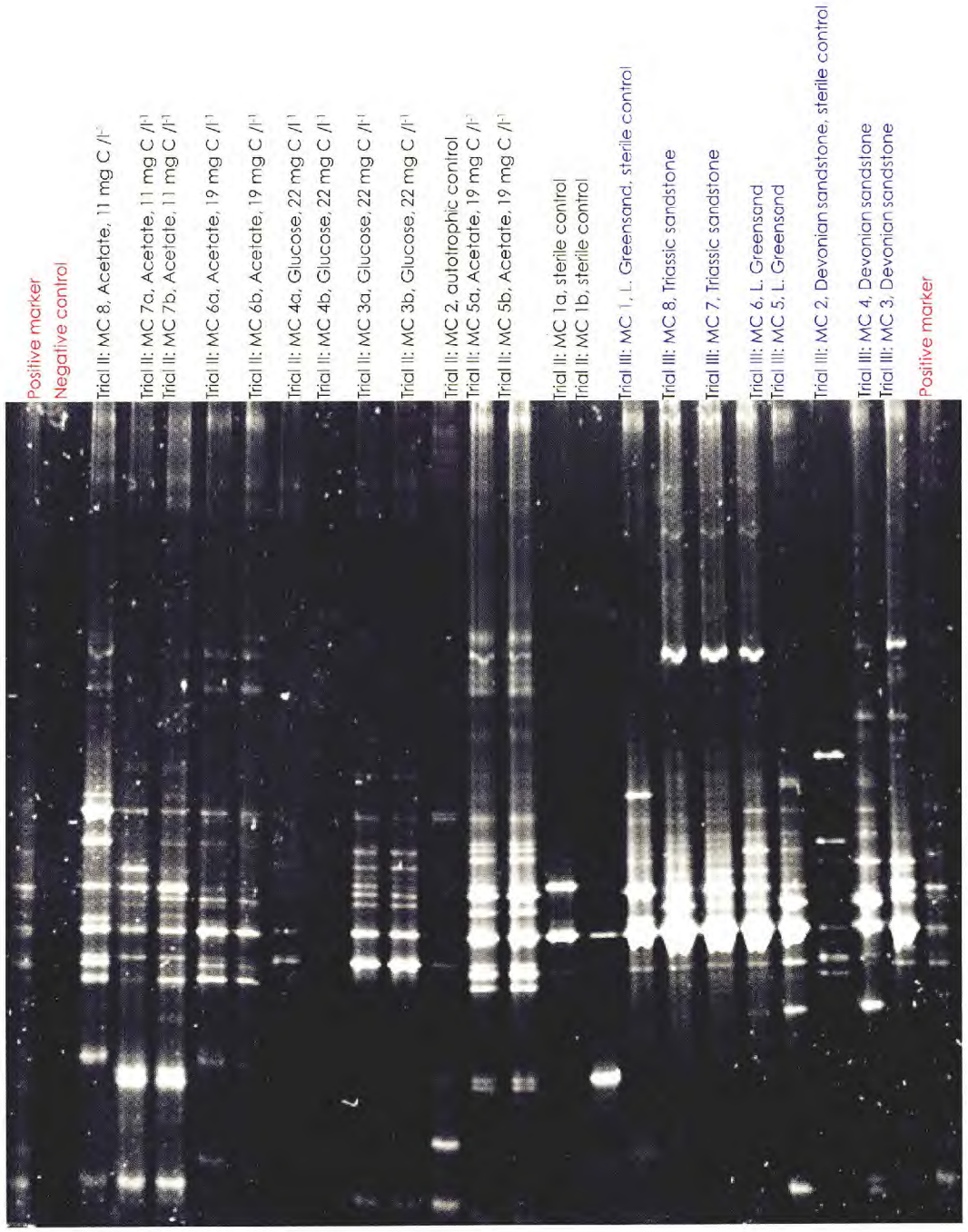


Figure 7.1: DGGE gel produced from DNA extracted from denitrifying microcosm communities and amplified using a nested PCR with pApH and VrVf primers

Table 7.1: Dice Coefficients (% similarity) for pair-wise comparisons of banding patterns for samples from microcosm Trial II: Microcosms prepared using Triassic sandstone and dosed with difference OC substrates and concentrations (see footnote)

SAMPLE ID CODES	Ac 11 MC8	Ac 11 MC7a	TS Ac 11 MC7b	Ac 19 MC6a	Ac 19 MC6b	G 22 MC4a	G 22 MC4b	G 22 MC3a	G 22 MC3b	autotrophic control MC2	Ac 19 MC5a	Ac 19 MC5b	sterile control MC1a
Ac 11 MC7a	35.3												
Ac 11 MC7b	48.8	64.3											
Ac 19 MC6a	62.7	33.4	53.1										
Ac 19 MC6b	42.4	19.2	41.2	70.5									
G 22 MC4a	37.6	35.4	45.8	43.4	31.3								
G 22 MC4b	23.1	7.6	11.1	14.2	7.1	17.7							
G 22 MC3a	45.6	26.1	44.7	56.5	45.3	55.2	12.9						
G 22 MC3b	48.7	27.7	42.4	57.1	44.0	51.4	17.6	87.5					
autotrophic control MC2	19.4	31.5	24.0	15.4	10.7	23.5	5.7	24.7	22.2				
Ac 19 MC5a	54.1	36.8	50.0	68.1	57.1	34.9	8.8	54.8	52.3	16.7			
Ac 19 MC5b	55.0	39.6	50.6	66.9	54.0	35.9	8.9	52.4	52.3	17.1	92.4		
sterile MC1a	17.4	14.2	9.3	24.7	25.1	15.0	0.0	30.0	31.1	6.7	26.5	29.1	
sterile control MC1b	5.7	6.7	4.1	6.0	6.1	8.8	0.0	6.5	7.0	12.4	7.6	8.2	14.2

Key: (Ac) denotes acetate; (G) denotes glucose; (11), (19), (22) denotes concentration (mg C l^{-1}); (MC) refers to microcosm identity number used throughout experiment and (a) or (b) indicates that two replicate sand samples were taken from an identical microcosm, and were labelled alphabetically

Table 7.2: Dice Coefficients (% similarity) for pair-wise comparisons of banding patterns for samples from microcosm Trial III: microcosms prepared using three different sandstone types and fed with sodium acetate at a concentration of 35 mg C l^{-1}

SAMPLE ID CODES	Lower Greensand sterile control MC 1	Triassic sandstone MC 8	Triassic sandstone MC 7	Lower Greensand MC 6	Lower Greensand MC 5	Devonian sandstone sterile control MC 2	DS Ac 35 MC4
Triassic sandstone MC 8	59.2						
Triassic sandstone MC 7	62.9	74.0					
Lower Greensand MC 6	55.0	70.9	80.1				
Lower Greensand MC 5	43.3	58.4	58.3	62.2			
Devonian sandstone sterile control MC 2	28.8	29.6	27.7	25.4	29.6		
Devonian sandstone MC4	37.7	48.4	47.9	48.7	66.7	26.5	
Devonian sandstone MC3	54.7	54.9	61.0	55.2	57.9	27.6	58.1

Key: Triassic sandstone = low Fe and Mn content; Lower Greensand = medium Fe and Mn content; Devonian sandstone = high Fe and Mn content (refer to Chapters 2 and 6)

Using the Dice Coefficients of similarity, data sets were compiled for statistical comparison using one-way ANOVA, as follows:

- Similarity values for duplicate sample pairs of the same microcosm (termed biological replicates, BRs), compared to the similarity values between pairs of samples taken from replicate microcosms of the same treatment (microcosm replicates, MRs);
- Similarity values from MR pairs compared to similarity values obtained from pairs of samples from different treatments (DTs), (data from Trial II, therefore considers only the differences in substrate type/substrate dose);
- Similarity values between pairs of samples fed 19 mg acetate-C l^{-1}, compared to similarity values between pairs of samples fed 22 mg glucose-C l^{-1} (i.e. two different substrates at similar OC concentrations);
- Similarity values between acetate microcosms paired with the autotrophic control, compared to similarities between glucose microcosms paired with the autotrophic control;
- Similarity between microcosms of the same sandstone, compared to similarities between microcosms of different sandstones.

The ANOVAs were completed using MINITAB (14) Statistical software. Prior to carrying out the one-way ANOVA tests, the data were checked for normality which revealed that there were potential outliers in the data set. After removing the outliers, which were data points involving the samples: MC 1a, MC 1b and MC 4b (Trial II), the similarity data was normally distributed. Visual inspection of the DGGE gel (Figure 7.1) showed that the samples which were removed as outliers had very few or no bands.

The P values of the ANOVA tests (without outliers) and mean similarities for data sets are detailed in Table 7.3. The results of the ANOVAs are summarised as follows:

- There was a significant difference in the similarities of biological replicates (BR) compared to microcosm replicates (MR), $P < 0.01$;
- There was a highly significant difference in the similarity between microcosm replicates (MR) compared to the similarity between samples from different treatments (DT), $P < 0.001$ (MR were more similar);
- The similarities between acetate (19 mg C l^{-1}) and glucose (22 mg C l^{-1}) treatments were not significantly different, $P > 0.05$;
- The degree of similarity between acetate (19 mg C l^{-1}) samples paired with the autotrophic control sample, compared to glucose (22 mg C l^{-1}) sample paired with the autotrophic control was significantly different, $P < 0.01$. The

glucose/autotrophic pairs were more similar than the acetate/autotrophic pairs;

- There was no significant difference between the similarities of microcosms filled with the same sandstone, compared to similarities of microcosms filled with different sandstones, $P > 0.05$.

Table 7.3: ANOVA results from comparisons of similarities in the microbial communities (implied from DGGE banding pattern) of various microcosm samples. (BR) denotes biological replicates, (MR) denotes microcosm replicates and (DT) denotes different treatment types, (defined further on p 181)

Comparison	P value (95 %)	Means of data sets
Similarities between BR vs Similarities between MR	<0.01	78.7 57.4
Similarities between MR vs Similarities between DT	<0.001	57.37 43.8
Similarities between acetate treatments (19 mg C l^{-1}) vs Similarities between glucose treatments (22 mg C l^{-1})	0.763	68.17 64.7
Similarities between acetate treatments (19 mg C l^{-1}) and autotrophic controls vs Similarities between glucose treatments (22 mg C l^{-1}) and autotrophic controls	<0.01	15.0 23.5
Similarities between microcosms filled with the same sandstone vs Similarities between microcosms filled with different sandstones	0.364	64.8 59.0

The implications of these statistical analyses are interpreted and compared to the findings of the alternative, Raup and Crick analysis, in Section 7.2.3.

7.2.2 Analysis of sample DGGE banding patterns using Raup and Crick Index

The Raup and Crick test was conceived for comparison of faunal similarity between different geographic locations (Raup and Crick, 1979) using presence/absence data. Since the test is based on presence/absence of biological taxa, it is also applicable to the analysis of DGGE banding patterns, for example as applied by Rowan *et al.*, (2003). In the case of these experiments, the aim of the DGGE analysis was to determine if different microcosm treatments, such as substrate type or sandstone type, had an effect on microbial community assemblage.

Conventional statistical analysis has two outcomes (at a specified α): H_0 is upheld; there is no significant difference between samples; H_0 is rejected; there is a significant difference between samples. Therefore, two compared communities would be determined as either similar (H_0 upheld) or dissimilar (H_0 rejected). The Raup and Crick test additionally considers that the two communities are randomly assembled, and that the similarity or dissimilarity between the communities may therefore be influenced by chance. In effect, the Raup and Crick test has three outcomes:

- two samples are significantly similar;
- two samples are significantly dissimilar;
- two samples are no more similar (or dissimilar) than would be expected from chance occurrence alone.

The latter outcome defines the null hypothesis for the Raup and Crick test: the degree of similarity measured between samples can be accounted for by the chance occurrence of the same two species in each sample (e.g. Rowan *et al.*, 2003). The incorporation of a chance element in the Raup and Crick test prevents common or widespread biological taxa from having a disproportionate influence on the measurement of similarity (Raup and Crick, 1979). This is relevant to the comparison of the microcosm communities, where microorganisms capable of denitrification are ubiquitous in the environment (Hawker and Linton, 1971) and, moreover, the same bulk source of sandstone was used to fill the microcosms in the majority of experiments.

7.2.2.1 Description of Raup and Crick Method

The Raup and Crick similarity index (S_{RC}) was calculated from presence/absence data transformed to binary digits using pooled information from microcosm Trial II and microcosm Trial III (i.e. every sample in the DGGE gel was compared to all other samples). The Raup and Crick test uses a random sampling method to simulate two samples from the pooled data set (K_{exp}), and these are compared to determine the number of common taxa (or in this case, common bands). This process is repeated 200 times by the statistical software used to complete the test and a frequency distribution graph of the results is compiled, as depicted schematically in Figure 7.2. The frequency distribution from the randomly generated comparisons (K_{exp}) is used as a probability distribution curve against which the similarities between real samples (K_{obs}) are compared. The reported similarity index (S_{RC}) is defined as the probability that the similarity between simulated samples is greater than or equal to the actual similarity between the real samples, i.e.: $S_{RC} = P\ K_{exp} \geq K_{obs}$, (e.g. Rowan *et al.*, 2003) and S_{RC} will have a value between 0.0 and 1.0.

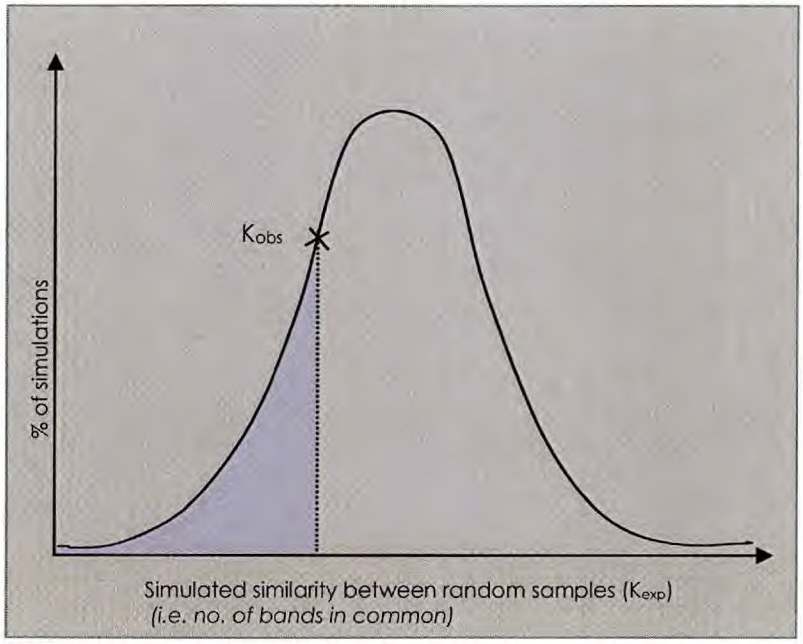


Figure 7.2: Schematic of frequency distribution curve used to compare K_{exp} with K_{obs}, after Raup and Crick, (1979). S_{RC} = the sum of K_{exp} which are $\leq K_{obs}$, i.e. those values which fall within the blue area. The S_{RC} is taken as a P value and of between 0.0-1.0

It is anticipated that the majority of sample-pair comparisons (K_{obs}) have a similarity of no greater than can be expected by chance alone, i.e., the similarity value S_{RC} will be between the 5 % tails of the K_{exp} frequency distribution generated from the random sampling simulations, shown in Figure 7.3 as 0.05 and 0.95 intervals (Raup and Crick, 1979). Conversely, S_{RC} values within the 5 % tails, contravene the null hypothesis, and it is assumed that the similarity between the two samples (K_{obs}) is either greater than or less than can be accounted for by chance alone. Similarity (S_{RC}) values greater than 0.95 are described as being 'significantly similar,' whereas S_{RC} less than 0.05 are considered 'significantly dissimilar.' In each of these cases, it is proposed that deterministic community selection has occurred (e.g. Rowan *et al.*, 2003).

The Raup and Crick similarity values for pair-wise comparison of microcosm samples were calculated using PAST statistical software (Hammer *et al.*, 2001). The results (S_{RC} values) are presented graphically in Figures 7.4 and 7.5, respectively, to enable visual inspection of the complete data set and additionally, have been tabulated for comparison on individual sample pairs in Tables 7.4 and 7.5. Once the S_{RC} values were compiled for the relevant sample combinations, the values were used to assess the similarity between single-microcosm replicate samples, between microcosm

replicate samples, and finally, between treatment types (denoted as BR, MR and DT respectively in the ANOVA tests).

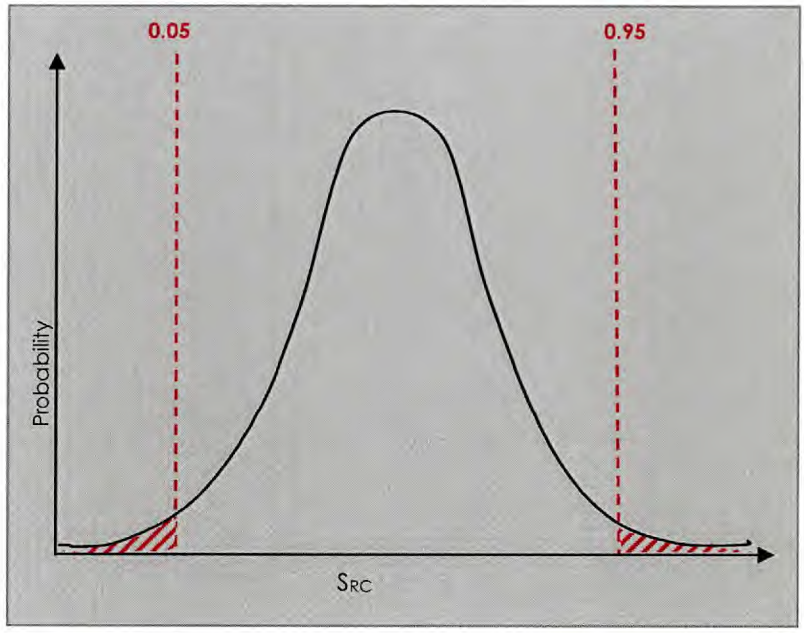


Figure 7.3: Conceptual probability distribution, used in the estimation of the Raup and Crick similarity index, showing 5 % tails at lower and upper ends of the distribution as shaded areas. $S_{RC} < 0.05$ indicates that the samples are significantly dissimilar; $S_{RC} > 0.95$ indicates that the samples are significantly similar; $0.05 < S_{RC} < 0.95$ indicates that the degree of similarity between the two samples may be accounted for by chance

7.2.2.2 Raup and Crick Similarity Results

The Raup and Crick comparisons showed that duplicate microcosm samples (BR, Trial II, e.g. MC 7a compared to MC 7b etc.) were generally significantly similar to each other ($S_{RC} = 1.0$). Exceptions to this were duplicate samples taken from the sterile control microcosm (MC 1a and MC 1b) and duplicates from MC 4 (G 22 mg C l^{-1}, MC 4a and MC 4b). The S_{RC} values were 0.91 and 0.94 for [MC 1a/MC 1b] and [MC 4a/MC 4b] respectively, therefore, the H_0 was rejected by a relatively small margin, as can be seen from Figure 7.4. These samples were also identified as being outliers, during the conventional DICE and ANOVA analysis of the DGGE banding patterns (Section 7.2.1).

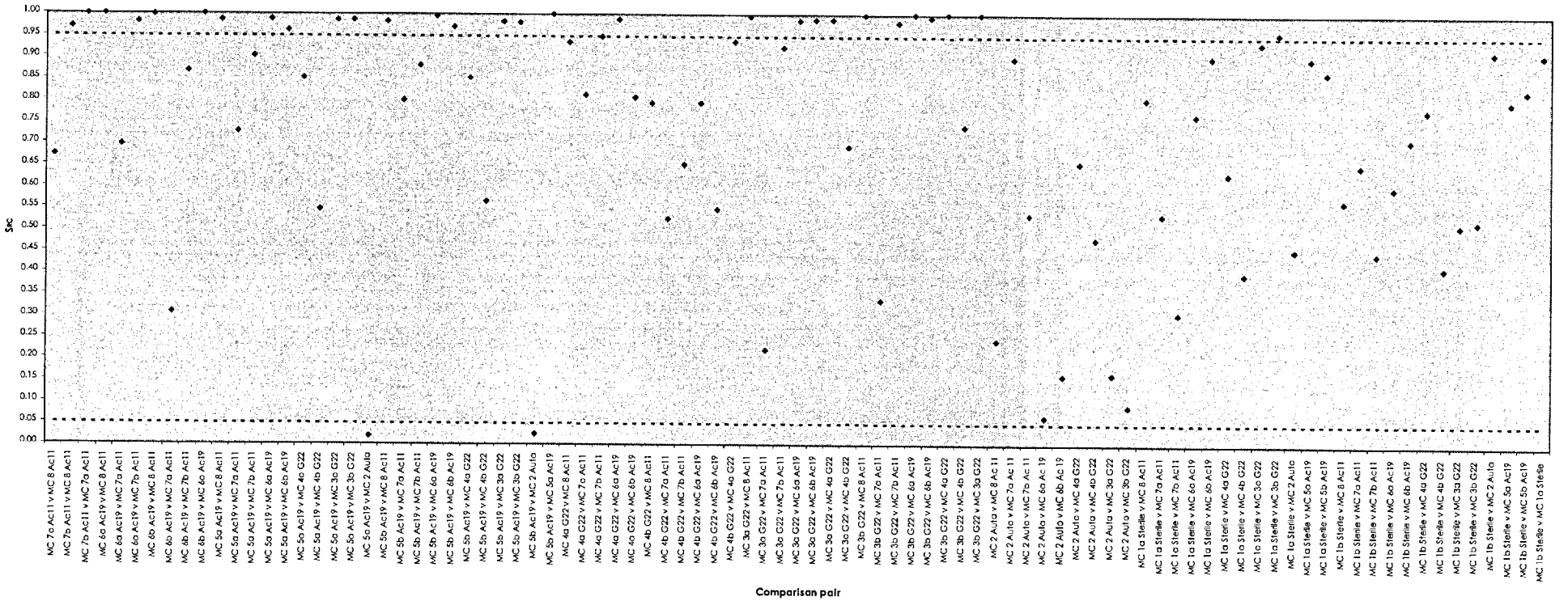


Figure 7.4: Pair-wise comparisons of Raup and Crick similarity values (S_{RC}) between samples collected during Trial II (using Triassic sandstone and various OC treatments). S_{RC} value < than 0.05 denotes significant dissimilarity; S_{RC} > 0.95 denotes significant similarity; 0.05 < S_{RC} < 0.95 denotes similarity no greater than would be expected by chance (H_0 upheld). Microcosm sample codes: (Ac) denoted acetate, (G) denotes glucose and numbers next to these initials denote concentration (mg C l^{-1})

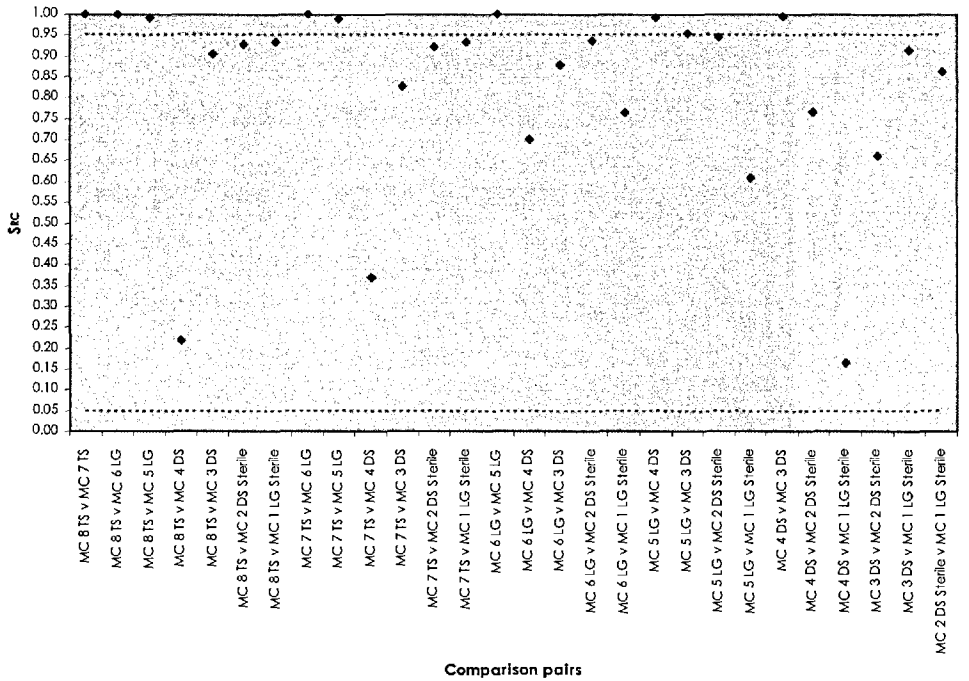


Figure 7.5: Pair-wise comparisons of Raup and Crick similarity values (S_{RC}) between samples collected during Trial III, fed acetate at 35 mg C l^{-1}. S_{RC} value < than 0.05 denotes significant dissimilarity; S_{RC} > 0.95 denotes significant similarity; 0.05 < S_{RC} < 0.95 denotes similarity no greater than would be expected by chance (H_0 upheld). Microcosm sample codes: (TS) denotes microcosms filled with Triassic sandstone, (LG) denotes Lower Greensand and (DS) denotes Devonian sandstone.

Table 7.4: Summary of S_{RC} for microcosm samples collected during Trial II (using Triassic sandstone and various OC types). Bold type indicates significant similarity **(S_{RC} >0.95)** or dissimilarity **(S_{RC} <0.05)**. Microcosm sample codes: (Ac) denoted acetate, (G) denotes glucose and numbers next to these initials denote concentration (mg C l^{-1})

SAMPLE ID	Ac 11 MC7a	Ac 11 MC7b	Ac 19 MC6a	Ac 19 MC6b	G 22 MC4a	G 22 MC4b	G 22 MC3a	G 22 MC3b	autotrophic control MC2	Ac 19 MC5a	Ac 19 MC5b	sterile control MC1a	sterile control MC1b
Ac11 MC8	0.67	**0.97**	**1.00**	**1.00**	0.94	0.80	**1.00**	**1.00**	0.24	**0.99**	**0.98**	0.80	0.57
Ac 11 MC7a		**1.00**	0.70	0.31	0.81	0.53	0.22	0.34	0.90	0.73	0.80	0.54	0.65
Ac 11 MC7b			**0.98**	0.87	**0.95**	0.55	0.92	**0.98**	0.54	0.90	0.88	0.31	0.45
Ac 19 MC6a				**1.00**	**0.99**	0.80	**0.99**	**1.00**	0.07	**0.99**	**1.00**	0.77	0.60
Ac 19 MC6b					0.81	0.55	**0.99**	**0.99**	0.16	**0.96**	**0.97**	0.90	0.71
G 22 MC4a						0.94	**0.99**	**1.00**	0.66	0.85	0.85	0.63	0.78
G 22 MC4b							0.69	0.74	0.48	0.55	0.57	0.40	0.41
G 22 MC3a								**1.00**	0.17	**0.99**	**0.98**	0.93	0.51
G 22 MC3b									0.09	**0.99**	**0.98**	**0.96**	0.52
autotrophic contol MC2										0.02	0.03	0.45	0.91
Ac 19 MC5a											**1.00**	0.90	0.80
Ac 19 MC5b												0.87	0.83
sterile control MC1a													0.91

Table 7.5: Summary of S_{RC} for microcosm samples collected during Trial III (fed acetate at 35 mg C l^{-1}). Bold type indicates significant similarity **(S_{RC} >0.95)** or dissimilarity **(S_{RC} <0.05)**. Microcosm sample codes: (TS) denotes microcosms filled with Triassic sandstone, (LG) denotes Lower Greensand and (DS) denotes Devonian sandstone

SAMPLE ID	TS MC7	LG MC6	LG MC5	DS sterile control MC2	DS MC4	DS MC3
LG sterile control MC1	0.93	0.77	0.61	0.86	0.17	0.91
2003 TS MC8	**1.00**	**1.00**	**0.99**	0.93	0.22	0.91
TS MC7		**1.00**	**0.99**	0.92	0.37	0.83
LG MC6			**1.00**	0.94	0.70	0.88
LG MC5				**0.95**	**0.99**	**0.96**
DS sterile control MC2					0.77	0.66
DS MC4						**1.00**

Comparison of samples taken from different microcosm but of identical treatments (e.g. treatment = glucose 22 mg C l^{-1}, replicate treatment microcosms = MC 3 and MC 4), including data from both Trial II and Trial III, also showed that the S_{RC} was significantly similar (S_{RC} >0.95) in most cases, for example as listed overleaf:

Trial II:

- Acetate 11 mg C l^{-1} treatment replicates (MC 7a,b and MC 8): S_{RC} = 0.67 and **0.97**,
- Acetate 19 mg C l^{-1} treatment replicates (MC 5 a, b and MC 6 a, b): S_{RC} = **0.99**, **1.0**, **0.96** and **0.97**,
- Glucose 22 mg C l^{-1} treatment replicates MC 3 a, b and MC 4 a (MC 4b removed from data set as outlier): S_{RC} = **0.99**, **1.0**,

Trial III:

- Devonian sandstone treatment replicates (MC 3 and 4): S_{RC} = **1.0**,
- Lower Greensand treatment replicates (MC 5 and MC 6) S_{RC} = **1.0**,
- Triassic sandstone treatment replicates (MC 7 and MC 8): S_{RC} = **1.0.**

The final comparisons were made between different treatments, which were the main comparisons of interest, for example, comparing MC 5 from Trial II (19 mg acetate-C l^{-1}) with MC 3 from Trial II (22 mg glucose-C l^{-1}) and so on. The results of the comparisons between treatment type (Figure 7.6) demonstrated that the majority of comparisons between treatment types showed no greater similarity than would be expected to occur by chance ($0.05 < S_{RC} < 0.95$). However, in some cases, comparison between two different treatment types demonstrated a significant similarity ($S_{RC} > 0.95$) these included comparisons between:

Trial II:

- Acetate 11 mg C l^{-1} and acetate 19 mg C l^{-1} (5/12 comparison pairs were similar),
- Acetate 11 mg C l^{-1} and glucose 22 mg C l^{-1} (3/9 comparison pairs were similar),
- Acetate 19 and glucose 22 mg C l^{-1} (9/11 comparison pairs were similar)

Trial III:

- Triassic sandstone and Lower Greensand (4/4 comparison pairs were similar),
- Lower Greensand and Devonian sandstone (2/4 comparison pairs were similar).

Moreover, in addition to these similarities, the data from microcosm Trial II also showed that there was a significant dissimilarity ($S_{RC} < 0.05$) between samples from MC 5 (Acetate 19 mg C l^{-1}) compared to the sample from MC 2 (autotrophic control).

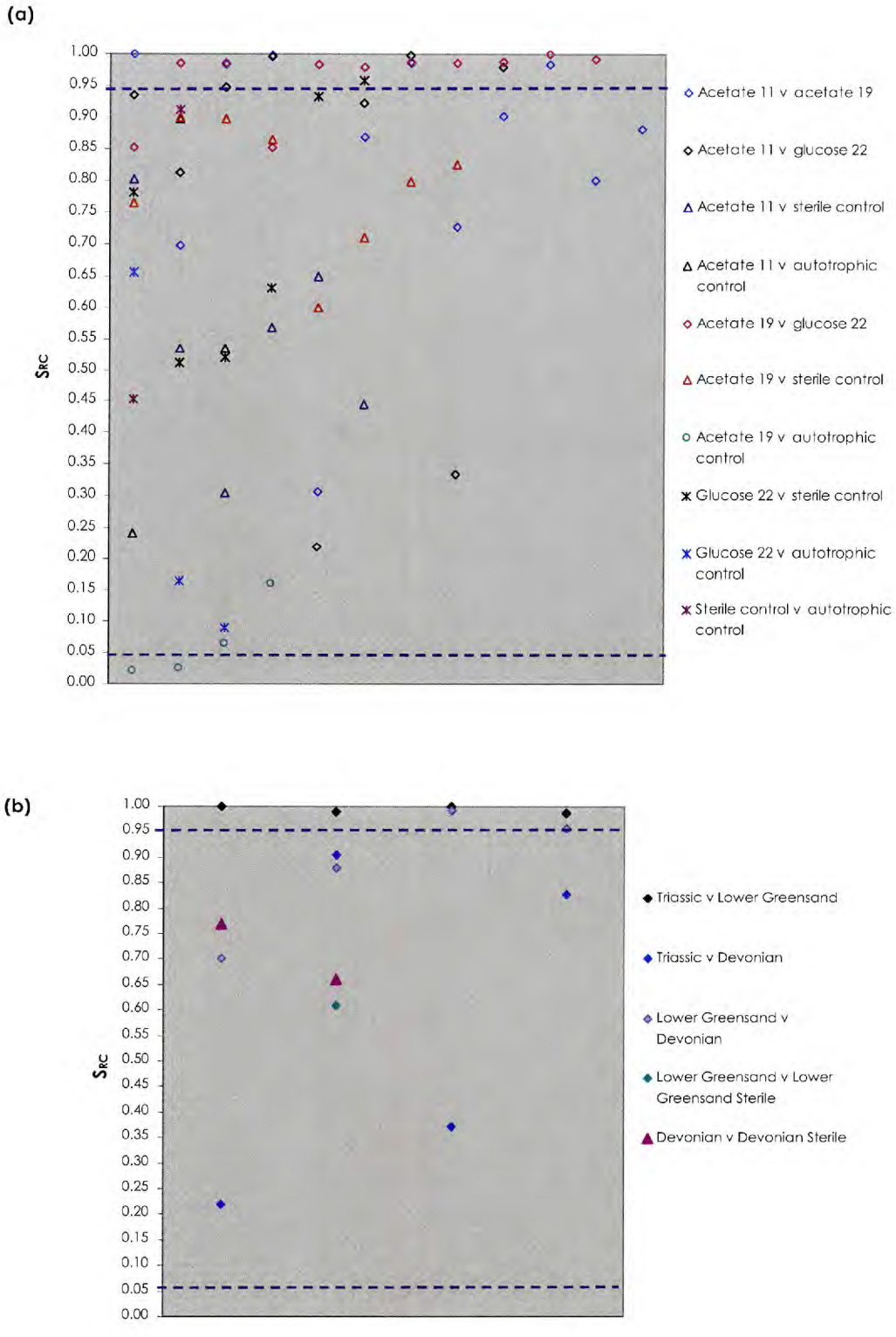


Figure 7.6: Raup and Crick similarity values (S_{RC}) for comparisons of different treatment types used during Trial II **(a)**; and Trial III **(b)**, with comparisons shown in legend. S_{RC} value <0.05 denotes significant dissimilarity; S_{RC} >0.95 denotes significant similarity; $0.05 < S_{RC} < 0.95$ denotes similarity no greater than would be expected by chance (H_0 upheld). Numbers in the legend for Trial II (a), indicate the concentration (mg C l^{-1}), the sample descriptions in the legend for Trial III describes the comparison of microcosms prepared with different sandstone types (all fed with acetate at a concentration of 35 mg C l^{-1})

7.2.3 Summary of Statistical comparisons of DGGE banding patterns

The ANOVA test of similarity values for biological replicates compared to microcosm replicates showed a significant difference between the means of the two data sets ($P < 0.01$). In this case, the biological replicates showed a greater degree of similarity than the samples from microcosm replicates, as would be anticipated, thus demonstrating the reproducibility of the DNA extraction and PCR-DGGE technique. This result implied that there was a degree of variation between replicate microcosms receiving the same treatment, which reflected the minor differences in denitrification chemistries observed between microcosm replicates (refer to Chapter 4). However, though the denitrification (C and N) concentration profiles for acetate treatments appeared more consistent between replicates compared to glucose treatments (Chapter 4), the degree of similarity between banding patterns of the acetate treatments (19 mg C l^{-1}) was not significantly different from the similarity observed between glucose treatments (22 mg C l^{-1}).

The Raup and Crick analysis found that biological replicates (BR) and microcosm replicates (MR) were more similar than could be expected by chance, (S_{RC} values > 0.95). From an ecological perspective, the Raup and Crick statistic infers deterministic similarity (S_{RC} >0.95), where external factors have influenced the structure of two communities in some way as to increase the degree of similarity between the two (e.g. Rowan *et al.*, 2003). Correspondingly, communities with significantly dissimilar S_{RC} values (< 0.05) are thought to have been caused to diverge by external factors, and communities with no more similarity than could be expected by chance are described as randomly assembled. In this regard, samples taken from replicate microcosms of the same treatment were deterministically similar, suggesting that treatment type caused microbial community structures from individual microcosms to become convergent.

Subsequently, an ANOVA test of similarities between microcosm replicates (MR) and similarities between different OC treatments (DT), showed a highly significant difference ($P < 0.001$) between the two data sets. The mean similarity between microcosm replicates was higher than the mean similarity between microcosms of different treatments (i.e. the microcosm replicates were more similar). In this instance, the ANOVA included only samples from the non-sterile microcosms amended with an OC source; therefore the type of OC treatment had a significant effect on the microbial communities in samples elucidated by the PCR-DGGE procedure.

The equivalent Raup and Crick indices amongst different treatments found that 41 % of comparisons between treatments: acetate 11 mg C l^{-1}/acetate 19 mg C l^{-1} were similar and 33.3 % of comparisons between acetate 11 mg C l^{-1}/glucose 22 mg C l^{-1} were similar. In these instances, where acetate and glucose samples from higher concentration treatments (19 and 22 mg C l^{-1} respectively,) were compared to the lower concentration acetate treatment (11 mg C l^{-1}), it was shown that the majority of the sample comparisons were found to be no more similar than could be expected by chance. However, when the higher acetate and glucose treatments were compared to each other (acetate 19 mg C l^{-1}/glucose 22 mg C l^{-1}), the Raup and Crick test found that 81.8 % of comparisons were significantly similar.

This reflected the statistical results presented in Chapter 4 (Section 4.3.5), regarding mean concentration of NO_3-N denitrified during the course of the experiment. For example, significant differences were detected between the acetate (19 mg C l^{-1}) and glucose (22 mg C l^{-1}) treatments compared to the acetate (11 mg C l^{-1}) treatment, respectively; conversely, no significant difference was found between acetate (19 mg C l^{-1}) compared to glucose (22 mg C l^{-1}). Therefore, the highly significant difference between communities from microcosms receiving different treatments may be ultimately due to the degree of denitrification activity that occurred within the microcosms.

An ANOVA comparing the similarity between the autotrophic control microcosm and acetate (19 mg C l^{-1}) and glucose (22 mg C l^{-1}) treatments, respectively, found that there was a significant difference in the two data sets ($P < 0.05$). In this instance, the similarity between glucose and autotrophic treatments was higher than the similarity between the acetate and autotrophic treatments, suggesting that the communities from the acetate and autotrophic treatments had diverged to a greater extent. Raup and Crick comparisons also highlighted significant dissimilarity ($S_{RC} < 0.05$), between the autotrophic control (MC 2, Trial II) and replicate samples from MC 5 (a, b) (acetate 19 mg C l^{-1}). This inferred that the addition of acetate (19 mg C l^{-1}) to MC 5 (Trial II) caused the community to become more dissimilar to the microcosm which received no OC, than would have been expected by chance. It is interesting, however, that the other replicate microcosm of this treatment pair (i.e. MC 6, 19 mg C l^{-1}, Trial II) was not significantly dissimilar to the autotrophic control.

In the samples collected during Trial III, where microcosms received identical OC amendments (acetate, 35 mg l^{-1}), but were prepared using different sandstones

(Lower Greensand and Triassic and Devonian sandstones), there was no significant difference ($P < 0.05$) in the similarities between any of the microcosms (excluding the sterile controls). Therefore, under a similar OC treatment regime, variation in sandstone type did not have a significant effect on the similarities of microbial communities within the microcosms.

The Raup and Crick comparisons between the various sandstone types also demonstrated that there were no significant dissimilarities between treatments.

7.3 ANALYSIS OF SEQUENCE DATA OBTAINED FROM DGGE GELS

7.3.1 Similarity of microcosm sample-Sequences to Known organisms

To characterise the microbial community recovered during the PCR/DGGE procedure, selected bands from the DGGE gels were excised and sent to a specialist laboratory for sequencing, as described in Chapter 3. Bands were excised from gels produced using both VrVf and pDpF primer pairs, as shown in Figure 7.7 (sample ID codes are described in the accompanying Table 7.6).

The sequence data from bands excised from both VrVF and pDpF gels, which was elucidated as a series of bases, were used to characterise the microbial community present in the samples by grouping the sequences with their 'nearest neighbours.' This was carried out by comparing the raw sequence data from the microcosm samples with known sequences, catalogued in the EMBL-EBI international database (http:/www.ebi.ac.uk) using the software package FASTA 3 (Pearson and Lipman, 1988). The details of the samples collected for sequencing and the nearest neighbours obtained are presented in Table 7.6 (band identity numbers refer to the annotations in Figure 7.7).

As can be observed from Figure 7.7, many of the bands that were amplified during the PCR procedure were common to all samples. The majority of the sequences recovered from the microcosm samples were similar to sequences of *β-proteobacteria,* predominantly species of the *Acidovorax* and *Burkholderia*. Additionally, sequences similar to *γ-proteobacteria* of the *Pseudomonas* genus were also present in the microcosm samples. Most of the species listed in the EMBL database were isolated from environmental samples, including contaminated sites and from activated sludge, as described in Table 7.6.

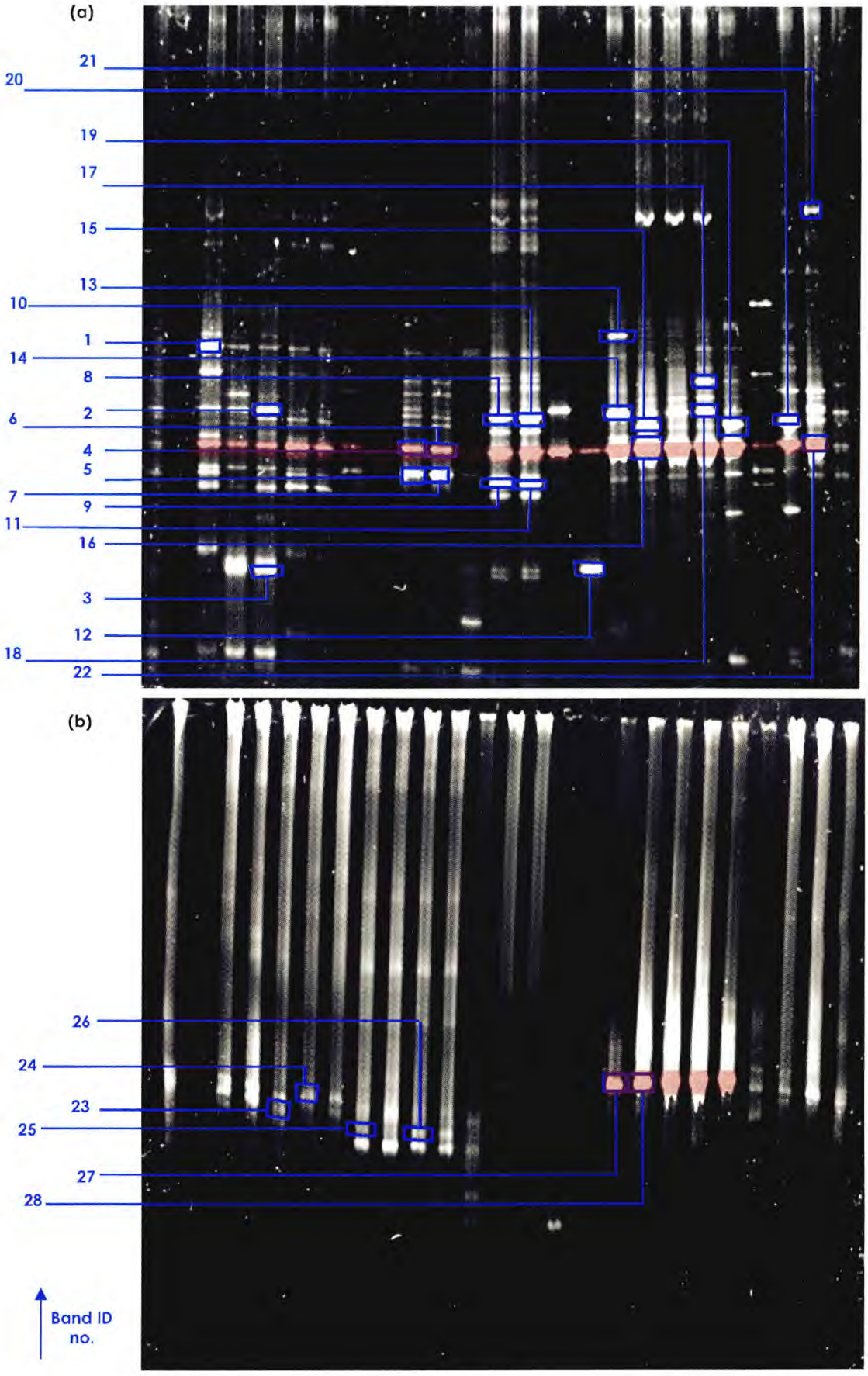


Figure 7.7: DGGE gels produced from PCR using **(a)** VrVf primers and **(b)** pDpF primers; highlighted bands were excised for sequencing, details presented in Table 7.6 according to the band identity number. Red shading highlights common band: *Acidovorax spp.*

Table 7.6: Nearest neighbours of the sequences obtained from DGGE gels performed with VrVf primers (band IDs 1-22) and pDpF primers (band IDs 23-28). Band identities correspond to the bands depicted in Figure 7.7

Band ID no.	Sample description	Nearest neighbours	Accession no.	Similarity between sample and 'neighbour'	Origin of neighbour
1	Trial II, MC 8, acetate – 11 mg C l^{-1}	Uncultured bacterium AT425	AY053477	99.4	Bacteria and Archaea physically associated with Gulf of Mexico gas hydrates
2	Trial II, MC 7b, acetate – 11 mg C l^{-1}	*Pseudomonas sp.* 5S2.F11	AY043562	99.3	British Columbia forest soils subjected to disturbance, from mature forest mineral soil
3	Trial II, MC 7b, acetate – 11 mg C l^{-1}	*Burkholderia sp.* NCPPB3025	AJ300684	98.7	UK, woodland rhizosphere environments, associated with *Allium cepa* (onion)
4	Trial II, MC 3a, glucose – 22 mg C l^{-1}	ultramicrobacterium strain D-7	AB008505	100	aerobic copiotrophic ultramicrobacteria from urban soil
		and			
		ultramicrobacterium strain MY14	AB008503	100	-
5	Trial II, MC 3a, glucose – 22 mg C l^{-1}	Uncultured beta proteobacterium clone CLi13	AF529324	99.4	PCE-contaminated site
		Uncultured bacterium clone SRS62DBA03	AF389436	99.4	Chlorinated-ethene-contaminated landfill groundwater
6	Trial II, MC 3b, glucose – 22 mg C l^{-1}	ultramicrobacterium strain D-7	AB008505	100	PAH contaminated soil, US
		ultramicrobacterium strain MY14	AB008503	100	"
		Janthinobacterium agaricidamnosum W1r3T	Y08845	100	Soft rot pathogen isolate
7	Trial II, MC 3b, glucose – 22 mg C l^{-1}	Uncultured beta proteobacterium clone CLi13	AF529324	98.7	PCE contaminated site
8	Trial II, MC 5a, acetate – 19 mg C l^{-1}	Uncultured gamma proteobacterium clone FTL 213	AF529099	100	TCE contaminated site
9	Trial II, MC 5a, acetate – 19 mg C l^{-1}	Uncultured *Dugannella* clone MT18	AF058385	99.3	Isolated from a negative DNA extraction control during mixed population studies
10	Trial II, MC 5b, acetate – 19 mg C l^{-1}	Uncultured gamma proteobacterium clone FTL 213	AF529099	100	TCE contaminated site
11	Trial II, MC 5b, acetate – 19 mg C l^{-1}	Uncultured *Dugannella* clone MT1B	AF058385	99.4	Isolated from a negative DNA extraction control during mixed population studies
		Uncultured eubacterium clone IAFR110	AF270945	99.4	-
12	Trial II, MC 1b, sterile control	Soil isolate KBS-EC4	AF183152	99.3	Long term ecological soil research site, Michegan, USA
13	Trial III, Lower Greensand, sterile control	Uncultured Acidobacterium clone 351B	AY571792	95.5	hydrocarbon-contaminated soil, Antarctica:Southern Victoria Land, Ross Island,
14	Trial III, Lower Greensand, sterile control	Uncultured gamma proteobacterium clone FLpost17	AF529132	96.6	TCE site undergoing ISBD treatment
15	Trial III, MC 3, Triassic sandstone	Uncultured gamma proteobacterium	AJ583182	84.8	ground water from a monitoring deep-well at a radioactive waste disposal site, Tomsk-7, Russia

Table 7.6 cont.

Band ID no.	Sample description	Nearest neighbours	Accession no.	Similarity between sample and 'neighbour'	Origin of neighbour
16	Trial III, MC 3, Triassic sandstone	Uncultured eubacterium clone IAFR110	AF270945	100	Related to *Acidovorax* spp. isolated from soil activated with pentachlorophenol (PCP) or with a wood preservative mixture containing PCP
17	Trial III, MC 6 Lower Greensand	*Pseudomonas frederiksberge,*	AJ249382	94.6	Isolated from soil at a coal gasification site, Denmark
		Pseudomonas borealis,	AJ012712	94.6	Isolated from pristine and contaminated soils, deep sediments and phytopathogenic isolates
		and other *Pseudomonas spp.*			
18	Trial III, MC 6 Lower Greensand	*Pseudomonas mephitic,*	AB021388	92.4	-
19	Trial III, MC 5 Lower Greensand	Uncultured gamma-proteobacterium	AJ583182	93.7	Ground water from a monitoring deep-well at a radioactive waste disposal site, Tomsk-7, Russia
		Acinetobacter sp. Phenon 1 strain LUH 4763	AJ275039	93.7	Isolated from human clinic specimen
20	Trial III, MC 4 Devonian sandstone	Uncultured gamma-proteobacterium	AJ583182	97.5	Ground water from a monitoring deep-well at a radioactive waste disposal site, Tomsk-7, Russia
21	Trial III, MC 3 Devonian sandstone	*Pseudomonas fluorescens* strain Q2-87	AJ278814	99.4	Pure culture
		Pseudomonas corrugata	AF348508	99.4	Isolated from sugar beet rhizosphere soil
		Pseudomonas folaasii	AF348507	99.4	Isolated from soil
		Pseudomonas kilonensis strain 520-20	AJ292426	99.4	Isolated from agricultural soil, Germany
		Pseudomonas brassicacearum strain 520-1	AJ292381	99.4	Isolated from agricultural soil, Germany
		Pseudomonas asplenii ATCC 23835T	AB021397	99.4	Pure culture
		Pseudomonas lundensis ATCC 49968T	AB021395	99.4	Pure culture
		Pseudomonas stutzeri strain 24a97	AJ312158	99.4	Isolated from marine sediment and soil
		Pseudomonas nitroreducens strain 24a97	AF494091	99.4	Pure culture
		Pseudomonas chloritidismutans	AY017341	99.4	Non-denitrifying chlorate
22	Trial III, MC 3 Devonian sandstone	Uncultured bacterium clone 38	AY250096	99.4	Coal-tar contaminated sediment of Hudson River, USA
		Uncultured eubacterium clone IAFR110	AF270945	99.4	Isolated from PCP contaminated soil, Canada
23	Trial II, MC 7b, acetate – 11 mg C l^{-1}	*Burkholderia sp.* AB2	AF219125	98.1	Strain: AB2
		Burkholderia cepacia	AF311971	98.1	Isolate: LS2.4, common plant-associated bacterium
		Burkholderia cepacia	AY741347	98.1	Strain ATCC 39277

Table 7.6 cont. Band ID no.	Sample description	Nearest neighbours	Accession no.	Similarity between sample and 'neighbour'	Origin of neighbour
23 continued	Trial II, MC 7b, acetate – 11 mg C l^{-1}	*Burkholderia thailandensis*	U91838	98.1	Strain: E264, a *Burkholderia pseudomallei*-like species
		Burkholderia pyrrocinia	AJ440715	98.1	Strain: R1904
		Burkholderia pseudomallei (Ara+ biotype)	AF093051	98.1	Strain: V683, exists in the environment especially in the soil biotype: Ara+
		Burkholderia sp. KBC-4	AY769905	98.1	Strain: KBC-4 *Burkholderia cepacia* complex in the environment
24	Trial II, MC 6a, acetate – 19 mg C l^{-1}	*Acidovorax sp.* 'smartab 133815'	AY093698	99.0	-
		Acidovorax sp. 040703/8067L	AY360350		-
25	Trial II, MC 4a, glucose – 22 mg C l^{-1}	*Burkholderia fungorum*	AJ544691	99.6	Strain W566, infection of deer and pig brain
		Burkholderia sp. LMG 16307	AF215706	99.6	*Burkholderia fungorum*, strain LMG 16307, isolated from the environment, animals and human clinical samples
		Burkholderia sp. ICD	AY134849	99.6	Strain ICD
		Burkholderia sp. A6.33GD	AF247492	99.6	Phenanthrene degrader, isolated from enrichments with phenanthrene
		Burkholderia sp. 56	AY177370	99.6	Phenanthrene-degrading soil column systems
		Burkholderia sp. CB2 (plus other strains associated with rhizobia of *Cyclopia sp.*)	AY178059	99.6	Strain CB2, specific host: *Cyclopia buxifolia* (rhizobia associated)
26	Trial II, MC 3a, glucose – 22 mg C l^{-1}	*Burkholderia fungorum*	AJ544691	99.6	Strain W566, infection of deer and pig brain
		Burkholderia fungorum LMG 16225T	AF215705	99.6	Isolate from *Phanerochaete chryosporium* (white rot fungi)
		Burkholderia fungorum LMG 16307	AF215706	99.6	Isolate from human cerebral spinal fluid, Sweden
		Burkholderia sp.	AF459796	99.6	Ant gut symbiont, Belgium
		Burkholderia sp. 56	AY177370	99.6	Phenanthrene-degrading soil column, Mexico and Arizona, USA
		Burkholderia sp. UCT 56	AY1708054	99.6	Rhizobia-associated isolate, South Africa
27	Trial III, MC 1, Lower Greensand Sterile Control	*Acidovorax defluvii* strain BS411	Y18616	98.6	Isolated from activated sludge, Germany
		Acidovorax sp. isolate G8B1	AJ012071	98.6	Enrichment isolate from activated sludge, Germany
		Acidovorax sp. isolate G7A1	AJ012070	98.6	"
28	Trial III, MC 8, Triassic sandstone	*Acidovorax defluvii* strain BS411	Y18616	98.6	Isolated from activated sludge, Germany
		Acidovorax sp. isolate G8B1	AJ012071	98.6	Enrichment isolate from activated sludge, Germany
		Acidovorax sp. isolate G7A1	AJ012070	98.6	"

One particular band which appeared present in all samples (except the failed sample MC 4b, Trial II), highlighted by red shading in Figure 7.7, appeared to be related to *Acidovorax spp.*, a member of the *Comamonadaceae* family (Hu *et al.*, 2001). This sequence was excised from samples including those treated with $HgCl_2$, therefore, it is likely that *Acidovorax* was originally present in the raw sandstone. *Acidovorax spp.* have been isolated from activated sludge (WWTPs) and soil environments contaminated with PAH and PCP (Table 7.6) and are expected to possess denitrifying capability (e.g. Khan *et al.*, 2002).

The higher resolution sequences excised from the pDpF gel indicated that many of the common bands were closely related to the *Burkholderia*, a soil bacterium commonly occurring as a symbiont in rhizosphere environs (Table 7.6). The pDpF sequences were additionally used to examine the evolutionary (clustering) relationships with similar organisms, using the phylogenetic tree-generating software package TREECON, as described by Rowan *et al.* (2003). This work was completed by the Centre for Molecular Ecology, University of Newcastle. The phylogenetic tree, presented in Figure 7.8, gives a more accurate indication of the relationship between the sequences obtained from the microcosm samples and known microbial species, based on an algorithm in the software that accounts for changes to the DNA sequence that might have occurred over evolutionary time.

Figure 7.8 demonstrates that most of the samples clustered with either the *Burkholderia* or the *Acidovorax* genus, with most similar relatives as follows:

- *Burkholderia vietnamienesis;*
- *Burkholderia* – symbiont of *A. aquaticus;*
- *Comamonus bacteria – C. terrigenia;*
- *Acidovorax delfluvii.*

7.3.2 Summary of Sequence Data

The DNA fingerprinting, PCR-DGGE technique was successfully applied to characterise some of the most commonly occurring organisms in the microcosm samples. Sequence data were elucidated for the most intense bands, hence, the organisms characterised by the sequencing were likely to represent the most abundant members of the microbial community in the microcosms. Members of the *Comamonadacea* family, including: *Acidovorax*, *Comamonas* and *Burkholderia*, were apparently abundant in the microcosms and organisms closely related to *Pseudomonas spp.* were also present.

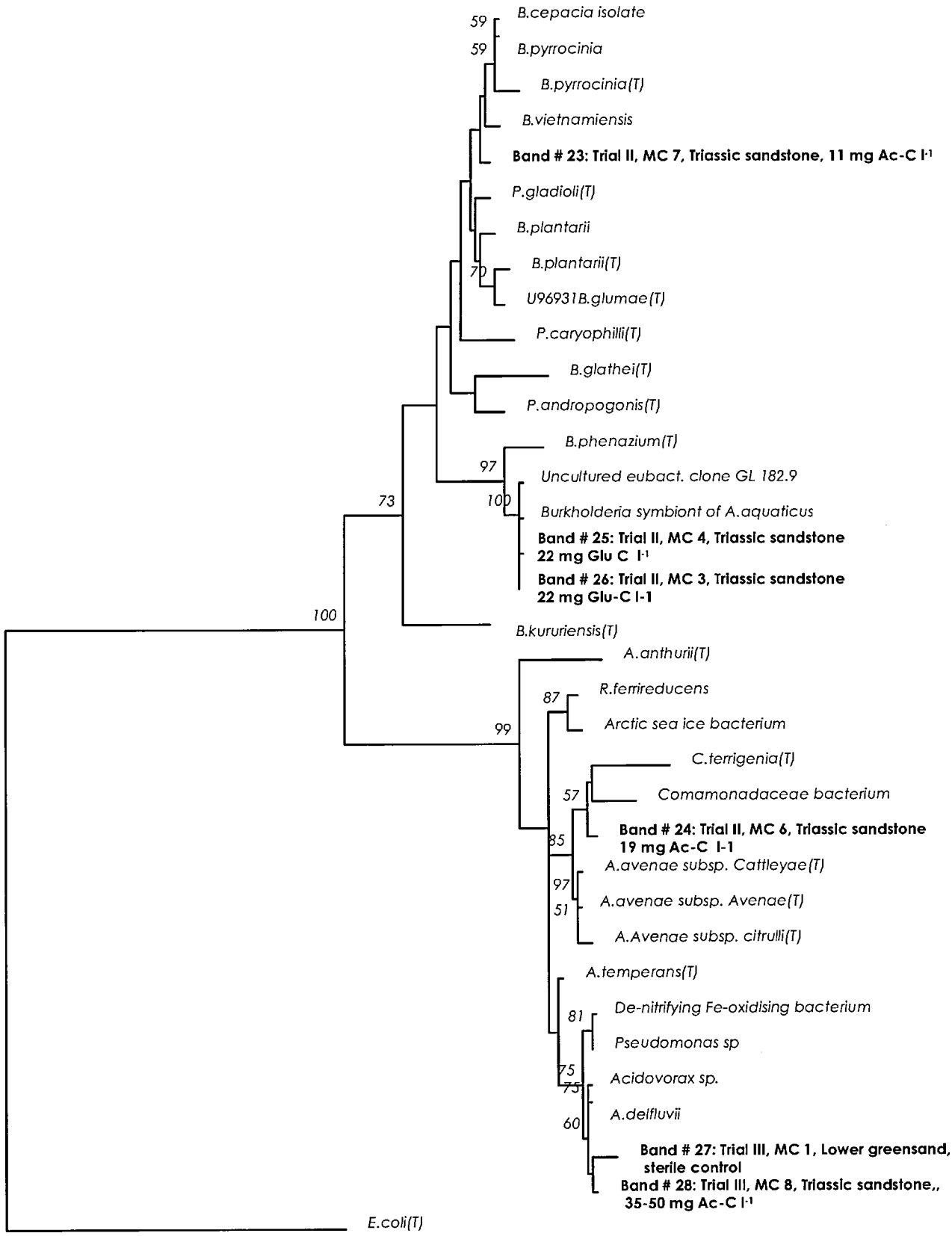


Figure 7.8: Phylogenetic tree indicating clustering relationships of microcosm samples (indicated in bold typeface with sample and DGGE band details), based on sequence analysis (*E.coli* was used as the outgroup). The distances of the branches indicate degree of similarity (not scaled) but the numbers on the branches indicate bootstrap values

Visual inspection of the DGGE gels suggested that organisms from these genus types were common to all treatments, regardless of OC substrate or sandstone type. This suggests that organisms capable of denitrification were predominant in the microcosms and in the case of the glucose-treated microcosms.

The denitrifying communities present in the microcosm samples from Trial III, where different sandstones were used, were apparently also dominated by members of the *Comamonadacea*, in spite of the potential variation in indigenous organisms in the source sandstone. Further investigation of the microbial communities with group-specific PCR techniques, however, would be required to elucidate DGGE and sequence data from more specific bacterial groups such as iron-oxidisers/iron reducers and sulphate-reducing bacteria.

Interestingly, there was a notable increase in the intensity of the bands from the samples collected during microcosm Trial III (refer to Figure 7.1), compared to samples from microcosm Trial II. A more intense (bright) band is generally understood to represent a more abundant number of organisms in the original sample, which would correlate with the increased denitrification activity observed (under a higher OC dose) in microcosms from Trial III, compared to Trial II (refer to Chapter 4). Therefore, the more rapid establishment of sustained complete denitrification in microcosms from Trial III may have influenced the microbial community structure, favouring a smaller number of species that became dominant at higher numbers.

7.4 DISCUSSION

A DNA extraction and PCR-DGGE protocol was successfully applied to natural sandstone where the indigenous microbial community had been enhanced with OC substrates, but the concentration of biomass remained relatively low overall (refer to Chapter 5). Additionally, the reproducibility of the DNA extraction and PCR-DGGE procedure was confirmed by relevant statistical analysis procedures.

The statistical analysis of the DGGE banding patterns demonstrated that the concentration of the OC amendment treatment had a significant effect on the inferred microbial community structure. Ultimately, this was attributed to the varying degree of denitrification observed in microcosms according to the concentration of OC input. For example, the microcosm that produced the highest overall denitrification efficiency during Trial II (MC 5) apparently had a

microbial community that was significantly divergent from the autotrophic microcosm, which did not receive OC. ANOVA comparing the similarity between communities of acetate and glucose microcosms with the autotrophic microcosms showed that the glucose microcosms were significantly more similar to the autotrophic microcosms than the acetate microcosms. Taken at face value, this might suggest that the difference was due to substrate type; however, the Raup and Crick test indicated that the 81% of pair-wise comparisons between acetate (19 mg C l^{-1}) and glucose (22 mg C l^{-1}) were deterministically similar. Consequently, it was concluded that the difference in similarities between acetate and autotrophic microcosms compared to glucose and autotrophic microcosms was again due to the degree of denitrification activity in these microcosms. A further, though qualitative, advocacy of this finding was the visibly brighter appearance of the bands in the samples collected after microcosm Trial III, (Figure 7.1). These microcosms received a higher OC dose (35-50 mg C l^{-1}) and exhibited more efficient denitrification overall, compared to microcosm in Trial II.

It is proposed that the DNA extraction and PCR-DGGE procedure recovered the most abundant species from the microcosm sandstone, which were apparently related to species of the *Comamonadacea*, particularly of the genus *Acidovorax*, which have been isolated from WWTPs (refer to Table 7.6). *Acidovorax spp.* were dominant in all microcosms, regardless of substrate type or sandstone type.

The similarities observed between acetate and glucose microcosm samples, identified by the Raup and Crick test, are contrary to observations that glucose (i.e. fermentable substrate) can select for a different microbial community compared to those substrates regarded more suitable for denitrification such as acetate, (Blaszczyk *et al.*, 1980; Mercado *et al.*, 1988; Devlin *et al.*, 2000). However, it is noted that in such reports, identification of bacteria has been primarily based on conventional microbial plating techniques, for example Blaszczyk *et al.* (1980), as opposed to the molecular methods used here. Therefore the different community structures elucidated may be a reflection of the different experimental methods, and their associated biases. Alternatively, it is possible that the microbial groups present in the sandstone microcosms are capable of respiration and fermentation.

During these experiments, the use of universal primers in the PCR procedure amplified the most dominant bacterial species, which were most closely related to facultative bacteria associated with denitrification and hydrocarbon degradation. However, as a subsequent experiment, so called 'group-specific' primers designed to amplify particular groups would elucidate subtle variations in microbial

community structure, with regards to (i) substrate type and (ii) sandstone type, as considered in Chapter 10. Nevertheless, universal primers and nested PCR to amplify different sized fragments of the 16S RNA gene provided a useful 'top-down' approach to characterise the dominant constituents of the microcosm microbial community.

CHAPTER 8

INFLUENCE OF ISBD ON THE BIOLOGICAL AND CHEMICAL PROPERTIES OF GROUNDWATER INTENDED FOR PUBLIC SUPPLY

8.1 OVERVIEW

8.2 RESULTS: SUSPENSIVE PROPERTIES OF DENITRIFIFED WATER

8.2.1 Bacterial Cell Counts

8.2.2 Effluent Turbidity

8.3 RESULTS: PHYSICO-CHEMICAL PROPERTIES OF DENITRFIED WATER

8.3.1 Dissolved Oxygen and Redox

8.3.2 pH

8.3.3 Conductivity

8.4 DISCUSSION

8.1 OVERVIEW

This Chapter provides a summary of the water quality of the denitrified microcosm effluents, analogous to treated groundwater from ISBD plants, with reference to regulatory standards for drinking water in the UK. Additional results relevant to water quality, such as residual NO_2^- and OC, and Fe and Mn are presented in Chapters 4 and 6 respectively. The general discussion at the end of the chapter considers the implications of these results with regards to post-denitrification treatments.

8.2 RESULTS: SUSPENSIVE PROPERTIES OF DENITRIFIED WATER

8.2.1 Bacterial Cell Counts

Effluent samples collected during microcosm Trial III were enumerated for colony forming units (CFU) using heterotrophic plate count (HPC) method (2 d and 5 d at 20 °C) and samples collected during Trial IV were sent to an external laboratory for enumeration (total viable counts, 3 d at 22 °C), as described in Chapter 3. The results are presented in Tables 8.1 and 8.2 for microcosm Trials III and IV, respectively. Large numbers of bacterial cells were generally present in microcosm effluents from both experiments (2.5 - >6.5 CFU $\log_{10}$ ml^{-1}).

During Trial III, control microcosms were treated with $HgCl_2$, and consequently, no colonies were observed in this case. In contrast, the biologically active (denitrifying) columns exhibited an initial increase in CFU during the first week of monitoring consistent with the establishment of microbial activity within the microcosms, and subsequently, an overall decreasing trend in bacterial counts was observed during the following 7 week period. The bacterial content of effluents from the Devonian sandstone microcosms were significantly smaller ($P < 0.01$) compared to effluents from the Lower Greensand and Triassic sandstone. The mean CFU $\log_{10}$ ml^{-1} counts (all sampling times) were: 5.9, 6.5 and 6.3, for Devonian sandstone, Lower Greensand and Triassic sandstone respectively.

Colony counts from samples collected between d 7-37, Trial III (the period of biomass establishment and onset of denitrification activity) were pooled and compared to residual effluent nutrient concentrations. Regression analysis showed no significant relationship between effluent DOC and CFU, but a significant positive relationship was observed between effluent TON and CFU ($P < 0.01$, $R = 0.5$), (Figure 8.1).

Table 8.1: Bacterial counts for effluents sampled during microcosm Trial III. Microcosms were prepared using various sandstone types, but all received an influent solution of 15 mg N l^{-1} and 35 mg acetate C l^{-1}, which was increased to 50 mg C l^{-1} on d 37. Reported bacterial counts are CFU log_{10} ml^{-1}

Time (experiment day)	Controls ($HgCl_2$) MC 1		Controls ($HgCl_2$) MC 2		Devonian sandstone MC 3		Devonian sandstone MC 4		Lower Greensand MC 5		Lower Greensand MC 6		Triassic sandstone MC 7		Triassic sandstone MC 8	
	2 d	5 d	2 d	5 d	2 d	5 d	2 d	5 d	2 d	5 d	2 d	5 d	2 d	5 d	2 d	5 d
3	0.0	0.0	0.0	0.0	6.0	6.1	5.2	5.2	>6.5	>6.5	>6.5	>6.5	5.4	5.6	5.6	5.6
7	0.0	0.0	0.0	0.0	5.9	6.1	5.7	6.1	6.9	7.0	6.7	6.8	6.6	6.7	6.6	6.7
10	0.0	0.0	0.0	0.0	5.9	6.5	5.7	6.3	6.3	6.9	5.9	6.9	5.7	6.8	5.7	6.9
13	0.0	0.0	0.0	0.0	6.9	6.9	5.9	5.9	6.7	6.7	6.8	6.8	6.3	6.3	6.7	6.7
17	0.0	0.0	0.0	0.0	5.8	6.4	5.2	6.3	6.4	6.6	6.2	6.7	5.5	6.6	5.6	6.4
22	0.0	0.0	0.0	0.0	5.4	5.8	5.4	5.5	5.9	6.6	5.9	6.5	5.6	6.5	5.9	6.4
25	0.0	0.0	0.0	0.0	5.7	6.3	5.4	6.4	6.5	6.9	6.0	6.5	6.3	6.7	6.3	6.4
29	0.0	0.0	0.0	0.0	5.0	5.8	4.3	5.4	5.7	6.8	5.7	6.5	5.5	6.7	5.2	6.4
32	0.0	0.0	0.0	0.0	5.3	5.4	5.2	5.4	6.3	6.5	6.2	6.4	6.4	6.5	6.4	6.5
37	0.0	0.0	0.0	0.0	4.7	5.5	4.9	5.3	5.7	6.0	6.1	6.2	5.5	5.9	5.3	5.8
42	0.0	0.0	0.0	0.0	7.0	7.0	4.9	5.4	5.4	6.4	6.0	6.6	5.9	6.3	5.5	6.5
46	0.0	0.0	0.0	0.0	5.7	6.0	5.5	5.9	6.1	6.3	6.3	6.5	5.3	6.5	-	-
48	0.0	0.0	0.0	0.0	4.3	5.9	4.8	5.7	5.3	6.1	5.5	6.1	5.4	6.0	5.3	6.1
53	0.0	0.0	0.0	0.0	-	5.3	-	5.3	-	5.8	-	5.8	-	5.7	-	5.6
Mean	0.0	0.0	0.0	0.0	5.7	6.1	5.2	5.7	6.1	6.5	6.1	6.5	5.8	6.3	5.8	6.3
Standard deviation	0.0	0.0	0.0	0.0	0.8	0.5	0.4	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.5	0.4

Table 8.2: Bacterial counts for microcosm feed solutions and effluents sampled during microcosm Trial IV. Microcosms were prepared using Upper Greensand and received an influent solution prepared using groundwater collected from the same site, amended with NO_3^- to give a final concentration of 15 mg N l^{-1}. The OC regime is described in the column headings. Reported bacterial counts are CFU log_{10} ml^{-1}

Time (experiment day)	Autotrophic controls			Glucose: 35 mg C l^{-1}			Glucose: 28 mg C l^{-1}		Glucose: 44 mg C l^{-1}		Acetic acid: 35 mg C l^{-1}		
	Feed soln.	MC 1 effluent	MC 2 effluent	Feed soln.	MC 3 effluent	MC 4 effluent	Feed soln.	MC 5 effluent	Feed soln.	MC 6 effluent	Feed soln.	MC 7 effluent	MC 8 effluent
20	4.0	4.1	3.9	2.6	4.1	4.1	2.8	4.2	3.0	4.1	-	4.1	3.9
21	3.8	3.9	3.9	4.4	4.2	3.9	4.3	3.5	-	3.9	3.8	3.9	3.9
22	5.0	5.4	4.7	6.4	4.4	3.7	6.3	4.7	5.9	3.3	5.7	3.6	4.6
25	3.3	3.8	3.4	3.4	3.8	3.7	3.6	4.0	3.9	3.9	3.5	3.7	3.6
28	4.5	6.0	4.9	5.5	5.5	4.2	5.2	5.3	5.2	5.4	5.4	5.3	5.3
Mean	4.1	4.6	4.2	4.4	4.4	3.9	4.5	4.3	4.5	4.1	4.6	4.1	4.3
Standard deviation	0.7	1.0	0.6	1.5	0.6	0.2	1.4	0.7	1.3	0.8	1.1	0.7	0.7

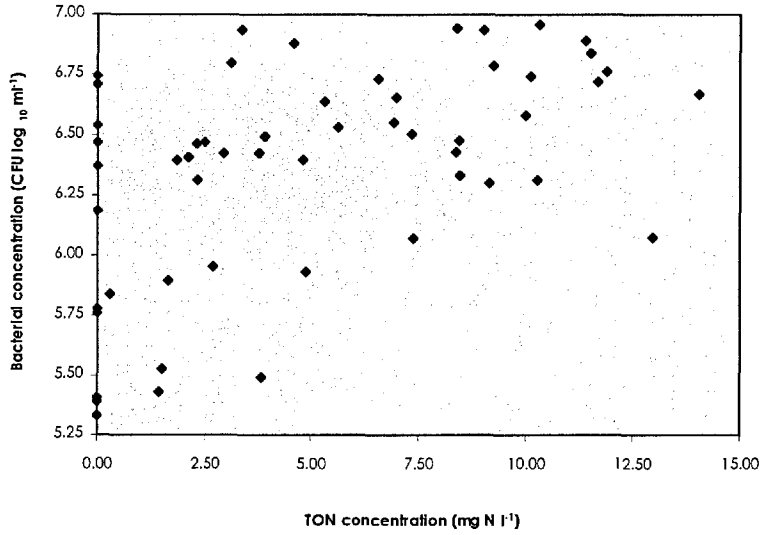


Figure 8.1: Relationship between effluent bacterial concentration (CFU ml^{-1} log_{10}) and residual effluent TON (mg N l^{-1}) during Trial III, d 7-37. Microcosms were composed of different sandstone types (not relevant to bacterial count data) but received the same influent solution of 15 mg N l^{-1} and 35 mg acetate-C l^{-1} during the period d 7-37.

This suggested that the concentration of bacterial cells present in the effluent decreased over time, consistent with the establishment of denitrification activity. Conversely, there was no significant relationship between residual DOC and bacterial counts, refer to Section 8.4.

Bacterial counts obtained from effluent samples collected during microcosm Trial IV did not show a significant relationship between the concentration of residual nutrients and bacterial counts (regression P value >0.05). Furthermore, ANOVA showed that there was no significant difference in effluent bacterial counts from different treatment regimes, including the autotrophic controls (P >0.05).

Effluents collected during Trial III were plated for identification purposes. Five isolates were recovered, as shown in Figure 8.2, and indicated that a mixed community was present in the effluents. Generally, these isolates were gram negative rods, consistent with the types of γ- and β- proteobacteria that were recovered from the microcosm sandstone (refer to Chapter 7). One set of samples (d 25) from microcosm Trial IV were additionally analysed for total coliforms and *Escherichia coli,* which are regulatory indicator organisms of drinking water quality (MAC = 0 ml^{-1}), (SI: 3184, 2000), but no coliforms or *E. coli* were counted in any of the samples (using the HPC method).

These results indicated that heterotrophic denitrification, stimulated by an OC dose, did not impair the microbial quality of the denitrified water once complete denitrification had become established. Prior to this, there was a transient increase in bacterial counts, related to residual nutrient concentration; however, in field application of this technique, this may be attenuated by the increased residence/travel time of the denitrified water plume within the aquifer.

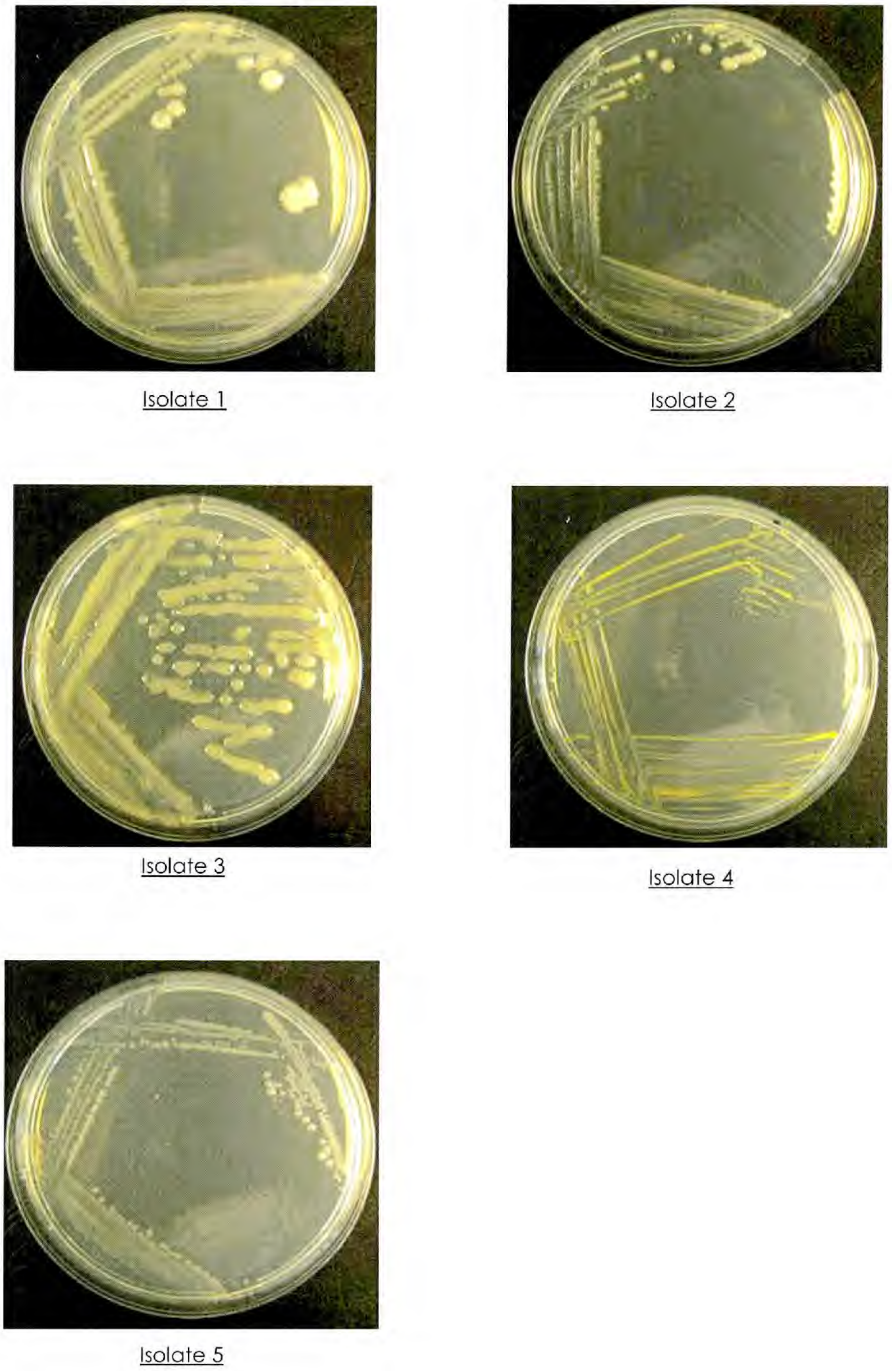


Figure 8.2: Streak plates showing bacterial isolates from effluent sampled during Microcosm Trial III (effluent sampled from MC 4: Devonian sandstone, 35 mg acetate-C l^{-1})

Pathogenic indicator organisms were not identified and once denitrification activity had established, there was no pronounced change in effluent biomass concentrations measured at the start and at the termination of the experiment, in the case of microcosm Trial III, and in the active treatments compared to autotrophic controls (no OC) in microcosm Trial IV.

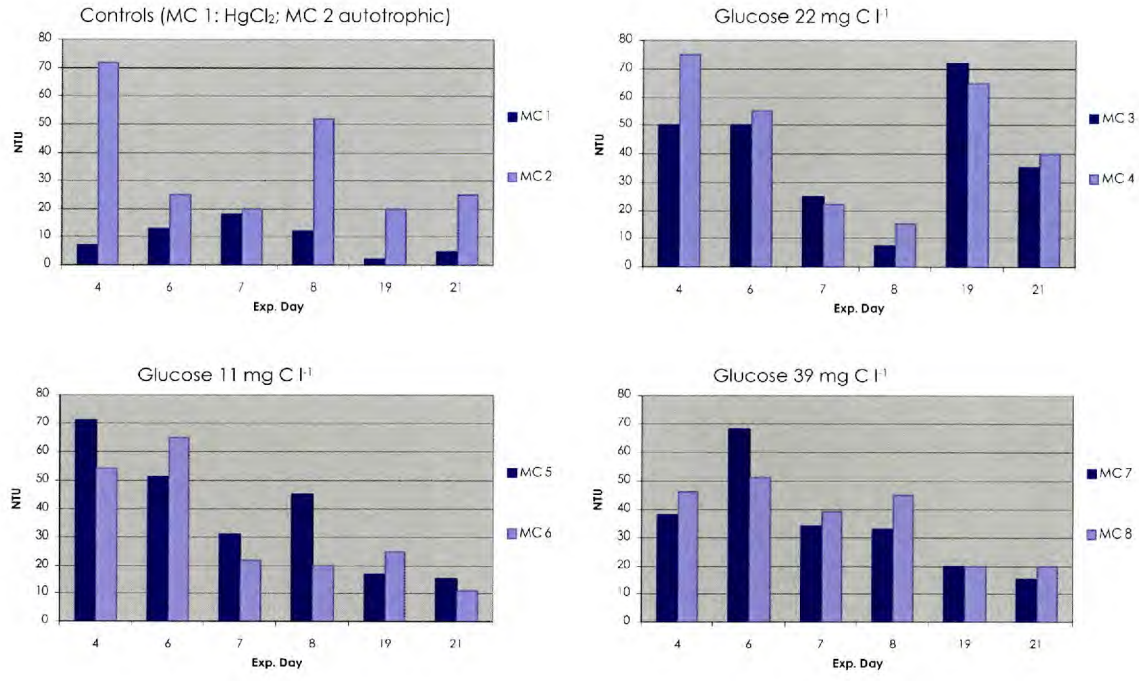
8.2.2 Effluent Turbidity

Turbidity provides a measure of the clarity of water, where impurities such as micro organisms, soluble coloured organics, fine particulates such as clay and silt or fine organic/inorganic matter suspended in the water, impair the clarity (US EPA, 1999). Turbidity is quantified according to the degree of light scattering that is caused by impurities when a beam of light is passed through the water sample (US EPA, 1999).

Turbidity in drinking water is aesthetically undesirable, but may also be of consequence to human health. For example, the particulate matter may compromise the effectiveness of disinfection methods, by effectively sheltering pathogenic micro-organisms. Furthermore, if turbidity is not removed, it may encourage proliferation of microorganisms in the water distribution network, by providing substrates for bacterial growth, for instance (US EPA, 1999). The turbidity of the microcosm effluents (denitrified water) in these experiments was measured according to standard methods, as described in Chapter 3. Turbidity was reported in nephelometric turbidity units (NTU), in accordance with drinking water regulations, which stipulate a MAC of 4 NTU (at consumers' taps) (SI: 3184, 2000).

During microcosm Trial I, considerable turbidity (up to 70 NTU) was measured in all microcosm samples, including those from the control microcosms, as depicted in Figure 8.3 a. The turbidity measurements were greater in the biologically active microcosms supplied with OC, (where NO_3^- reduction activity occurred), compared to the sterile control ($HgCl_2$ treated; MC 1), indicating that the turbidity was influenced by microbiological activity. Visual inspection of the effluents showed fine particles of silt to be suspended in the effluent samples and this likely to be the main explanation for the high turbidity recorded. Qualitative XRD analysis on samples of Triassic sandstone confirmed the presence of fine clay fractions such as chlorite, kaolinite, illite and smectite. Subsequently, measures were taken to remove as much of the silt/clay fraction from the Triassic sandstone as possible, in further microcosm experiments (refer to Chapter 3).

(a) Microcosm Trial I



(b) Microcosm Trial II

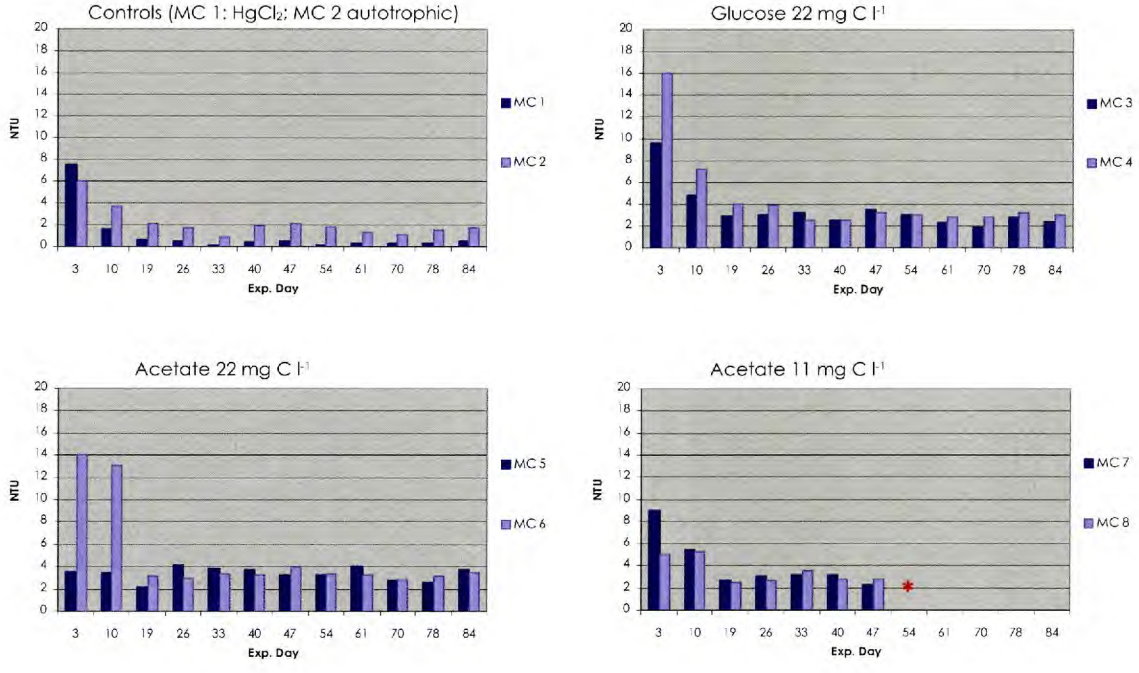
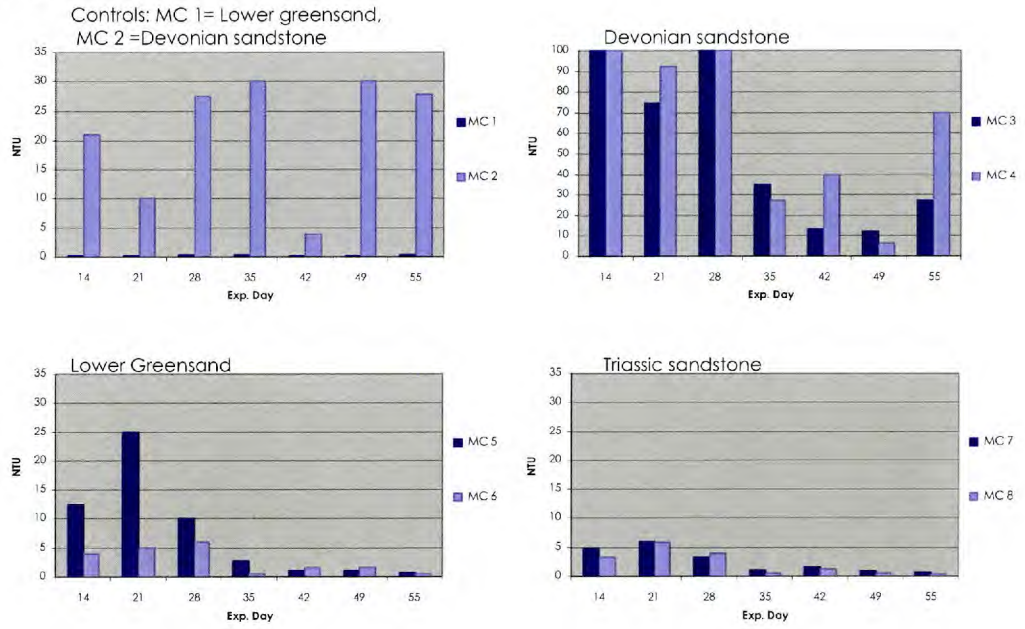


Figure 8.3: Turbidity of denitrified effluents from Triassic sandstone microcosms amended with different OC types and concentrations during **(a)** Microcosm Trial I and **(b)** Microcosm Trial II, Control treatments were either sterilised using $HgCl_2$ or were autotrophic (did not receive OC)

* *this treatment was terminated on d 51*

(a) Microcosm Trial III



(b) Microcosm Trial IV

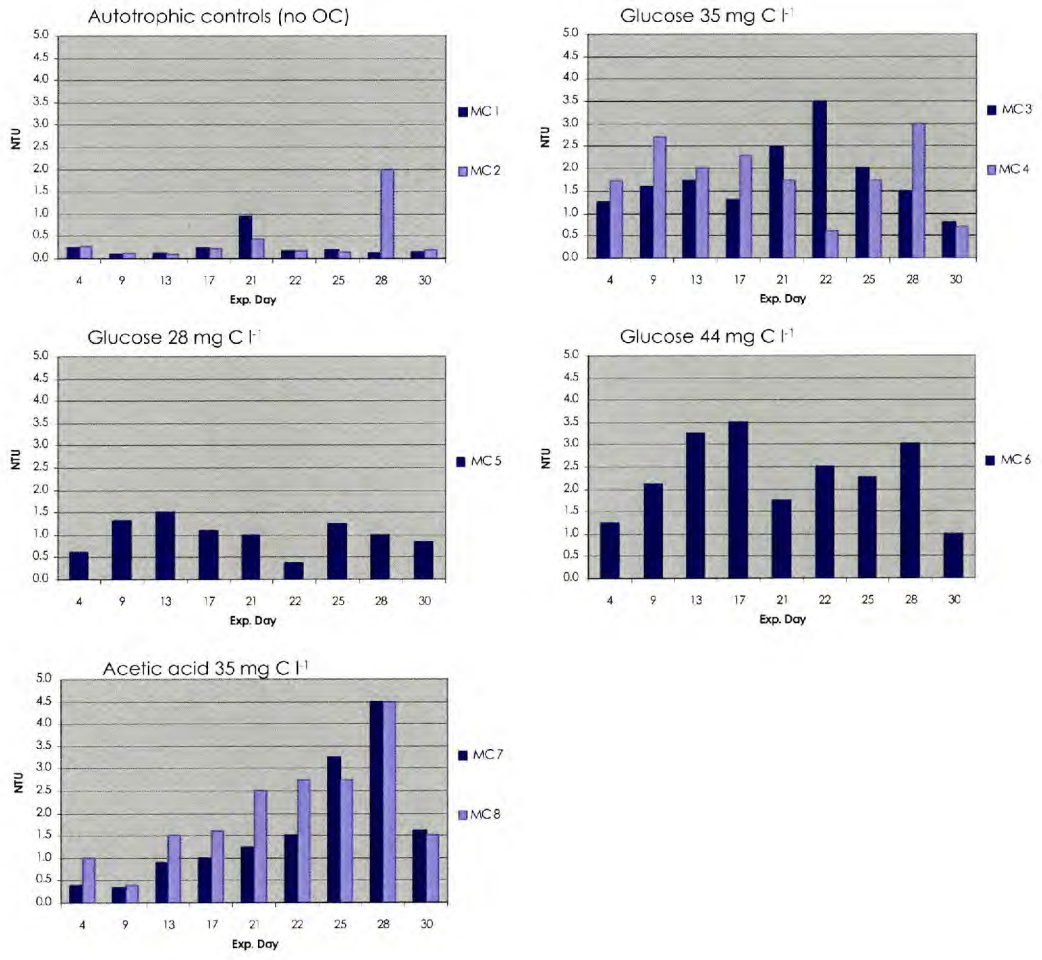


Figure 8.4: Turbidity of denitrified effluents from various sandstone microcosms; **(a)** Trial III: microcosms were filled with different sandstone but received 35 mg C l^{-1} of acetate throughout, controls (MC 1/MC 2) were sterilised with $HgCl_2$; **(b)** Trial IV: microcosms were prepared with Upper Greensand but received varying OC concentrations and types, controls were autotrophic

The results of microcosm Trial II (Figure 8.3 b) demonstrated that the removal of the fine particulate fraction from the sandstone prior to use in the microcosm markedly reduced effluent turbidity. Following an initial period of acclimation during the first 10 d of the experiment, the turbidity in the control microcosms decreased to ≤ 2.0 NTU in MC 2 (no OC), and < 1.0 NTU in MC1 (sterilised with $HgCl_2$). In the microcosms amended with OC, the turbidity after d 10 was consistently between 2-4 NTU, less than the UK MAC (SI: 3184, 2000), in all treatment types.

In microcosm Trial III (Figure 8.4 a), three different types of sandstone were used to pack the microcosms, and the various sandstone types influenced effluent turbidity. Effluents from Devonian sandstone microcosms were comparatively more turbid than effluents from the other sandstone microcosms (Figure 8.4 a), in both the sterilised control (MC 2) and biologically active treatment replicates (MC 3 and 4). This was attributed in part to the flushing out of sand particles during operation, since the Devonian sandstone was processed (dried and crushed) to facilitate microcosm packing (refer to Chapter 3). In all sandstone types, higher turbidity was recorded in effluents from the biologically active microcosms (i.e., those that were fed OC and not sterilised). However, the turbidity was within drinking water standards after approximately 3-4 weeks, consistent with the time taken to achieve complete denitrification.

In Trial IV (Figure 8.4 b), which comprised microcosms packed with Upper Greensand and fed with a solution based on raw groundwater, the NTU values were also greater in the microcosms amended with OC compared to the autotrophic controls (MC 1 and MC 2), without OC additions. Turbidity in the effluents from the glucose microcosms (MC 3-MC 6), was within the drinking water MAC (4 NTU), generally fluctuating between 0.5 and 3.5 NTU. In contrast, the acetic acid effluents showed an increasing trend in turbidity with incubation time, ultimately exceeding the MAC on d 28. However, the turbidity decreased markedly following the reduction in input OC dose and concomitant decreased in effluent OC breakthrough (Figure 8.4 b).

An ANOVA and subsequent Tukey pair-wise comparisons showed that generally, the turbidity in the biologically active microcosms, supplied with OC, was significantly larger than the turbidity observed in either sterilised or autotrophic controls, throughout all microcosm trials. This was quantified by ANOVA and Tukey pair-wise comparisons (using the GLM procedure outlined in Chapter 4, $P < 0.05$). Exceptions to this were pair-wise comparisons between: the sterile Lower greensand treatments in Trial II (sterile control = MC 1, active treatments = MC 5 and 6), the low acetate treatment (MC 7 and 8) compared to the autotrophic control (MC 2) in Trial II and the

low glucose treatment (MC 5) compared to the autotrophic controls (MC 1 and 2) in Trial IV. In these cases, the active treatments showed no significant difference in effluent turbidity compared to controls, ($P > 0.05$). Interestingly, however, these treatments gave the lowest denitrification efficiencies in their respective experiments and led to the speculation that turbidity was in some way related to the occurrence and possibly the degree of denitrification that occurred in the microcosms.

To investigate this, regression analysis was used to define the relationships between residual TON (inferring denitrification) and effluent turbidity. However, the results were conflicting; no significant relationship was observed during Trial II, in Trial III, the TON showed a significant positive correlation with turbidity ($P < 0.05$, $R = 0.62$), but in Trial IV, TON was negatively correlated with turbidity ($P < 0.05$, $R = 0.42$). Therefore, although the turbidity did appear to be related to heterotrophic denitrification activity, the relationship could not be further elucidated as discussed further in Section 8.4.

8.3 CHEMICAL QUALITY OF DENITRIFIED WATER

8.3.1 Dissolved Oxygen and Redox

Denitrification requires anaerobic conditions to proceed, as described in Chapter 1, and it is anticipated that during ISBD, consumption of DO will occur in the region adjacent to the OC injection point (e.g. Mercado *et al.*, 1988). The depletion of DO within the aquifer could produce denitrified water with a suppressed DO concentration, compared to untreated water. Whilst DO is not a regulated parameter with regards to drinking water quality (SI: 3184, 2000), it is desirable that ISBD does not significantly reduce the DO concentration of the water source, as this may incur other chemical transformations in the groundwater. For example, changes in the ionic composition of the groundwater, caused by significantly altered DO concentration, may lead to subsidiary reactions such as corrosion processes within the water distribution network.

The DO concentration of the microcosm effluents was not be measured in real time but for qualitative purposes, DO was measured in effluents (collected under ambient oxygen conditions over a period of 24 h) during microcosm Trial II; the results are presented in Figure 8.5. The results showed that DO concentrations were reduced in effluents from denitrifying microcosms compared to the autotrophic control microcosm, which did not receive OC.

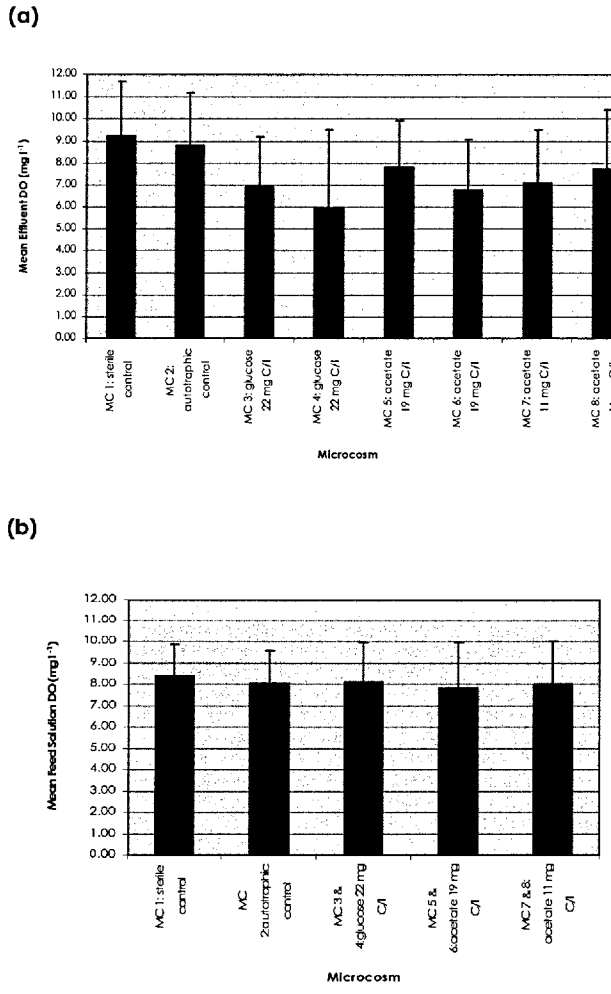


Figure 8.5: Effects of various denitrification treatments on DO measured in microcosm effluents **(a)**, and **(b)** initial DO measured in microcosm feed solutions, during Trial II. Bars represent mean minima and maxima

Furthermore, redox (mV) was measured in all microcosm trials, at the top of the sand column and was also increasingly depressed over time, as the microcosms tended towards more reducing conditions, as demonstrated in Figure 8.6. Whilst these microcosm parameters cannot be extrapolated to ISBD conditions in the field, they demonstrate the intrinsic effect of ISBD on groundwater DO concentration. It is likely that under engineered ISBD conditions, however, there will be a degree of dilution between the denitrified water plume and the unamended groundwater at the point of abstraction, particularly under operational regimes where the OC dosing is intermittent. Generally, therefore, depressed DO concentration is not expected to present a water quality issue (e.g. Mercado *et al.*, 1988).

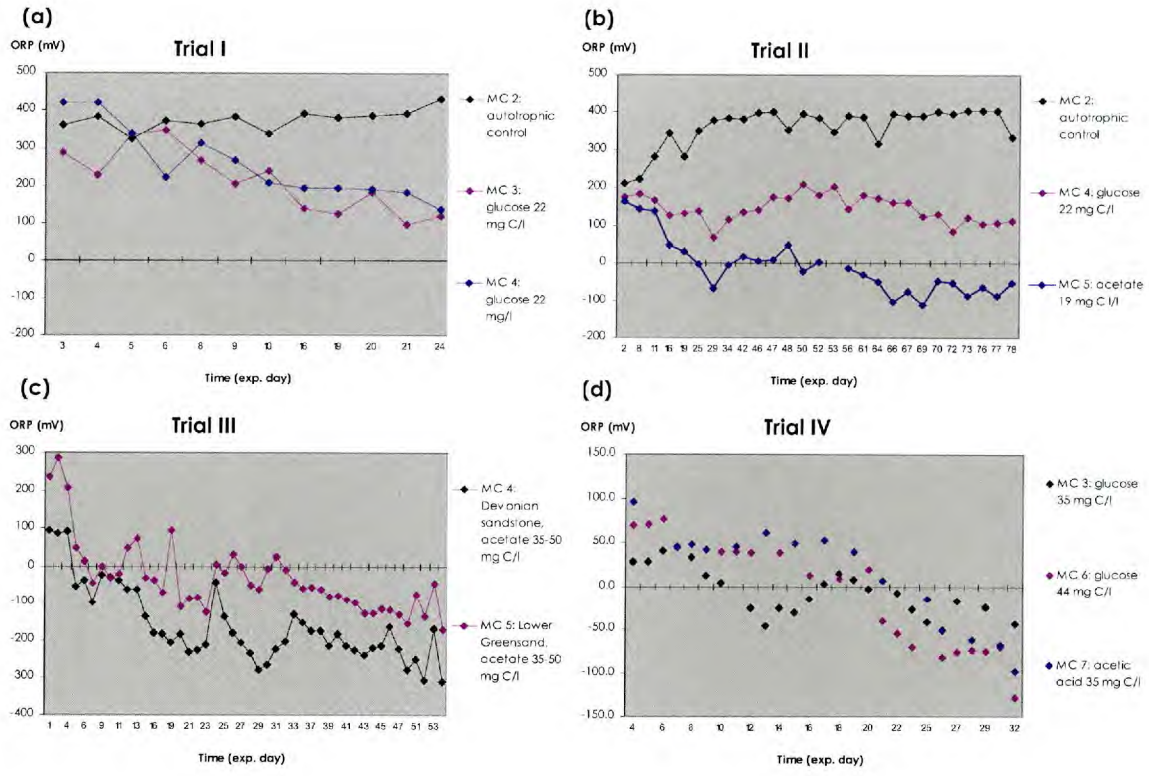


Figure 8.6: Effect of heterotrophic denitrification on redox potentials measured in various sandstone and OC supply conditions (redox values (mV) are not absolute since redox measurements made with probes are qualitative). OC supply regimes are indicated legends and sandstone was Triassic sandstone unless otherwise indicated. Redox values Trial IV are discreet, since the redox probes were moved between microcosms to facilitate measurements

8.3.2 pH

Denitrification, as a form of microbial respiration may increase groundwater alkalinity and pH due to the HCO_3^- and/or OH^- ions that are produced by the reaction (e.g. Blaszczyk *et al.*, 1980; ITRC, 2002; Rust *et al.*, 2000), for example:

i) $5CH_3COO^- + 8NO_3^- + 3H^+ \Rightarrow 4N_2 + 10HCO_3^- + 4H_2O$
(Devlin *et al.*, 2000)

ii) $^5/_8CH_3COO^- + NO_3^- \Rightarrow {}^1/_2N_2 + {}^5/_4CO_2 + {}^1/_8H_2O + {}^{13}/_8\ OH^-$
(ITRC, 2002)

iii) $5CH_3COOH + 8NO_3^- \Rightarrow 2CO_2 + 4N_2 + 8HCO_3^- + 6H_2O$
(Devlin *et al.*, 2000)

The actual reaction products were not quantified in the microcosm experiments, however, increased pH was observed in the effluents of microcosms amended with

acetate (microcosm Trials II and III), but not in effluents of microcosms amended with acetic acid (Trial IV) or glucose (Trial I, II and IV), as presented in Figure 8.7 and 8.8. In the acetate microcosms, the effluent pH increased by approximately 2 units over the duration of the experiments, in conjunction with denitrification activity; in Trial II, effluent pH from acetate treatments was greater than that in control effluents by up to 4 pH units, approaching pH 9.5. However, the increased pH remained within the UK specified limits of pH 6.5-10.0 (SI: 3184, 2000). The effluents from acetic acid (Trial IV, and glucose (Trials I, II and IV) treatments, remained at approximately neutral pH during the experimental incubation period.

The rise in pH value of denitrified water depends on the relative quantities of CO_2, OH^- and subsequently HCO_3^- and CO_3^{2-} produced by the denitrification reaction (e.g. Dtril *et al.*, 1995). For example, according to Drtil *et al.* (1995), dissimilatory denitrification produces CO_2 and OH^-, which subsequently react to give HCO_3^- leaving some residual CO_2. The increase in HCO_3^- concentration raises alkalinity (acid-neutralising capacity) and pH. However, when the amount of OH^- produced during denitrification exceeds the amount of CO_2 produced, some OH^- will remain in solution following reaction of OH^- with CO_2 to form HCO_3^-. In this case, the remaining OH^- may react further with HCO_3^- producing CO_3^{2-}, potentially causing solution pH to rise in excess of pH 8.3.

The varying ratios of CO_2 and OH^- produced from dissimilatory denitrification (and related assimilatory reactions) may be influenced by OC substrate type. For example, Drtil *et al.* (1995) demonstrated relationships between production of CO_2/OH^- and:

(i) the degree of oxidation of the carbon atoms in un-dissociated substrates, such as glucose and acetic acid;
(ii) the number of C, H and O atoms for dissociated substrates such as acetate.

According to Drtil *et al.* (1995), both acetic acid and glucose-denitrification produce HCO_3^- and CO_2, and the pH of the microcosm effluent solution should therefore not exceed 8.3. Conversely, acetate denitrification, which involves the reaction of two anions (CH_3COO^- and NO_3^-), produces more OH^- ions than CO_2 and therefore CO_3^{2-} ions may be generated, raising the pH to > 8.3.

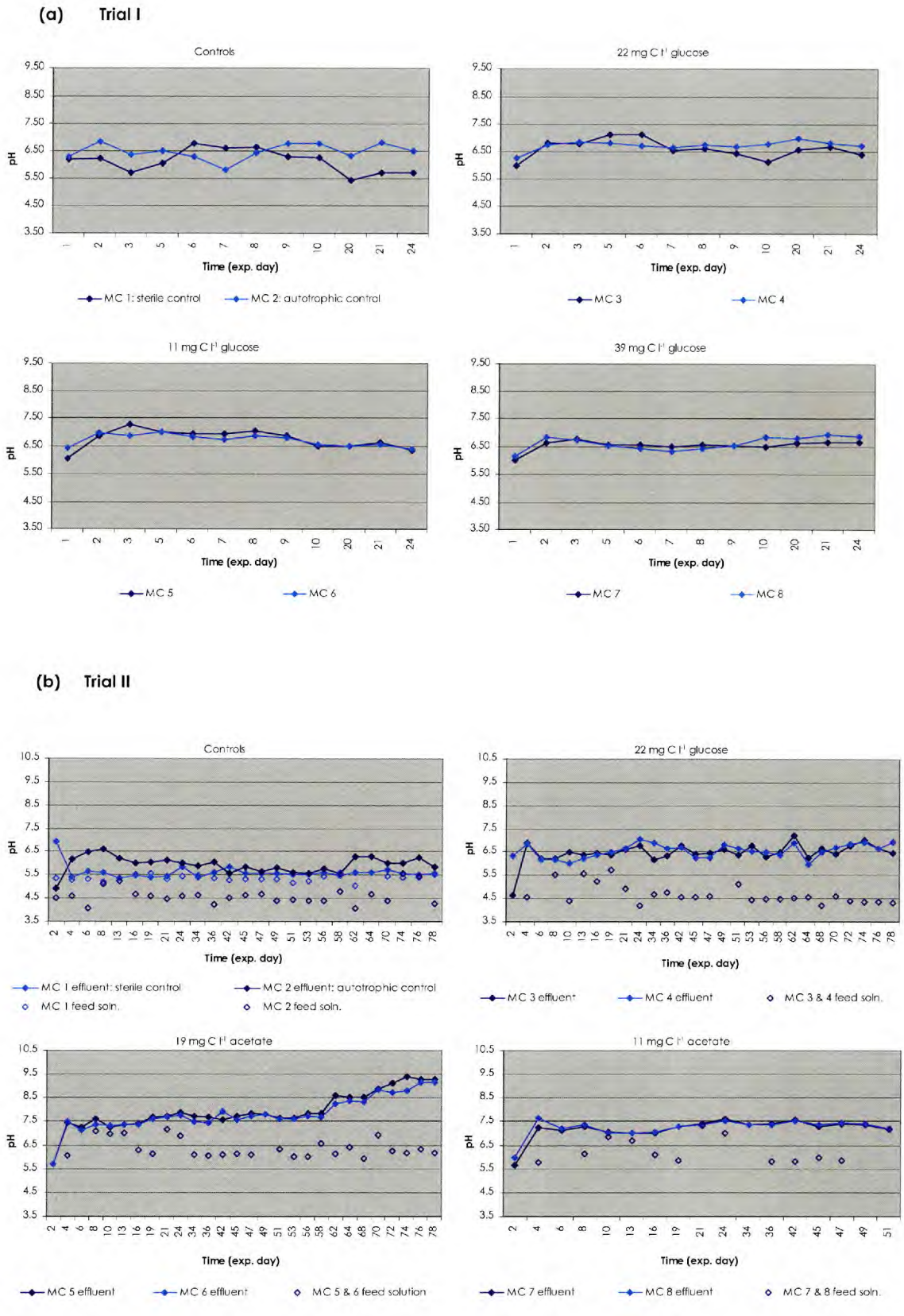


Figure 8.7: Effect of denitrification on pH of microcosm effluents, compared to their corresponding feed solutions, measured during Trial I **(a)**, and Trial II **(b)**, where the effects of glucose and acetate amendments on denitrification activity in Triassic sandstone microcosms were investigated

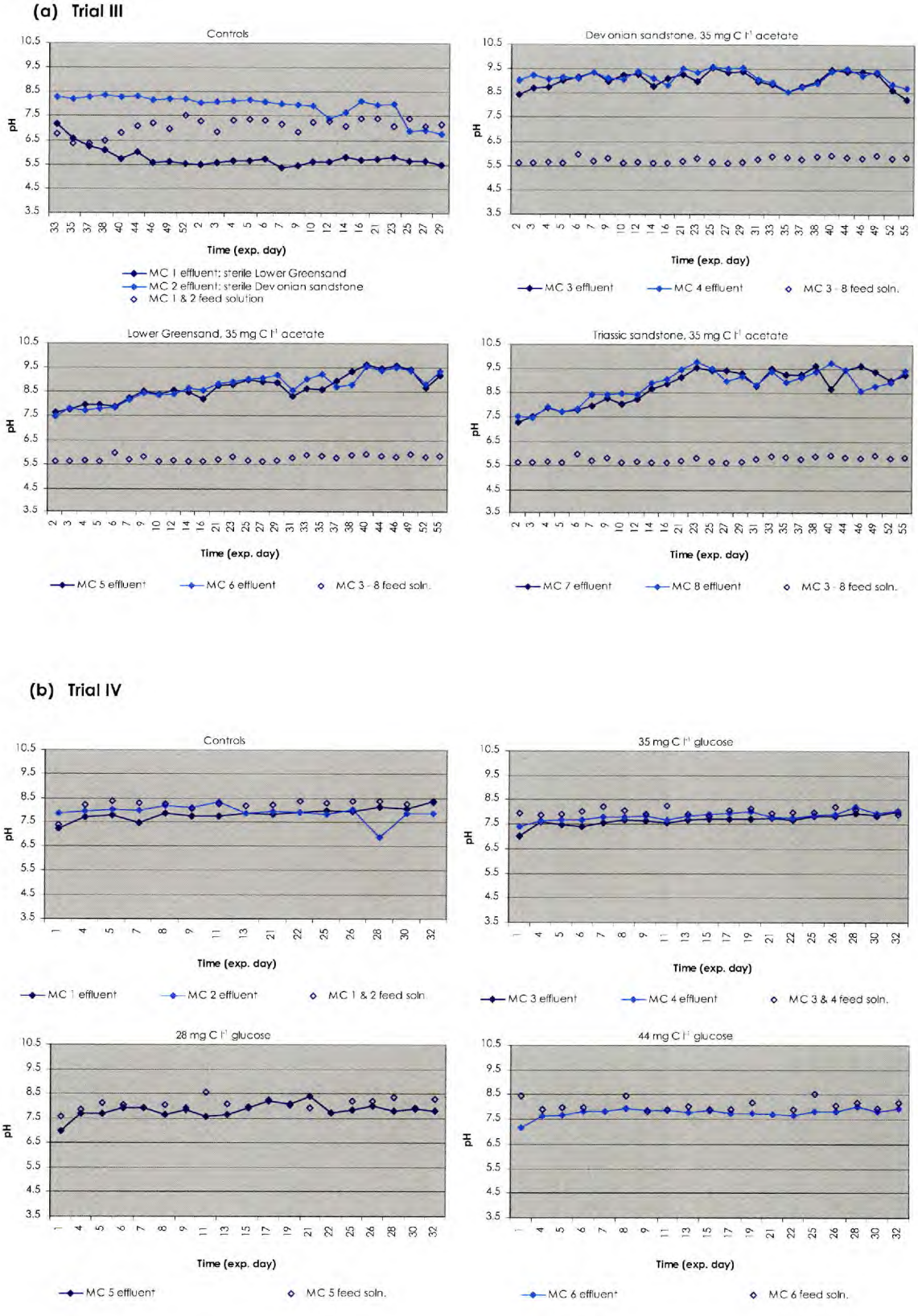


Figure 8.8: Effect of denitrification on pH of microcosm effluents, compared to their corresponding feed solutions, measured during Trial III **(a)**, investigating the effects of sandstone type on quality of denitrified water; and Trial IV **(b)**, investigating the effects of denitrification on water quality of natural groundwater collected from an Upper Greensand aquifer

In the case of the glucose treatment, the maintenance of near neutral pH during denitrification of mixed microbial communities has also been attributed to the contribution of acidity from fermentation reactions to the overall solution pH (e.g. Wilderer *et al.*, 1987; Drtil, 1995), and this may have been the case here.

The pH results presented here are in agreement with the predictions of Drtil *et al.* (1995) and also the empirical data of Mosheni-Bandpi *et al.* (1999), who noted that the use of acetic acid reduced the extent of pH increases. The large increase in effluent pH measured in acetate treatments was therefore probably attributed to release of OH^- ions and the formation of CO_3^{2-}.

Most authors (e.g. Hamon and Fustec, 1991; Dahab, 1993, ITRC, 2002) assume that the natural buffering capacity of most aquifers is sufficient to maintain groundwater at approximately neutral pH, under the increased $HCO_3^-/CO_2/OH^-$ loads during ISBD treatment. However, as these experiments demonstrate, this may also depend on OC substrate type. Acetate-mediated denitrification is more likely to require buffering, either by natural rock dissolution processes, or with an extraneous buffer, (described in Chapter 1), compared to either glucose or acetic acid, for example. In the field, additional alleviation of pH rises owing to dilution of the denitrified water plume at the abstraction well could also be expected.

8.3.3 Conductivity

Conductivity is a measure of the amount of dissolved inorganic ions in aqueous solution and is a regulated drinking water parameter with a MAC of 1500 µS/cm (DWI web site, 2005). During Trial IV, where natural groundwater was used to supply microcosms packed with Upper greensand from the same site, electrical conductivity in the effluent and feed solutions were monitored; results are presented in Figure 8.9. During the experimental period between d 9 and 15, however, microcosms were supplied using a batch of groundwater that had been frozen during storage, causing the precipitation of groundwater minerals out of solution. Therefore, the electrical conductivity values measured during this period were artificially low compared to values recorded during the remained of the trial.

Generally, the results showed that in the autotrophic control (no OC) and glucose microcosms, effluent conductivity was similar to the feed solutions. Therefore, neither physico-chemical processes such as dissolution nor biological metabolism of OC, in the case of the glucose treatments, influenced the conductivity.

Contrastingly, microcosms treated with acetic acid consistently gave lower conductivity compared to the influent solution. Thus, a proportion of the dissolved ions in the feed solution were apparently precipitated within the sandstone of the acetic acid microcosms which could be linked to the more alkaline pH of this treatment, compared to the other types of OC (refer also to pH values for acetic acid microcosms, Figure 8.8 b). On the whole, however, the conductivities of the effluent solutions were not markedly different from the feed solutions.

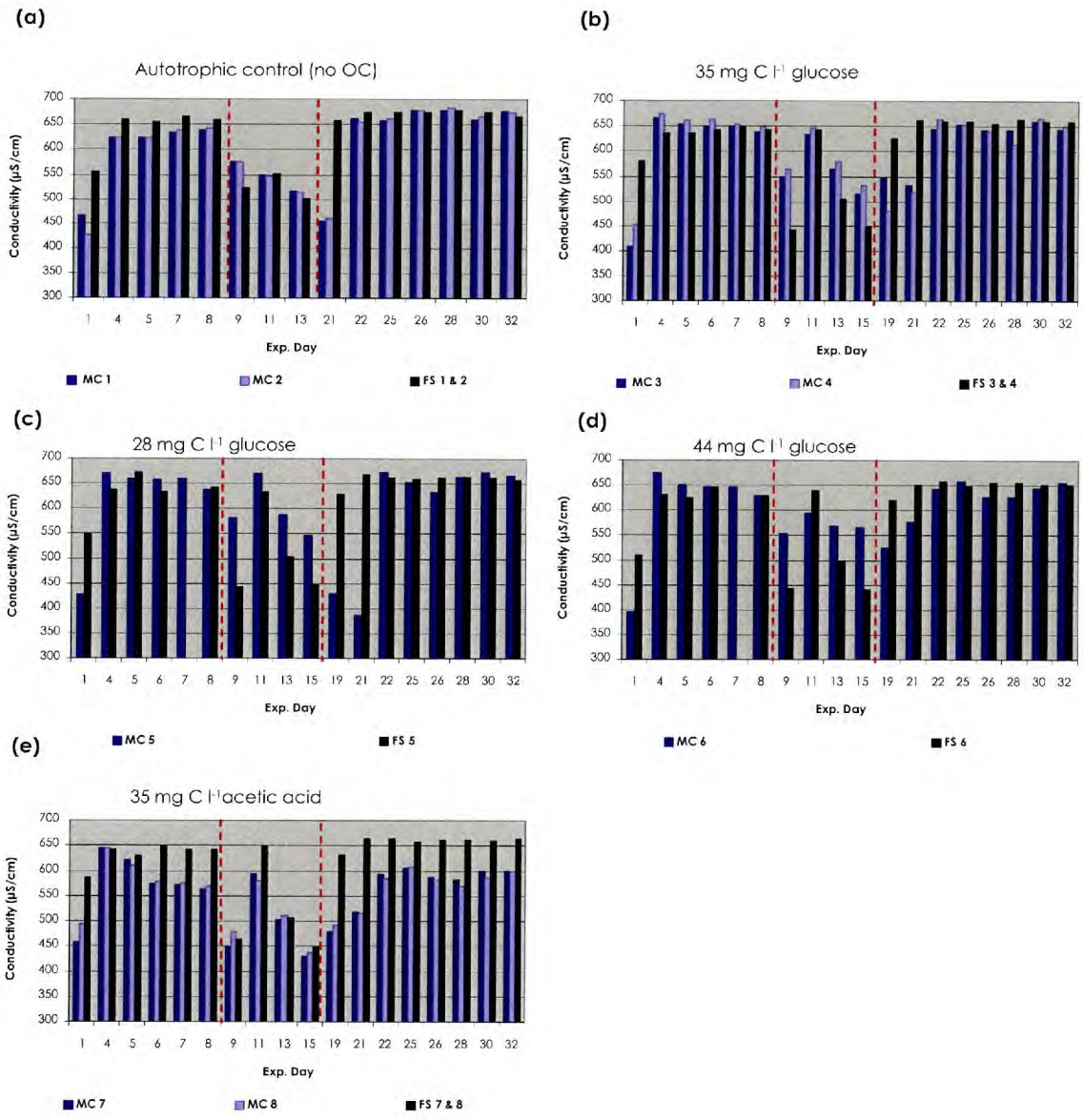


Figure 8.9: Effect of denitrification activity on the electrical conductivity measured in microcosm effluents during Trial IV, compared to their respective feed solutions (treatment conditions indicated on graphs). The period between the red lines denotes a period of low conductivity in both feed solutions and effluents, due the precipitation of groundwater minerals during storage (freezing), as described in Section 8.3.3

8.4 DISCUSSION

The results presented in this chapter demonstrate that stimulated heterotrophic denitrification could potentially affect the following water quality characteristics of the denitrified water:

- Bacterial content, during start up period,
- Turbidity,
- DO (and redox potential),
- pH.

However, although heterotrophic denitrification does influence these water quality parameters, it is pertinent to highlight that generally, the effects are too subtle to pose a risk to public supply resources with regards to drinking water standards. Rather, the relationships between denitrification activity and water quality determinands are of only of scientific interest.

For example, the bacterial concentration in the denitrified water showed a transient increase which was significantly proportional to the concentration of TON in the effluent (results from Trial III). Hence, the concentration of bacteria generally decreased once TON was absent from the effluent. This was initially taken as an indication that the residual TON nutrient in the effluent was providing a substrate for additional heterotrophic biomass growth. If this were the case, then a source of effluent OC would also be necessary. This might have been possible during the first 10 days of the experiment, when the microcosm biomass was just becoming established and both nutrients were present in the effluents, however, during the remaining incubation period, TON and residual influent OC (acetate) were not generally detected in the effluent at the same time (refer to Chapter 4). Furthermore, when considerable breakthrough of acetate occurred (following the increased influent dose on d 37 to remove all TON from effluent), the bacterial concentration of the effluents were not apparently affected, for example, no significant relationships were found using regression analysis.

Therefore, the bacterial concentration in the denitrified water was not simply a function of residual nutrient concentration, for example based on the intuitive assumption that bacterial cells proliferated in the effluent. Alternatively, the effluent bacterial concentrations may reflect physical phenomena such as the removal of biomass from the microcosm sand column under the flow through regime. For example, in this case, the positive correlation of bacterial concentration with effluent TON (where both decreased over time) could indicate that once denitrification

activity established, the biomass developed into a more cohesive biofilm (e.g. Characklis and Cooksey, 1983) with increased structural resistance to transportation out of the sand column, influenced by pore water flow. The consequences of biofilm development during the start up phase of denitrification activity in ISBD or indeed, in drinking water filter applications requires further investigation, for example with regards to drinking water quality.

Ultimately, however, the bacterial concentrations measured during Trial IV demonstrated that there was no significant difference in effluent biomass between autotrophic controls and heterotrophic microcosms. Therefore, from a qualitative perspective, ISBD is not expected to impair the biological quality of the denitrified water.

Similarly turbidity also appeared to be related to the degree of denitrification occurring within the microcosms. For instance, heterotrophic denitrifying microcosms exhibited significantly larger effluent turbidity than the control microcosms (sterile and autotrophic); with exceptions being those microcosms where denitrification efficiency was relatively low (refer to Section 8.2.2), which were not significantly different from the controls.

However, since each microcosm trial had different experimental conditions, it was difficult to develop this association into a definitive relationship. Indeed, in Trials II and III, the turbidity decreased over the incubation period, consistent with the bacterial concentration data and perceived physico-chemical processes such as removal of fine particulate fractions from the sand column and chemical dissolution of minerals, whilst the column equilibrated. In contrast, effluent turbidity from microcosms in Trial IV was generally higher, which may have been a factor of the distinct microcosm conditions, involving natural groundwater and aquifer material that had been dried and ground prior to use in the microcosms (refer to Chapter 3). Moreover, the effluents from the acetic acid microcosms exhibited an inverse relationship between turbidity and TON concentration, which could not be fully elucidated with the experimental data available. It was considered that the increasing turbidity might be related to a product of acetic acid metabolism, since the investigations of EPS content (Chapter 5) showed that acetic acid microcosms had higher carbohydrate content (relative to protein) compared to the glucose treatments, which conversely had a higher protein content (relative to carbohydrate). However, further characterisation of the chemical species present in the effluents would be necessary to resolve this. Again, this may be of scientific interest but in relation to ISBD application, turbidity generally remained below the drinking water quality standard.

With regards to the affects of heterotrophic denitrification on DO concentration and water pH, (i.e. decreased DO and increased pH), these determinands in particular should be attenuated/buffered in the aquifer. For example, with dilution from un-denitrified water at the abstraction point (Mercado *et al.*, 1988), or buffering from aquifer minerals in the case of pH (Dahab, 1993). In addition, pH increases may be avoided through selection of an appropriate substrate, as these results demonstrate.

Alternative chemical aspects of water quality pertinent to ISBD, such as residual NO_2^-, OC breakthrough, and soluble Fe and Mn, which are potentially serious drinking water contaminants have been reviewed in previous Chapters (4 and 6 respectively). It was concluded that residual NO_2^- and OC breakthrough should not occur when the C/N ratio of the ISBD system is controlled at optimal concentrations. Furthermore, under optimal C/N ratio, Fe and Mn concentrations in the denitrified groundwater should not exceed regulatory standards, given the dilution of the denitrified water plume at the abstraction well.

Therefore, the results of these microcosm experiments suggest that the post-abstraction requirements for denitrified water from ISBD would not be beyond the scope of the usual post-abstraction treatments, particularly once attenuation with travel time through the aquifer and dilution factors have been considered. Previously, post-abstraction treatments including: filtration, disinfection and aeration have been advocated, to produce a potable water source from ISBD systems that is compliant with drinking water standards (e.g. Janda *et al.*, 1988; Mercado *et al.*, 1988; Hamon and Fustec, 1991; Dahab, 1993). However, these publications have not included specific details of the water quality parameters included here, and it is not clear whether these treatments are in excess of routine groundwater treatment processes. Furthermore, in some of these publications, there is additional evidence to suggest that the ISBD systems, at field scale, did not achieve optimal C/N ratios. For instance:

- Janda *et al.* (1988) described OC breakthrough, indicating that uniform dispersion of injected substrate was not achieved;
- Mercado *et al.* (1988) concluded that the denitrification reaction was not efficient, hence, C/N ratio was not optimal;
- Hamon and Fustec (1991) experienced severe biofouling with ethanol as the OC substrate (resulting in the termination of the trial), demonstrating that uniform distribution and mixing of OC within the aquifer was not achieved and again, the C/N ratio was sub-optimal.

This demonstrates the fundamental importance of optimisation of the OC injection, in terms of achieving the desired C/N ratios in the field. Additionally, this implies that ISBD process engineering is paramount not only to the denitrification yield, previously the primary focus of system optimisation, but also to water quality.

For example, the results presented in this chapter and in Chapter 4, show that water quality should be an important consideration for substrate selection, in addition to denitrification yields and consumable costs, indeed, costs incurred for comparatively expensive OC compounds, may be recuperated through reduced post-abstraction treatment requirements, if their use results in a higher quality denitrified water. During these experiments, glucose was used because it is an acceptable substance for injection into aquifers, under the UK Groundwater Regulations, and its use was supported by the Drinking Water Inspectorate (DWI) during a feasibility study commissioned by the Environment Agency and Severn Trent Water (Cartmell *et al.*, 2000). Acetate was additionally selected for investigation since it is typically cited as one of the most efficient substrates for aquifer denitrification (e.g. Devlin *et al.*, 2000; ITRC, 2002). However, acetic acid, which has not been commonly applied, perhaps owing to its comparatively higher purchase cost (e.g. Mosheni-Bandpi *et al.*, 1999), gave the best performance results in these experiments, in terms of denitrification yield, influent C/N dosage requirements and denitrified water quality.

Therefore, various design steps may be taken to facilitate high quality denitrified water quality at the outset. If the ISBD plant is operated with water quality as a critical consideration, then post-abstraction treatment requirements should be minimal compared with treatment requirements for water denitrified in biological reactors and should be consistent with the usual treatment processes used for raw groundwater.

CHAPTER 9

SUMMARY DISCUSSION AND CONCLUSIONS

9.1 OVERVIEW OF NITRATE CONTAMINATION IN THE UK

9.2 SUMMARY DISCUSSION OF EXPERIMENTAL RESULTS

9.3 SUGGESTIONS FOR FURTHER WORK

9.4 CONCLUDING REMARKS AND RECOMMENDATIONS FOR ISBD APPLICATION IN THE UK

9.1 OVERVIEW OF NITRATE CONTAMINATION IN THE UK

As discussed in Chapter 1, the increasing trend in the NO_3^- concentration of aquifers exploited for drinking water supplies has led to an increasing requirement for NO_3^- removal treatment. Owing to the protracted time scales involved in NO_3^- transport in groundwater, the present NO_3^- concentrations are forecast to increase in the coming decades (Canter, 1997), before the effects of current preventative land use management strategies are realised.

Biological denitrification in large scale reactors (at treatment plants) has become a popular method of NO_3^- removal European countries such as France and Germany (Mateju *et al.*, 1992). However, the water produced requires extensive post-denitrification treatment to remove residual organics and biomass and therefore have high costs, owing to reactor operation and maintenance, compared to ISBD (refer to Chapter 1). In this regard, this conventional type of biological denitrification in reactors may be more suited to large scale operation where the costs of post-denitrification treatments are more likely to be justified. In the UK, compliance with the drinking water NO_3^- MAC for groundwater sources has been achieved either by blending high NO_3^- groundwater with lower NO_3^- supplies, or alternatively, has been processed using ion exchange (refer to Chapter 1). However, the blending of groundwater resources is not a environmentally sustainable and ion exchange is expensive, with considerable power, consumable and maintenance requirements. Additionally, ion exchange has associated problems with regards to the disposal of the concentrated waste streams generated by the process in a fashion that is economic and environmentally acceptable.

A collection of papers published in the late 1980s/early 1990s demonstrated that heterotrophic denitrification could occur in the aquifer, when stimulated with an appropriate organic carbon source. Thus, the concept of using a small section of aquifer as a biological reactor for ISBD was proposed (Janda *et al.*, 1988; Mercado *et al.*, 1988; Hamon and Fustec, 1991). This scheme would potentially be as effective at removing NO_3^- as biological denitrification in above-ground reactors, but without the associated operational costs, and was considered as being suitable for the treatment of municipal water supplies and at the smaller rural level, as reviewed in Chapter 1. However, though the proposed ISBD schemes were conceptually sound, published examples of this treatment described a variety of application problems at field scale, including OC breakthrough, production of H_2S at the abstraction well (Janda *et al.*, 1988), low denitrification yield (Mercado *et al.*, 1988), but most serious, clogging of

the injection wells due to biofouling which typically resulted in the whole abstraction facility becoming inoperable (e.g. Hamon and Fustec, 1991).

In light of these early results, research and development was apparently focused toward process engineering, for example, assessing different plant configurations, different OC substrates and different OC dosing regimes, such as intermittent compared to continuous injection. During this time, bioremediation as a treatment method for clean up of contaminant plumes became extensively adopted, but use of the method for drinking water treatment was not widely accepted (Dahab, 1993), presumably because of the reticence of the regulators to permit the introduction of extraneous substances into the aquifer environment, particularly those exploited as a potable resource (Dahab, 1993). Furthermore, to date there is a scarcity of quantitative data relating denitrification kinetics (C input and NO_3-N denitrified) to water quality, and biofouling, though work has been carried out to compare intermittent with continuous dosing regimes as a means of preventing this.

These factors have been inhibitive to the development of ISBD technology in the UK, but paradoxically, it has been difficult to secure a field site for the necessary pilot scale research to develop ISBD. Therefore, this PhD project focussed on advancing the technology to the field (pilot) scale, by producing a quantitative body of data to demonstrate the potential water quality issues and biofouling risks pertinent to ISBD under conditions representative of UK aquifers (e.g. temperature, geological type etc.).

9.2 SUMMARY DISCUSSION OF EXPERIMENTAL RESULTS

The results of the microcosm studies and detailed discussions, interpreting the experimental data and comparing it to published information, are presented in Chapters 4-8. This section draws on these detailed discussions to provide a summary of the experimental results from a more holistic perspective, highlighting the relevant issues for application of ISBD in the UK.

Heterotrophic denitrification was stimulated in natural sandstones by supplying the indigenous microbial populations with various OC substrates, glucose, acetate and acetic acid, at temperatures representative of UK aquifer conditions and without the need for additional extraneous nutrients. Therefore, it was shown that bacterial inoculation and extraneous nutrients, which had been advocated by some authors, (Fourmentraux-Chevron *et al.*, 1999) were not required.

The microcosm experiments demonstrated that the C/N ratio achieved in the sandstone matrix was the most influential parameter terms of denitrification yield and also with respect to the quality of the denitrified water. The optimal OC injection regime for ISBD must therefore be calculated so that groundwater C/N ratio is optimal. Measured groundwater concentrations of DO and NO_3^- at the specific site of ISBD application should be used to determine the OC injection dose, according to the reaction stoichiometry, with a supplementary quantity for biomass assimilation. The biomass assimilation fraction is typically estimated at the outset, for example, during these trials; a biomass assimilation factor of 15 % of the total stoichiometric dose was added, according to previous estimates described in the literature (ITRC, 2002). However, in the case of these experiments, this was shown to be inefficient.

For example, under this dosage regime (stoichiometric requirement for DO removal + NO_3^- removal + 15 %) in Trial II, NO_3^- reduction to NO_2^- became the dominant biochemical reaction in the microcosm with generally < 5 mg N l^{-1} denitrified to N_2 (refer to Chapter 4, section 4.3). NO_3^- reduction to NO_2^- is indicative of insufficient OC (electron donor), where NO_3^- reduction dominates since NO_3^- reductase is thought to be more competitive than NO_2^- reductase (Almeida *et al.*, 1995; Burns *et al*,. 1995; as discussed in Chapter 1). In this instance, the electron shortage produced similar denitrification kinetics, regardless of substrate type; similar denitrification results were observed in both acetate and glucose treatments (Chapter 4, section 4.3). Furthermore, the microbial community information elucidated using molecular techniques, showed that the dominant members of both acetate and glucose fed communities were similar (Chapter 7).

At this level, the denitrifying community existed at the threshold of C-limitation, and therefore was sensitive to slight changes in C availability. For example, it was proposed that in these experiments, differential DO concentration in the influent solution caused an alternating pattern of periods of NO_3^- reduction to NO_2^-, and periods of no NO_3^- reduction activity at all. Similar concentration dynamics were also reported by Dahab (1993), as a result of DO penetration into the laboratory aquifer model. Under these conditions, increased incubation time simply increased the magnitude of the NO_3^- reaction to NO_2^-, and no net denitrification was observed. This could only be achieved with an increased influent OC dose of 15 mg C l^{-1} (refer to Chapter 4).

These experiments showed that sustained NO_2^- production therefore represents a considerable threat to groundwater quality, where concentrations severely in excess

of the drinking water MAC (0.15 as NO_2-N; SI: 3184, 2000) were observed. The carbon limiting conditions were primarily due to an underestimation of the amount of carbon consumed in biomass assimilation, and in subsequent trials, higher doses were used, based on the empirical data from microcosm Trial II, rather than on the reaction stoichiometry.

With higher OC doses, 35-40 mg C l^{-1}, NO_2^- accumulation was transient as anticipated and complete denitrification was achieved (refer to Chapter 4). At these higher doses, when the system was no longer C-limited, differences in efficiency between the various OC substrates became apparent. For example, complete denitrification (100 % yield) was not sustained in microcosms supplied with glucose, although it was achieved incidentally, in spite of increasing the influent dose to the point of breakthrough (Chapter 4, Section 4.5.4). It was speculated that the less efficient denitrification activity observed in the microcosms supplied with glucose may have been due to competition for electrons from fermentative bacteria Conversely, complete denitrification was sustained in acetate-fed microcosms at an influent concentration of 35-40 mg C l^{-1}, and in the acetic acid treatments, the influent dose could actually be reduced once complete denitrification had been established (Chapter 4, Section 4.5.5).

When acetic acid was used as the carbon substrate, the start up stage was comparatively shorter; 13 days compared to 21 ± 2 d with glucose or acetate. During the start up phase, (time taken for the microbial community to become established), the treated water is expected to contain residual NO_2^- which subsequently decreased following the onset of complete denitrification (Hamon and Fustec, 1991; Bates and Spalding 1998; Abdelouas *et al.*, 1999; Nutall *et al.*, 1999; refer to Chapter 4, Section 4.6.4). The microcosm results also showed that bacterial concentration in the effluents appeared to increase during the start up phase, but showed no significant difference compared to the autotrophic control microcosm once complete denitrification was established (Chapter 8, Section 8.2.1). Interestingly, the bacterial content of the microcosm effluents did not appear to be affected by prolonged periods of carbon breakthrough, where intuitively, it was presumed that the OC nutrient might result in bacterial proliferation (refer to Chapters 4, Section 4.4 and Chapter 8, Section 8.2.1).

Furthermore, it was shown that under OC rich conditions, simulated during periods of OC breakthrough, denitrification to N_2 prevailed as the dominant denitrification pathway. The alternative denitrification reaction, DNRA, was not evident, since NH_4^+ was not detected in any microcosm effluent (refer to Chapter 4, Section 4.6.3).

In addition to carbon breakthrough, a residual DOC concentration of ± 4 mg l^{-1} was detected in all microcosm effluents, (except sterile controls), including both autotrophic and heterotrophic denitrifying microcosms. This residual OC was defined as the lowest concentration observed in effluents from heterotrophic microcosms and the background concentration detected in effluents from autotrophic control microcosms, which were not supplied with OC. The nature of this residual was not determined in the scope of these experiments, but demonstrated that heterotrophic denitrification did not contribute additional residual organic carbon, compared to the autotrophic control, once complete denitrification activity was established.

Other aspects of water quality that were affected by heterotrophic denitrification activity were: pH, conductivity, turbidity, DO and soluble Fe^{2+} and Mn^{2+} concentrations. In the case of pH, conductivity and turbidity, substrate type appeared to be the decisive factor, whereas soluble Fe and Mn concentrations were influenced more by substrate concentration.

For example, microcosms supplied with acetic acid and glucose produced effluents of near neutral pH throughout the experiment, whereas pH in effluents from the acetate microcosms increased to approximately pH 9.5 (Chapter 8, Section 8.3.2). Similarly, acetate has been previously observed to increase conductivity during ISBD operation (ITRC, 2002), presumably due to its ionic form. Alternately, the conjugate acid of this salt, acetic acid, did not alter the conductivity of the denitrified water, compared to influent values, and neither did glucose (Chapter 8, Section 8.3.3). Turbidity was also apparently influence by substrate type, where the microbial metabolism of acetic acid was related to increased effluent turbidity over the experimental incubation time. However, the inverse was observed in glucose and acetate microcosms, and turbidity decreased over the duration of the experiment (Chapter 8, Section 8.2.2). Therefore, selection of OC substrate was shown to be of importance with regards to final water quality of denitrified water, in addition to being important to denitrification kinetics.

The water chemistry of oxidised aquifer species such as Fe^{3+}, Mn^{4+} could not be completely defined from the experimental results. However, it was suggested that the reductive dissolution of Fe^{3+} and Mn^{4+} was only detected in appreciable concentrations when the OC influent dose was in excess of the denitrification requirements, to the point of carbon breakthrough (Chapter 6). This carbon excess situation would in any case represent a failure of the ISBD system and plant configuration should be designed to avoid this. Under optimal denitrification

operating conditions, a small quantity of Mn^{4+} dissolution was observed, and soluble Mn^{2+} was detected in the effluents (Chapter 6, Section 6.2.1), this was also observed by Hamon and Fustec, (1991) and Abdelouas *et al.* (1999). Reduction of SO_4^{2-} was not detected in the microcosms, in accordance with (Mosheni-Bandpi *et al*,. 1999). The small qualities of $Mn^{2+}_{(aq)}$ present in the denitrified water, were not in excess of drinking water standards (SI: 3184, 2000) and moreover, the dilution of denitrified water plumes with unamended groundwater during the abstraction processes would further decrease the concentration. The dilution of denitrified water with un-treated groundwater would occur as a consequence of the ISBD plant figuration, described in Chapter 1 (Section 1.7.2). This is also reported to increase the dissolved oxygen concentration of the water, which becomes depleted during denitrification, to that of the ambient groundwater (Mercado *et al.*, 1988).

With regards to biofouling, the hydraulic conductivity of the heterotrophic denitrifying microcosms was decreased during the experimental incubation periods, compared to the controls; however, this reduction was small compared to that observed in laboratory sand columns by Vandevivere and Baveye, 1992 a). The observed reduction in hydraulic conductivity in the heterotrophic microcosms may also have been exacerbated by production and entrapment of gas bubbles, (refer to Chapter 5, Section 5.2.1). Indeed, the production of gasses resulted in changes to the rheological properties of the sand column and ultimately, preferential flow channels were formed.

Pertinently, no clogging was detected in the porous sandstone matrix; however, this did occur in the feed solution inlet tubing of the glucose microcosms. This anecdotal evidence suggested that the risk of biofouling to the ISBD plant could be minimised with selection of an appropriate substrate, for example, from these experiments, biofilm deposits were only observed in the inlet apparatus of microcosms fed with glucose or acetate (accumulating to the extent of complete blockage in the glucose microcosms), as described in Chapter 5, Section 5.3. Therefore, acetic acid emerged as a superior substrate from a biofouling perspective. Interestingly, there were also differences in the nature of the EPS recovered from glucose and acetic acid microcosms, where glucose microcosms had higher protein content in the EPS and acetic acid had higher carbohydrate content.

Previously, authors have advocated the use of an intermittent dosing regime to minimise biofouling risks (Hamon and Fustec, 1991; Peyton, 1996).

However, whilst this was not specifically investigated in these experiments, the results did indicate that phases of operational recess, where microcosm pumping was terminated for up to 48 h, were followed by transient increases in effluent DOC, though denitrification capability was complete resumed (within 24 hours; refer to Chapter 4, Section 4.6.8). Therefore, the implication of intermittent substrate injection for water quality requires further investigation.

9.3 SUGGESTIONS FOR FURTHER RESEARCH

In the UK, ISBD now needs to progress from the laboratory scale to the field stage, although continued laboratory microcosm studies are necessary to further quantify the biochemical reactions with specific regard to parameterisation of ISBD models. For these purposes, however, the microcosms would need to be more sophisticated than those used for these experiments.

For example, a series of permanently fixed taps or rubber septa vertically spaced along the length of the microcosm should be incorporated, to facilitate sampling of the interstitial solution, in addition to the final microcosm effluent. This would allow more precise monitoring of the microcosm water chemistry, and hence, the progression of the denitrification reaction, along the spatial length of the sand column. Additional ports for the permanent location of on-line probes to monitor DO, redox, pH, temperature and pressure should also be built in (this would require additional data logging equipment). Furthermore, the delivery of microcosm feed solution could be altered to allow the OC substrate to be injected into the microcosm separately from the NO_3^- solution to facilitate monitoring of biofouling in conditions more representative of the field. It would be advantageous to increase the length of the sandstone column, to compare the changes in the microcosm water chemistry with additional residence time within the sand matrix. However, this could make it increasingly difficult for the gas bubbles produced nearer the base of the microcosms to ascend and be vented at the top. Therefore, an extended microcosm would require a gas-venting mechanism incorporated along the column length, or two standard length microcosms in sequence could be utilised, where the effluent from the first would be subsequently passed through the second microcosm. Finally, a series of sampling ports of similar design to those in the existing microcosm apparatus spaced uniform would facilitate the sampling of sandstone material, for example collected as a core (e.g. 1 g wet weight).

With an improved microcosm apparatus, experiments to further quantify the biochemistry of the denitrifying microcosms could be carried out as listed below:

- Quantification the effect of OC injection and denitrification on the mineral speciation within the denitrification treatment zone and subsequently, within the more oxygenated zone towards the abstraction point, with relevance to Fe, Mn and SO_4^{2-}. This would require quantitative speciation of the microcosm mineral content, before and after the experiment and it would be imperative that the groundwater and the aquifer material was collected from a site of similar chemistry (and may additionally incorporate an equilibrium period where the water was recycled through the column). The interstitial solution could be sampled from the microcosm taps into a nitrogen flushed vessel (which may incorporate a 0.45 µm filter) and subsequently, analysed for total Fe and Mn in addition to Fe and Mn ions. Additionally, an analytical method capable of measuring DOC to a higher resolution than 1 mg C l^{-1} would be required to help unify the mass balance.
- Quantification and speciation of biomass at different spatial locations within the microcosm, to create a more comprehensive representation of the microbial community interactions within the sandstone. Ultimately, more quantitative knowledge regarding the behaviour of the microbial community in response to environmental factors such as nutrient availability would afford more informed control over the ISBD process. Such information might explain the various differences in denitrification yield and water quality exhibited when different OC substrates are used, for instance. Furthermore, it might help explain why certain ISBD trials have failed, for example with regards to biofouling. This analysis of the microbial community would be facilitated by the collection of cores from the microcosm which could subsequently be analysed using a community fingerprint approach, since these experiments demonstrate that this kind of analysis is feasible with sandstone samples (Chapter 7). It may be also be possible to further define the microbial community with more in depth analysis using group-specific probes and moreover, to further develop the qualitative analysis into a quantitative technique such as fluorescent *in situ* hybridisation.
- Quantification of the effects of specific OC injection regimes (i.e. continuous and intermittent) on the microbial ecology of the ISBD system and therefore on the water quality. For instance, though intermittent injection regimes might promote further penetration of the OC substrate into the aquifer (Peyton, 1996), these experiments shown that after such periods of forced endogenous respiration, the DOC concentration of the denitrified water was temporarily increased. In addition, previous research has shown that intermittent dosing regimes caused

transient increases in the amount of N_2O liberated from laboratory microcosms (Cartmell, 1997). Since N_2O is a greenhouse gas and monitored under air quality emissions budgets (refer to Chapter 1), this should be investigated further.

In addition, the microcosms could be used to develop existing rapid monitoring techniques specifically in terms of ISBD application, to increase the use of feed-back mechanisms for control of the ISBD plant. For instance, a rapid method of bacterial enumeration in the denitrified water would be particularly useful to monitor water quality at the ISBD site. Although the HPC is used as the reference method for water during treatment (Boulos *et al.*, 1999), this is a relatively intensive laboratory procedure and would not be suitable for elucidating the bacterial counts of denitrified water from ISBD plants on a quick turn-around monitoring basis. Initial steps were taken in the context of the experiments reported here, towards the application of a direct count staining method, using the fluorescent stain LIVE/DEAD® *Bac*Light™, which stains both dead and viable cells. The *Bac*Light™ kit has been applied to wastewater operations (Vollertsen *et al.*, 2001), water quality evaluation of raw and potable water resources (Boulos, *et al.*, 1999; Hoefel, *et al.*, 2003) and bottled waters (Ramalho *et al.*, 2001). Published results have indicated that bacterial counts made with the *Bac*Light™ kit are comparable with counts made using the more conventional Acridine Orange stain (Boulos *et al.*, 1999), however, the *Bac*Light™ kit has the advantage of not requiring any specific sample preparation or incubation, compared to Acridine Orange and Dapi stains (Boulos *et al.*, 1999), and thus is more versatile as a rapid laboratory technique.

In terms of application of ISBD in the UK, more work is needed at the field scale to advance the technology. Specifically, a demonstration programme is required to show the efficacy of the treatment and enable process engineering at the field scale. This might involve installation of a scaled down ISBD plant for research purposes, or may involve the collaboration with a Water Utility at an existing borehole, but in this case, it may be prudent to select a site which would not threaten an existing public supply. At this scale, a more representative study of the carbon injection regime could be conducted with regards to intermittent dosing regime and its affect on water quality, for example with regards to the microbial biomass concentration. Alternatively, borehole regeneration schemes involving biocides and bleaches could be investigated.

In an alternative approach to ISBD based on the heterotrophic denitrification reaction, it would be prudent to further investigate the water quality issues associated with autotrophic denitrification. Although some research has been published with

regards to the kinetics of the reaction in limestone reactors supplemented with elemental sulphur, this type of biological denitrification has generally been disregarded owing to the comparatively lower reaction rates (refer to Chapter 1). Since some of the UK aquifers used to provide drinking water resources are located within limestone or chalk aquifers, autotrophic denitrification could prove successful in these instances.

9.4 CONCLUDING REMARKS AND RECOMMENDATIONS FOR ISBD APPLICATION IN THE UK

The results from the microcosm experiments highlighted the importance of precise control over the C/N ratio within in the denitrification treatment zone, to facilitate maximum control over the denitrification reaction. The engineering of ISBD systems, for the purposes of NO_3^- removal for potable supply, therefore requires a prescribed approach, accounting for and responding to fluctuations in the groundwater chemistry at the site. However, whilst the specified C/N ratios may be easily reproduced under laboratory conditions, it is clearly more difficult to achieve the desired C/N ratio in the field and there are numerous reports of successful laboratory bioremediation experiments that subsequently fail when engineered at field scale (Sturman *et al.*, 1995). In this regard, it is essential to account for the different processes that affect ISBD at different scales, for example, at the laboratory scale, C/N ratio is paramount, but at field scale, this in turn becomes a function of the mixing of injected OC and groundwater NO_3^- within the aquifer.

The mixing of injected OC with NO_3^- in the groundwater is governed *in situ* by aquifer hydrogeology (Devlin and Barker, 1994), and in porous media such as aquifer matrices, mixing is facilitated by physical processes such as dispersion and diffusion. These processes can be conceptualised and modelled in porous media that is homogenous, and subsequently, the hydrogeology of the ISBD site can be characterised and a suitable ISBD plant designed accordingly. Thus, in homogenous media, it may be possible to achieve the uniform distribution of OC (Tompkins *et al.*, 2001) and subsequent mixing with NO_3^-, which is essential to the success of ISBD. The majority of previous research into ISBD has previously concentrated on relatively isotropic, granular matrix, single porosity aquifers such as un-cemented sandstone, or shallow aquifers of alluvial and gravely deposits (for example, refer to Table 1.5, detailing ISBD field trials, Chapter 1).

However, the intrinsically heterotrophic nature of aquifer rock even within essentially granular rock types, such as sandstone, complicates solute transport processes (Devlin and Barker, 1994) and leads to inaccuracies in hydrogeological transport models. If the aquifer matrix within the ISBD reaction zone has areas of anisotropy, for instance caused by the presence/absence of intercalating minerals such as Calcite; the dispersion of solutes within the matrix may differ from the ideal. As a consequence of this, groundwater within the ISBD treatment zone may flow at different velocities or along preferential flow paths, potentially by-passing much of the aquifer matrix. In anisotropic aquifer environments, it is therefore difficult to ensure uniform distribution of substrate and necessary residence time of the OC amendment within the aquifer and hence, to control denitrification activity (Mateju *et al.*, 1992).

In situ biological denitrification is therefore suited to homogenous/isotropic aquifers, of essentially granular matrices and primary porosity. It is vital that the plant is designed and operated according to the specific site and that comprehensive hydrogeological modelling of the solute transport and mixing processes within the ISBD treatment zone are completed before pilot operations are installed. Previous proposals of ISBD in the UK appear to have involved the adaptation of existing public supply wells for the pilot trial (e.g. Cartmell *et al.*, 2000), thus failure of the pilot trial would render the existing supply well inoperable. As a variation of this approach, it is suggested that the treatment could be used at existing facilities, with smaller, subsidiary wells, therefore removing the threat to the main public supply. In this instance a smaller treatment could be used to provide low NO_3^- water for blending with the main resource so that the final concentration of NO_3^- would be compliant with the drinking water standard, as opposed to the use of ISBD to completely denitrify groundwater abstracted from the main supply well. Furthermore, a smaller sized plant configuration should reduce the risk of sub-optimal dispersion and mixing of injected OC, caused by anisotropic rock formations, due to the smaller overall volume of rock utilised. Under this approach, if a site exists with suitable hydrogeology, it could be exploited by a series of small scale ISBD systems to supply a larger volume of low NO_3^- water for blending at larger water supply treatment works. This concept should additionally be further investigated as a recommendation of this work.

In conclusion, ISBD can provide an environmentally sustainable and economic alternative to above-ground treatments for NO_3^- removal from groundwater, such as ion exchange and reactor-based biological denitrification. However, a diversification of the original concept and flexibility of approach is essential, with potential applications considered on the merits of the individual site.

As part of the PhD project, original contributions to the current knowledge of ISBD reactions have been made as follows:

- PCR and DGGE methods have been successfully applied to sandstone collected from UK aquifers, with relatively low biomass concentrations. DNA sequences recovered from the microcosm samples were compared to an international microbial sequence database, and the closest matches were reported, to infer the likely speciation of the dominant community members.
- This molecular approach also enabled elucidation of the microbial community structure of the most abundantly represented species. Differences in community structure were primarily due to the concentration of OC that each community received, and were not affected by either geological sandstone type or substrate type or, as previously suggested on the basis of culture based identification methods.
- Finally, the use of pressure sensors for hydraulic conductivity determination and methods previously used to detect EPS in activated sludge have been successfully applied to the sandstone matrix, in the context of laboratory microcosm studies of low substrate-loading biological denitrification for drinking water purposes.

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APPENDIX 1: Details of Aquifer Resources from Fovant Site

1.1: Drilling details and Local groundwater Conditions, (details from Clark, L. *et al*, 2004)

The drilling of Borehole 4A started on the 3rd February and was completed on the 6th February. The borehole was drilled to 3.10m at 200mm diameter and temporary casing was set at that depth. Drilling then progressed at 150mm diameter by the rotary technique using a button bit and air-mist flush through the Chalk to the water table. The water is recorded as struck at 19.20m but rising to 14.90m from the surface. The base of the Chalk in the driller's log is at 17.10m. The drilling continued in the Greensand Aquifer to 21m and then four cores of the sandstone were taken (Table 1). Groundwater characteristics from the water at the drilled borehole are reported in Table 2.

Table 1

Core Number	Cored Interval (m)	Core Recovery (%)
1	21 to 22.5	53
2	22.5 to 23.85	52
3	23.85 to 25.35	87
4	25.35 to 26.85	91

Table 2

GROUNDWATER QUALITY. LABORATORY ANALYSES (Source: Clark, L. *et al.*, 2004)							
Date	Time	T Coli/100ml	F Coli/100ml	PI Count 37,2	PI Count 22,3	Turbidity	Conduct 20 C
22-Mar-04	13:33	0	0	1	138	0.64	440
23-Mar-04	13:40	0	0	0	54	<0.25	435
24-Mar-04	13:55	0	0	0	118	<0.25	448
25-Mar-04	14:30	0	0	1	138	<0.25	421
Date	Time	pH	Total hard	Chloride	Na mg/L	Ca mg/l	Mg mg/l
22-Mar-04	13:33	7.4	249	15	7	90.4	1.4
23-Mar-04	13:40	7.3	246	15	7	95.9	1.5
24-Mar-04	13:55	7.4	231	15	6	89.9	1.4
25-Mar-04	14:30	7.3	232	16	6	90.4	1.4
Date	Time	K mg/l	Nitrite	Nitrate as N	Ammonia	Phosphate	Fe
22-Mar-04	13:33	0.8	<0.01	5.7	<0.01	0.05	0.01
23-Mar-04	13:40	0.9	0.005	6	<0.01	<0.03	0.08
24-Mar-04	13:55	0.8	<0.003	5.65	<0.01	0.03	0.01
25-Mar-04	14:30	0.8	<0.003	5.66	<0.01	0.05	0.01
Date	Time	Mn	Cu	Zn	Alkalinity MO	TOC	
22-Mar-04	13:33	<0.005	<0.01	0.02	180	1.7	
23-Mar-04	13:40	<0.005	<0.01	0.03	213	2.2	
24-Mar-04	13:55	<0.005	<0.01	0.02	208	1.4	
25-Mar-04	14:30	<0.005	<0.01	0.02	161	1.4	
ON-SITE QUALITY MONITORING							
Date	Time	Conductivity	pH	Dissolved	EH (Redox)	Temp	
		mg/L		Oxygen mg/L	mV	oC	
24-Mar-04	09:00	411	7.23	7.3	216.7	10.3	
24-Mar-04	15.15	432	7.32	8.2	230.2	10.5	
25-Mar-04	08:00	464	7.32	6.8	179.7	10.3	
25-Mar-04	16:00	464	7.32	6.7	233.7	10.3	
26-Mar-04	08:30	474	7.32	6.3	258.9	10	

1.2: LITHOLOGICAL LOG OF FOVANT CORES

Core drilled on: 04-02-04
Log carried out on: 06-02-04
Photographed on: 07-02-04

The core was approximately labelled by the driller in 4 sections (water table at ~ 20 m):

- **Core 1;** Uppermost section – '21.0 – 22.5 mbgl'
- **Core 2;** Upper middle section – '22.5 – 23.85 mbgl'
- **Core 3;** Lower middle section – '23.85 – 25.35 mbgl'
- **Core 4;** Lowest section – '25.35 – 26.85 mbgl'

In general, the core was very broken up and some of the core cases were filled with broken up pebbles. There were traces of clay mineral throughout. The colours of each core were also scored for reference (British Standard Colour Chart for Soils: BS381C). Lithological details for each core are recorded in Table 3, geological descriptions from Dr. M. DeFreitas, Dept. of Civil Engineering, Imperial College, London, UK, pers. comm.

Table 3:

Length along sample	**Description (Core 1 ~ '21.0 - 22.5 M' – total core length = 90 cm)**
0–50 cm	Soft glauconitic sand (disturbed by drilling?) colour#442 light bronze green. Weak, some clay mineral present
50-60 cm	Crumbled region of weakly cemented glauconitic sand
60-90 cm	Weakly cemented calcareous glauconitic sand, bedded and laminated, highly porous, appears free of clay minerals, moderately strong.
Length along sample	**Description (Core 2 ~ '22.5 - 23.85 M' – total core length = 86 cm)**
0-60 cm	Core very disturbed. Cobble and pebble size pieces of well cemented dark glauconitic sand, carbonate-free, mixed with smaller fragments of glauconitic sand cemented with calcium carbonate. Colour# 278 light olive green and 283 aircraft grey green
50-86 cm	Revealing presence of jointed cemented sections, all much disturbed by drilling. Evidence of calcareous cemented sections mixed with clayey minerals and sand. Some rusty coloured iron oxide striations. Colour# 278 light olive green, 411 middle brown, 627light aircraft grey in calcareous sections
Washed out clay and sand	When the core was washed to observe the rock, these clays sand chunks were collected. Colour# 222 light bronze green
Length along sample	**Description (Core 3 ~ '23.85 – 25.35 M' – total core length = 86 cm)**
0-20 cm	Mixture of dark green, coarse, hard/strong well cemented glauconitic sand with small traces of calcium carbonate. Colour# 283 aircraft grey green
20-22 cm	Clayey sand horizon. Colour# 283 aircraft grey green
22-45 cm	Green silty fine glauconitic sand, laminated calcium carbonate cement. Mixed with sections of well cemented calcareous brown sandstone, possibly from higher up the borehole where it would have been weathered. Clay minerals present. Colour# 283 light aircraft green and 630 French grey
45-55 cm	Laminated shelly medium grained sandstone. Dark laminations – possible traces of organic material. Clayey. Colour# middle brown.
55-86 cm	Increasingly well cemented with calcium carbonate to produce hard/strong nodular cemented sandstone. No clay, quite porous. Colour# 389 Camouflage beige, 676 Light weatherwork grey, 630 French grey. Clay/sand band at very end.

Length along sample	Description (Core 4 ~ '25.35 – 26.85 M' – total core length = 135 cm)
0–20 cm	Fine grained moderate – well cemented glauconitic sand. Colour# 258 Light buff, 389 Camouflage beige and 630 Light French grey
20-45 cm	Well cemented calcareous sandstone containing in-filled burrows of dark quartz rich faecal deposits. Colour# 411 Middle brown, 642 Night and 671Middle graphite.
45-70 cm	Well cemented calcareous laminated fine grain sandstone. Moderately strong. Colour# 411 Middle brown.
70-90 cm	Laminated cemented calcareous light brown sandstone with occasional Chert pebbles. Colour# 630 French Grey
90-106 cm	As above, without Chert pebbles.
106-135 cm	Well cemented slightly laminated calcareous sandstone, sometimes modular. Extremely clayey/fine sands at extreme bottom.

APPENDIX 2: Raw denitrification data (for Chapter 4)

2.1 RAW C and N CONCENTRATION DATA (Microcosm Effluents)

Trial I		MC 1 (mg l^{-1})				MC 2 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
04-02-02	2	11.69	11.69	0.00	23.00	11.57	11.57	0.00	4.90
05-02-02	3	13.14	13.14	0.00	23.11	13.04	13.04	0.00	2.90
06-02-02	4	12.69	12.69	0.00	23.90	16.51	16.51	0.00	5.10
07-02-02	5	16.39	16.39	0.00	22.10	16.95	16.95	0.00	3.64
08-02-02	6	14.83	14.83	0.00	23.88	15.32	15.32	0.00	2.30
09-02-02	7	15.69	15.69	0.00	25.81	14.64	14.13	0.00	-
10-02-02	8	20.84	20.84	0.00	27.20	17.11	17.11	0.00	3.90
19-02-02	15	17.47	17.47	0.00	26.99	12.49	12.49	0.00	-
22-02-02	18	17.80	17.80	0.00	27.80	17.48	17.48	0.00	2.80

Trial I		MC 3 (mg l^{-1})				MC 4 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
04-02-02	2	11.45	11.45	0.00	22.40	10.94	10.94	0.00	21.70
05-02-02	3	12.77	12.77	0.00	16.20	13.16	13.16	0.00	16.20
06-02-02	4	15.15	15.15	0.00	17.50	15.98	15.98	0.00	18.50
07-02-02	5	14.81	14.81	0.00	-	14.38	14.38	0.00	-
08-02-02	6	15.42	15.42	0.00	10.00	15.16	11.77	3.39	3.30
09-02-02	7	15.17	11.44	3.73	-	13.58	6.11	7.47	-
10-02-02	8	17.91	11.96	5.95	4.70	16.54	8.16	8.37	4.30
19-02-02	15	7.35	2.38	4.97	-	3.49	1.40	2.09	-
22-02-02	18	10.43	2.26	8.17	3.70	11.27	3.83	7.44	2.70

Trial I		MC 5 (mg l^{-1})				MC 6 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
04-02-02	2	10.16	10.16	0.00	14.10	10.56	10.56	0.00	12.60
05-02-02	3	12.44	12.44	0.00	7.10	12.87	12.87	0.00	7.50
06-02-02	4	14.86	14.86	0.00	3.20	14.85	14.85	0.00	4.50
07-02-02	5	16.25	16.25	0.00	-	14.66	14.66	0.00	-
08-02-02	6	17.20	17.20	0.00	2.70	17.36	17.36	0.00	3.30
09-02-02	7	17.05	17.05	0.00	-	17.17	13.82	3.35	-
10-02-02	8	16.93	16.93	0.00	4.80	15.56	15.50	0.07	6.80
19-02-02	15	15.39	11.37	4.02	-	15.10	11.52	3.58	-
22-02-02	18	14.62	10.02	4.60	2.50	15.58	11.67	3.92	3.70

Trial I		MC 7 (mg l^{-1})				MC 8 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
04-02-02	2	11.40	11.40	0.00	36.40	10.21	10.21	0.00	34.65
05-02-02	3	12.44	12.44	0.00	35.00	12.45	12.45	0.00	31.60
06-02-02	4	14.66	14.66	0.00	26.10	-	-	-	17.30
07-02-02	5	14.17	13.92	0.24	-	14.92	14.45	0.47	-
08-02-02	6	14.93	11.03	3.91	16.70	14.97	12.54	2.43	10.88
09-02-02	7	13.96	5.98	7.98	-	-	-	-	-
10-02-02	8	15.27	7.67	7.60	6.80	14.31	4.97	9.34	6.50
19-02-02	15	5.84	0.90	4.94	-	5.27	0.93	4.34	-
22-02-02	18	4.71	0.26	4.45	4.70	3.04	1.31	1.73	2.80

Trial II		MC 1 (mg l^{-1})				MC 2 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
29-05-02	3	15.63	15.63	0.00	25.00	10.69	10.69	0.00	2.15
30-05-02	4	13.45	13.45	0.00	25.68	14.31	14.27	0.00	1.77
31-05-02	5	12.25	12.25	0.00	25.67	12.01	11.98	0.00	1.95
01-06-02	6	15.00	15.00	0.00	20.70	11.75	11.71	0.00	1.71
02-06-02	7	16.06	16.03	0.03	19.82	15.40	15.40	0.00	1.51
03-06-02	8	16.51	16.51	0.00	21.75	16.80	16.78	0.00	1.93
05-06-02	10	14.41	14.40	0.01	23.47	15.95	15.94	0.00	2.00
08-06-02	13	12.49	12.49	0.00	18.50	15.62	15.62	0.00	1.19
11-06-02	16	13.82	13.82	0.00	21.73	20.44	20.44	0.00	1.51
14-06-02	19	15.14	15.14	0.00	17.24	15.78	15.78	0.00	2.55
16-06-02	21	16.10	16.10	0.00	20.11	16.07	16.07	0.00	3.01
18-06-02	23	15.74	15.74	0.00	22.35	16.02	16.02	0.00	2.75
21-06-02	26	15.77	15.77	0.00	20.85	15.74	15.74	0.00	1.94
23-06-02	28	15.85	15.85	0.00	20.63	15.93	15.93	0.00	1.90
26-06-02	31	15.43	15.43	0.00	20.56	16.10	16.10	0.00	2.57
28-06-02	33	16.45	16.45	0.00	17.60	14.18	14.18	0.00	3.24
16-07-02	36	16.77	16.77	0.00	20.27	16.14	16.14	0.00	2.18
20-07-02	40	14.96	14.96	0.00	21.03	15.35	15.35	0.00	2.45
22-07-02	42	14.41	14.41	0.00	18.55	13.85	13.85	0.00	1.04
25-07-02	45	14.87	14.87	0.00	19.23	14.16	14.16	0.00	1.79
27-07-02	47	14.94	14.94	0.00	19.76	11.65	11.65	0.00	1.94
29-07-02	49	11.77	11.77	0.00	17.50	13.91	13.91	0.00	1.30
31-07-02	51	16.63	16.63	0.00	18.65	15.35	15.35	0.00	1.88
03-08-02	54	13.42	13.42	0.00	17.06	15.02	15.02	0.00	1.51
08-08-02	56	16.79	16.79	0.00	19.97	20.30	20.30	0.00	1.29
10-08-02	58	11.58	11.58	0.00	21.00	15.05	15.05	0.00	0.91
12-08-02	60	14.34	14.34	0.00	18.96	15.00	15.00	0.00	1.78
25-08-02	64	15.53	15.53	0.00	22.62	14.84	14.48	0.00	1.40
27-08-02	66	15.11	15.11	0.00	21.18	15.67	15.67	0.00	1.55
31-08-02	70	11.96	11.96	0.00	19.98	14.97	14.97	0.00	1.58
02-09-02	72	15.55	15.55	0.00	20.21	14.88	14.88	0.00	0.00
04-09-02	74	15.99	15.99	0.00	24.23	11.89	11.89	0.00	0.00
10-09-02	76	15.45	15.45	0.00	20.70	15.41	15.41	0.00	3.12
12-09-02	78	14.48	14.48	0.00	20.73	16.03	16.03	0.00	1.54
13-09-02	79	14.70	14.70	0.00	26.94	15.73	15.73	0.00	1.47
14-09-02	80	15.38	15.38	0.00	21.18	15.01	15.01	0.00	2.12
16-09-02	81	15.12	15.12	0.00	22.91	15.20	15.20	0.00	1.56
20-09-02	84	15.16	15.16	0.00	20.07	15.09	15.09	0.00	1.48

Trial II		MC 3 (mg l^{-1})				MC 4 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
29-05-02	3	11.18	11.11	0.07	20.47	14.46	14.43	0.03	24.33
30-05-02	4	13.09	12.98	0.11	14.08	13.11	13.05	0.06	20.42
31-05-02	5	16.25	16.00	0.25	4.64	15.85	14.57	1.28	4.73
01-06-02	6	14.72	14.51	0.21	15.34	12.25	12.22	0.03	5.17
02-06-02	7	15.29	13.33	1.96	9.61	6.63	4.39	2.24	3.00
03-06-02	8	8.42	5.84	2.58	6.02	15.08	12.69	2.39	3.35
05-06-02	10	14.47	11.88	2.59	10.22	9.71	8.54	1.17	1.80
08-06-02	13	10.90	1.56	9.34	5.15	11.27	7.17	4.10	4.34
11-06-02	16	9.61	4.01	5.60	9.50	11.76	7.13	4.63	2.66
14-06-02	19	12.83	1.34	11.49	5.56	9.47	4.96	4.51	4.33
16-06-02	21	9.60	7.13	2.47	8.49	6.09	3.82	2.27	5.24
18-06-02	23	5.43	2.63	2.80	5.18	9.28	6.90	2.38	7.07
21-06-02	26	10.20	7.41	2.79	1.67	11.82	8.71	3.11	1.23
23-06-02	28	13.69	10.74	2.95	2.07	15.89	13.51	2.38	1.51
26-06-02	31	13.72	3.39	10.33	-	10.39	5.24	5.15	1.37
28-06-02	33	13.98	2.91	11.07	2.02	10.19	2.69	7.49	1.79
16-07-02	36	14.41	9.08	5.33	9.04	10.18	7.76	2.42	12.97
20-07-02	40	12.42	4.50	7.92	3.24	8.51	3.86	4.67	4.23
22-07-02	42	9.43	6.10	3.32	6.26	9.99	7.49	2.50	8.14
25-07-02	45	10.95	1.58	9.37	2.66	12.86	4.98	7.88	6.82
27-07-02	47	14.80	11.17	3.63	3.55	14.13	2.06	12.07	4.43
29-07-02	49	14.18	0.37	13.81	1.67	15.17	0.17	15.00	3.20
31-07-02	51	15.99	7.27	8.72	2.00	15.50	3.00	12.49	2.40
03-08-02	54	11.09	0.61	10.48	3.52	12.05	3.37	8.68	2.90
08-08-02	56	14.18	10.52	3.66	17.21	10.57	9.84	0.73	17.48
10-08-02	58	11.95	9.86	2.09	1.68	11.42	7.55	3.87	2.31
12-08-02	60	14.51	10.55	3.96	5.33	14.41	11.16	3.25	6.32
25-08-02	64	13.47	7.40	6.07	10.61	13.52	7.83	5.69	11.99
27-08-02	66	14.15	8.59	5.56	16.06	13.44	13.44	0.59	12.73
31-08-02	70	10.37	2.33	8.04	6.67	8.29	0.82	7.47	2.19
02-09-02	72	7.81	1.79	7.98	4.71	4.51	1.03	4.94	1.02
04-09-02	74	1.37	0.17	1.20	3.03	0.88	0.08	0.80	2.42
10-09-02	76	9.97	0.09	9.88	12.41	6.57	0.18	6.93	10.53
12-09-02	78	10.19	2.44	7.74	8.30	5.29	4.28	1.01	2.02
13-09-02	79	6.48	0.06	6.41	7.56	6.51	0.09	6.42	7.70
14-09-02	80	3.60	0.01	3.59	3.87	4.67	0.00	4.67	5.88
16-09-02	81	3.45	0.04	3.41	3.45	4.03	0.43	3.60	4.46
20-09-02	84	2.84	0.52	2.32	3.02	3.21	0.00	3.21	3.64

Trial II		MC 5 (mg l^{-1})				MC 6 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
29-05-02	3	14.24	13.87	0.37	16.89	14.60	14.60	0.04	17.05
30-05-02	4	13.76	12.36	1.40	14.12	12.65	12.50	0.15	15.64
31-05-02	5	13.90	13.35	0.55	11.30	14.26	13.78	0.48	11.54
01-06-02	6	13.65	11.29	2.36	7.63	14.02	13.31	0.71	8.30
02-06-02	7	6.86	4.38	2.48	6.93	14.96	13.35	1.61	8.55
03-06-02	8	13.48	12.90	0.58	2.34	13.45	6.47	6.98	3.85
05-06-02	10	14.87	14.76	0.11	3.86	14.81	13.98	0.83	1.89
08-06-02	13	8.34	7.21	1.13	3.61	14.96	14.87	0.09	1.06
11-06-02	16	10.05	8.32	1.73	1.19	15.46	14.68	0.78	1.35
14-06-02	19	11.13	8.83	2.30	2.84	12.53	4.85	7.68	3.31
16-06-02	21	9.42	7.22	2.20	5.45	7.37	3.22	4.15	6.49
18-06-02	23	10.08	7.64	2.44	4.35	12.50	9.73	2.77	5.02
21-06-02	26	9.27	6.77	2.50	1.91	11.37	8.53	2.84	4.55
23-06-02	28	11.88	3.22	8.66	5.18	12.71	9.71	3.00	3.12
26-06-02	31	13.52	6.50	7.02	4.97	13.44	2.91	10.54	-
28-06-02	33	9.40	4.07	5.33	6.05	14.51	5.12	9.39	5.27
16-07-02	36	11.00	3.25	7.75	3.65	15.48	3.95	11.53	4.77
20-07-02	40	10.50	5.98	4.52	2.02	11.72	1.86	9.86	2.64
22-07-02	42	14.50	4.34	10.16	2.52	10.99	7.31	3.68	2.18
25-07-02	45	15.00	3.45	11.55	1.39	13.49	1.87	11.53	2.47
27-07-02	47	12.86	1.95	10.91	1.12	15.00	2.68	12.32	1.93
29-07-02	49	12.62	0.91	11.81	1.47	14.50	1.38	13.11	2.13
31-07-02	51	14.10	2.84	11.22	1.54	13.19	0.90	12.28	1.92
03-08-02	54	7.89	6.06	1.83	1.89	13.63	1.49	12.14	2.35
08-08-02	56	8.59	7.06	1.53	11.15	9.82	7.37	2.45	5.36
10-08-02	58	9.43	6.57	2.86	2.67	12.95	8.17	4.78	4.48
12-08-02	60	7.57	0.68	6.90	2.59	11.86	7.36	4.50	3.03
25-08-02	64	7.39	0.70	6.69	2.37	0.54	1.46	8.55	2.26
27-08-02	66	7.52	1.10	6.42	3.57	0.00	1.38	8.65	2.80
31-08-02	70	7.48	0.85	6.62	2.07	1.56	0.30	7.56	2.21
02-09-02	72	5.39	0.70	5.85	2.72	0.00	0.76	5.51	2.89
04-09-02	74	4.17	0.00	4.17	2.16	4.62	2.02	2.60	3.15
10-09-02	76	7.11	0.18	6.93	9.89	5.18	0.32	4.85	9.63
12-09-02	78	3.90	3.90	0.00	2.89	4.97	4.97	0.00	2.50
13-09-02	79	2.33	0.12	2.22	3.09	2.75	0.04	2.71	3.33
14-09-02	80	0.99	0.56	0.43	6.48	1.03	0.13	0.9	4.88
16-09-02	81	0.86	0.09	0.77	6.04	1.54	0.06	1.48	5.04
20-09-02	84	0.52	0.22	0.30	5.96	0.87	0.00	0.87	5.11

Trial II		MC 7 (mg l^{-1})				MC 8 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
29-05-02	3	10.08	10.03	0.05	10.68	14.47	14.43	0.04	9.60
30-05-02	4	12.86	12.71	0.15	3.59	12.35	12.24	0.11	4.24
31-05-02	5	15.14	14.48	0.66	2.74	14.77	13.54	1.23	4.20
01-06-02	6	15.14	13.76	1.38	2.34	14.18	14.87	0.31	2.22
02-06-02	7	14.96	14.07	0.89	1.96	14.79	12.96	1.83	1.74
03-06-02	8	15.08	12.81	2.27	2.24	14.72	13.71	1.01	2.36
05-06-02	10	15.44	15.39	0.05	1.60	15.38	15.30	0.08	1.79
08-06-02	13	14.30	14.25	0.05	2.33	15.10	15.10	0.00	3.40
11-06-02	16	15.61	13.87	0.08	1.79	14.95	14.73	0.22	2.39
14-06-02	19	14.36	14.33	0.04	4.19	13.50	13.28	0.22	4.55
16-06-02	21	15.34	13.35	1.98	5.44	15.92	14.58	1.34	5.84
18-06-02	23	8.60	6.50	2.10	3.98	14.65	12.52	2.13	4.64
21-06-02	26	14.16	11.94	2.12	6.92	14.22	12.05	2.17	6.21
23-06-02	28	14.24	12.17	2.07	5.13	14.83	12.69	2.14	5.55
26-06-02	31	14.52	13.45	1.07	4.35	14.69	14.07	0.62	4.49
28-06-02	33	14.74	14.39	0.35	3.24	14.09	13.74	0.35	3.02
16-07-02	36	16.26	14.25	2.01	3.54	12.79	11.26	1.53	2.70
20-07-02	40	13.26	12.45	0.81	1.78	14.01	13.39	0.71	2.05
22-07-02	42	13.61	13.13	0.48	1.63	14.05	13.94	0.11	1.83
25-07-02	45	14.61	11.81	2.79	2.08	12.32	10.17	2.15	2.11
27-07-02	47	15.00	11.54	3.46	1.59	13.22	10.04	3.18	2.05
29-07-02	49	15.01	13.37	1.64	1.42	14.82	13.82	1.01	2.43
31-07-02	51	19.13	19.13	0.00	1.20	14.87	14.63	0.24	1.71

Trial III		MC 1 (mg l^{-1})				MC 2 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
02-02-03	2	15.24	15.24	0.00	28.27	15.11	15.11	0.00	31.05
03-02-03	3	15.30	15.30	0.00	33.87	15.06	15.06	0.00	34.14
04-02-03	4	15.10	15.10	0.00	34.35	14.86	14.86	0.00	32.91
05-02-03	5	15.62	15.62	0.00	30.57	14.98	14.98	0.00	33.34
06-02-03	6	15.01	15.01	0.00	31.46	15.35	15.35	0.00	32.18
10-02-03	9	12.74	12.74	0.00	33.15	15.59	15.59	0.00	35.67
11-02-03	10	14.81	14.81	0.00	35.17	13.67	13.67	0.00	33.64
13-02-03	12	16.02	16.02	0.00	31.27	15.71	15.71	0.00	33.13
15-02-03	14	12.28	12.28	0.00	21.08	15.17	15.17	0.00	31.33
17-02-03	16	12.96	12.96	0.00	25.71	12.19	12.19	0.00	33.74
24-02-03	21	14.89	14.89	0.00	4.15	14.74	14.74	0.00	5.52
26-02-03	23	15.50	15.50	0.00	30.92	15.74	15.74	0.00	31.78
28-02-03	25	16.14	16.14	0.00	28.26	17.35	17.35	0.00	29.68
02-03-03	27	14.12	14.12	0.00	31.50	14.18	14.18	0.00	32.02
04-03-03	29	13.98	13.98	0.00	31.67	13.75	13.75	0.00	32.25
06-03-03	31	14.23	14.23	0.00	33.54	15.24	15.24	0.00	32.73
08-03-03	33	14.93	14.93	0.00	38.54	15.19	15.19	0.00	38.94
10-03-03	35	14.40	14.40	0.00	42.25	14.79	14.79	0.00	43.02
12-03-03	37	15.03	15.03	0.00	22.26	14.47	14.47	0.00	30.39
13-03-03	38	12.25	12.25	0.00	36.59	14.73	14.73	0.00	47.36
15-03-03	40	14.98	14.98	0.00	52.42	13.58	13.58	0.00	52.51
19-03-03	44	14.46	14.46	0.00	38.78	14.66	14.66	0.00	39.37
21-03-03	46	14.03	14.03	0.00	51.09	15.33	15.33	0.00	49.24
24-03-03	49	16.03	16.03	0.00	45.93	13.95	13.95	0.00	45.56
27-03-03	52	14.83	14.83	0.00	41.25	15.16	15.16	0.00	39.94
30-03-03	55	14.48	14.48	0.00	42.37	14.72	14.72	0.00	42.48

Trial III		MC 3 (mg l^{-1})				MC 4 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
02-02-03	2	11.39	11.40	0.00	36.12	1.73	14.73	0.00	35.95
03-02-03	3	14.46	14.45	0.01	27.81	15.92	15.92	0.00	30.25
04-02-03	4	13.79	13.63	0.16	25.06	1.67	13.76	0.00	29.61
05-02-03	5	11.31	11.31	0.00	19.39	13.99	13.99	0.00	25.14
06-02-03	6	7.36	7.36	0.00	13.92	12.95	12.92	0.03	24.17
10-02-03	9	9.46	7.47	2.00	15.56	9.87	9.85	0.02	19.06
11-02-03	10	8.42	6.67	1.74	11.43	9.11	8.78	0.34	13.87
13-02-03	12	8.37	5.02	3.35	8.36	4.84	3.73	1.12	3.90
15-02-03	14	10.38	8.34	2.04	13.10	5.53	3.68	1.85	2.82
17-02-03	16	8.33	5.52	2.81	11.97	10.23	9.22	1.00	22.27
24-02-03	21	0.36	0.29	0.07	7.01	3.82	3.41	0.41	6.08
26-02-03	23	0.14	0.14	0.00	9.87	8.42	5.01	3.40	14.93
28-02-03	25	2.33	2.33	0.00	4.44	1.81	0.92	0.89	13.19
02-03-03	27	3.16	1.20	1.95	2.48	2.90	0.88	2.02	4.10
04-03-03	29	0.30	0.30	0.00	7.77	0.76	0.68	0.08	2.71
06-03-03	31	1.43	1.43	0.00	8.17	0.04	0.04	0.00	6.36
08-03-03	33	1.00	1.00	0.00	2.67	0.05	0.05	0.00	7.00
10-03-03	35	2.27	1.70	0.57	2.16	3.29	1.87	1.43	3.07
12-03-03	37	1.49	1.49	0.00	6.14	0.16	0.16	0.00	2.07
13-03-03	38	3.91	3.91	0.00	18.21	0.18	0.18	0.00	4.52
15-03-03	40	1.33	1.33	0.00	22.16	0.85	0.85	0.00	14.38
19-03-03	44	0.18	0.18	0.00	19.93	0.41	0.24	0.16	20.02
21-03-03	46	0.03	0.03	0.00	23.95	0.00	0.00	0.00	20.66
24-03-03	49	0.05	0.05	0.00	20.08	0.00	0.00	0.00	19.72
27-03-03	52	0.25	0.25	0.00	20.41	0.07	0.07	0.00	17.52
30-03-03	55	0.01	0.01	0.00	17.56	0.00	0.00	0.00	19.36

Trial III		MC 5 (mg l^{-1})				MC 6 (mg l^{-1})			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
02-02-03	2	6.89	6.89	0.00	19.09	14.94	14.94	0.00	36.03
03-02-03	3	14.63	14.63	0.00	27.49	14.02	14.02	0.00	26.65
04-02-03	4	13.32	13.32	0.00	16.65	13.96	13.96	0.00	-
05-02-03	5	12.14	10.61	1.53	16.93	12.80	12.00	0.80	18.50
06-02-03	6	10.29	8.42	1.87	12.65	11.52	10.52	1.01	15.39
10-02-03	9	3.31	2.28	1.03	8.06	8.83	7.05	1.77	20.92
11-02-03	10	8.99	6.08	2.91	7.03	11.39	8.82	2.56	16.57
13-02-03	12	10.12	5.81	4.31	3.74	9.23	4.34	4.89	5.51
15-02-03	14	10.65	5.33	5.32	6.30	8.94	6.45	2.49	8.47
17-02-03	16	9.97	3.96	6.00	11.76	6.99	2.55	4.43	4.47
24-02-03	21	6.90	1.77	5.13	4.14	7.33	1.85	5.48	6.91
26-02-03	23	4.60	1.10	3.50	5.28	5.49	1.33	4.16	7.50
28-02-03	25	3.37	0.84	2.54	3.75	5.61	1.54	4.07	5.85
02-03-03	27	6.59	1.38	5.21	3.09	4.19	0.96	3.23	3.43
04-03-03	29	3.14	0.90	2.24	5.76	3.90	0.91	2.99	7.97
06-03-03	31	2.31	0.48	1.83	5.59	3.79	0.37	3.42	7.59
08-03-03	33	1.88	0.67	1.22	1.74	3.35	1.03	2.32	4.84
10-03-03	35	4.04	0.73	3.30	1.68	4.23	1.35	2.88	2.34
12-03-03	37	2.70	0.98	1.71	1.78	0.43	0.34	0.09	3.69
13-03-03	38	0.31	0.31	0.00	8.90	0.18	0.18	0.00	11.04
15-03-03	40	0.31	0.31	0.00	16.44	0.54	0.54	0.00	16.80
19-03-03	44	0.05	0.05	0.00	9.90	0.01	0.01	0.00	19.26
21-03-03	46	0.00	0.00	0.00	25.62	0.00	0.00	0.00	25.28
24-03-03	49	0.00	0.00	0.00	18.37	0.00	0.00	0.00	18.86
27-03-03	52	0.01	0.01	0.00	15.39	0.00	0.00	0.00	16.91
30-03-03	55	0.00	0.00	0.00	16.02	0.02	0.02	0.00	18.03

Trial III		MC 7	(mg l^{-1})			MC 8	(mg l^{-1})		
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
02-02-03	2	15.12	15.12	0.00	34.51	11.08	11.08	0.00	30.27
03-02-03	3	14.45	14.45	0.00	30.68	14.28	14.28	0.00	30.36
04-02-03	4	14.22	14.22	0.00	27.20	13.97	13.93	0.04	24.51
05-02-03	5	14.16	13.04	1.12	18.69	11.61	10.37	1.24	12.88
06-02-03	6	14.01	12.07	1.94	18.74	11.67	9.42	2.25	14.94
10-02-03	9	5.72	4.14	1.59	11.59	0.88	0.53	0.35	5.68
11-02-03	10	11.88	10.01	1.87	19.82	4.58	1.84	2.74	5.01
13-02-03	12	8.43	4.57	3.87	5.82	6.57	3.07	3.49	3.78
15-02-03	14	6.79	4.27	2.53	5.57	6.41	2.88	3.53	3.79
17-02-03	16	5.28	2.00	3.27	3.07	4.81	1.01	3.80	3.10
24-02-03	21	2.51	0.78	1.73	4.63	2.93	1.08	1.85	4.84
26-02-03	23	0.30	0.15	0.15	5.75	0.01	0.01	0.00	3.76
28-02-03	25	0.19	0.10	0.09	3.75	2.11	0.87	1.24	2.62
02-03-03	27	1.31	0.60	0.71	3.77	0.92	0.55	0.37	3.76
04-03-03	29	0.35	0.30	0.05	5.64	0.44	0.37	0.07	3.65
06-03-03	31	0.03	0.03	0.00	5.43	0.05	0.05	0.00	7.38
08-03-03	33	0.03	0.03	0.00	2.62	0.04	0.04	0.00	5.17
10-03-03	35	3.03	2.69	0.35	2.52	1.20	0.73	0.47	2.99
12-03-03	37	1.63	1.06	0.57	2.66	0.86	0.58	0.28	2.40
13-03-03	38	0.26	0.26	0.00	10.88	0.27	0.27	0.00	12.26
15-03-03	40	0.29	0.29	0.00	8.29	0.42	0.42	0.00	14.14
19-03-03	44	0.05	0.05	0.00	17.67	0.08	0.08	0.00	16.55
21-03-03	46	0.00	0.00	0.00	44.69	0.00	0.00	0.00	50.70
24-03-03	49	0.00	0.00	0.00	17.47	0.01	0.01	0.00	17.22
27-03-03	52	0.01	0.01	0.00	13.62	0.13	0.13	0.00	10.15
30-03-03	55	0.00	0.00	0.00	16.19	0.02	0.02	0.00	16.44

Trial IV		MC 1	(mg l^{-1})			MC 2	(mg l^{-1})		
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
20-02-04	1	15.38	15.38	0.00	3.24	15.76	15.76	0.00	4.19
23-02-04	4	15.45	15.45	0.00	4.73	15.43	15.43	0.00	3.93
24-02-04	5	16.13	16.13	0.00	3.61	15.79	15.79	0.00	2.72
25-02-04	6	15.52	15.52	0.00	2.99	15.344	15.344	0.00	2.86
26-02-04	7	14.38	14.38	0.00	1.11	14.08	14.08	0.00	2.34
27-02-04	8	14.21	14.21	0.00	2.49	13.48	13.48	0.00	2.28
28-02-04	9	14.78	14.78	0.00	4.07	14.74	14.74	0.00	2.14
01-03-04	11	14.36	14.36	0.00	3.08	14.87	14.87	0.00	1.98
03-03-04	13	14.36	16.64	0.00	2.93	15.51	15.51	0.00	3.77
05-03-04	15	-	-	-	-	-	-	-	-
07-03-04	17	-	-	-	-	-	-	-	-
09-03-04	19	-	-	-	-	-	-	-	-
11-03-04	21	10.32	10.32	0.00	2.97	12.81	12.81	0.00	1.73
12-03-04	22	12.71	12.71	0.00	2.78	13.80	13.80	0.00	1.84
15-03-04	25	11.48	11.48	0.00	3.17	15.68	15.68	0.00	5.49
16-03-04	26	16.01	16.01	0.00	4.73	15.92	15.92	0.00	3.59
18-03-04	28	17.59	17.58	0.00	3.00	17.51	17.51	0.00	3.38
20-03-04	30	17.14	17.14	0.00	4.96	17.09	17.09	0.00	4.70
22-03-04	32	15.84	15.84	0.00	3.28	15.10	15.10	0.00	5.77

Trial IV		MC 3	(mg l^{-1})			MC 4	(mg l^{-1})		
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
20-02-04	1	14.89	14.89	0.00	29.07	14.75	14.75	0.00	24.03
23-02-04	4	13.17	12.28	0.89	20.44	8.11	5.24	2.87	7.79
24-02-04	5	14.40	11.22	3.18	20.19	7.72	3.28	4.45	2.35
25-02-04	6	11.26	6.29	4.97	13.84	8.67	5.49	3.18	2.92
26-02-04	7	5.81	3.79	2.02	3.63	6.99	2.44	4.55	2.69
27-02-04	8	3.60	1.11	2.49	3.27	5.89	1.28	4.61	3.46
28-02-04	9	1.49	0.00	1.49	2.22	4.00	0.16	3.84	2.80
01-03-04	11	0.94	0.22	0.72	2.42	4.31	0.70	3.62	2.90
03-03-04	13	1.40	0.85	0.55	3.41	4.98	1.04	3.94	3.65
05-03-04	15	0.01	0.01	0.00	4.44	4.49	0.86	3.63	2.68
07-03-04	17	0.10	0.00	0.10	7.19	1.83	0.11	1.72	3.20
09-03-04	19	0.00	0.00	0.00	7.90	0.00	0.00	0.00	4.32
11-03-04	21	0.00	0.00	0.00	3.96	4.11	0.00	4.11	2.62
12-03-04	22	1.83	0.00	1.83	3.15	8.62	2.31	6.31	2.37
15-03-04	25	0.00	0.00	0.00	8.05	3.98	0.19	3.79	6.37
16-03-04	26	0.00	0.00	0.00	3.62	3.80	0.13	3.67	3.13
18-03-04	28	4.84	3.41	1.43	3.33	4.74	2.87	1.87	3.53
20-03-04	30	12.03	7.53	4.50	2.28	16.95	9.74	7.21	2.42
22-03-04	32	0.00	0.00	0.00	5.34	7.09	6.81	2.80	3.52

Trial IV		MC 5 ($mg\,l^{-1}$)				MC 6 ($mg\,l^{-1}$)			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
20-02-04	1	14.87	14.87	0.00	22.07	14.90	14.90	0.00	32.56
23-02-04	4	10.08	4.83	5.25	5.56	7.56	5.17	2.39	15.54
24-02-04	5	10.05	3.76	6.29	2.57	3.76	1.56	2.20	6.09
25-02-04	6	10.53	2.93	7.60	2.49	2.73	0.60	2.13	2.38
26-02-04	7	9.42	3.30	6.12	2.23	3.10	0.75	2.35	-
27-02-04	8	10.87	2.46	8.41	2.55	1.59	0.36	1.23	2.89
28-02-04	9	10.03	4.14	5.90	2.06	1.75	0.00	1.75	5.11
01-03-04	11	9.17	0.00	9.17	1.92	0.27	0.27	0.00	3.78
03-03-04	13	10.65	3.12	7.53	3.33	4.15	2.02	2.13	9.36
05-03-04	15	6.56	1.59	4.97	2.24	0.39	0.00	0.39	13.38
07-03-04	17	9.71	2.66	7.05	2.30	4.85	3.07	1.78	11.76
09-03-04	19	8.90	5.38	3.52	2.41	4.94	3.95	0.99	17.13
11-03-04	21	8.75	2.16	6.59	2.66	4.85	2.81	2.04	18.74
12-03-04	22	12.57	5.12	7.45	2.26	4.21	1.68	2.53	
15-03-04	25	10.17	3.46	6.71	3.49	6.09	4.05	2.05	20.35
16-03-04	26	1.92	0.03	1.89	3.32	0.37	0.37	0.00	8.36
18-03-04	28	14.94	9.36	5.58	2.69	-	-	-	-
20-03-04	30	15.34	10.16	5.18	2.07	7.82	5.98	1.84	11.41
22-03-04	32	15.84	10.56	5.28	2.33	10.04	8.05	1.99	17.23

Trial IV		MC 7 ($mg\,l^{-1}$)				MC 8 ($mg\,l^{-1}$)			
Date	Exp day.	TON	NO_3^--N	NO_2^--N	DOC	TON	NO_3^--N	NO_2^--N	DOC
20-02-04	1	15.01	15.01	0.00	27.51	15.11	15.11	0.00	27.87
23-02-04	4	8.41	5.38	3.03	17.73	7.97	4.80	3.17	17.51
24-02-04	5	6.38	1.54	4.84	10.52	3.83	0.55	3.28	9.42
25-02-04	6	0.54	0.00	0.54	3.74	0.40	0.00	0.40	5.73
26-02-04	7	0.39	0.00	0.39	3.12	0.38	0.00	0.37	5.07
27-02-04	8	0.39	0.00	0.39	4.58	0.38	0.00	0.38	5.64
28-02-04	9	0.42	0.00	0.42	4.30	0.40	0.00	0.40	5.35
01-03-04	11	2.09	1.41	0.68	3.73	0.00	0.00	0.00	2.10
03-03-04	13	0.87	0.71	0.16	4.32	0.00	0.00	0.00	9.45
05-03-04	15	0.00	0.00	0.00	9.21	0.00	0.00	0.00	9.68
07-03-04	17	0.00	0.00	0.00	11.51	0.00	0.00	0.00	11.65
09-03-04	19	0.00	0.00	0.00	14.88	0.00	0.00	0.00	14.80
11-03-04	21	0.00	0.00	0.00	7.44	0.00	0.00	0.00	10.63
12-03-04	22	0.00	0.00	0.00	9.84	0.00	0.00	0.00	9.85
15-03-04	25	0.00	0.00	0.00	15.15	0.00	0.00	0.00	15.77
16-03-04	26	0.00	0.00	0.00	5.85	0.00	0.00	0.00	9.10
18-03-04	28	0.00	0.00	0.00	5.44	0.00	0.00	0.00	4.75
20-03-04	30	0.00	0.00	0.00	2.40	0.00	0.00	0.00	2.35
22-03-04	32	0.00	0.00	0.00	4.90	0.00	0.00	0.00	3.84

2.2 MASS BALANCE CALCULATIONS

Trial I			MC 1		Sterile control							
Date	Exp day.	Measured influent NO_3^--N ($mg\,l^{-1}$)	Nominal influent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_2^--N ($mg\,d^{-1}$)	Mass TON denitrified (from nominal input) ($mg\,d^{-1}$)	Mass TON in effluent ($mg\,d^{-1}$)	Measured effluent DOC ($mg\,l^{-1}$)	Nominal influent DOC ($mg\,d^{-1}$)	Effluent DOC ($mg\,d^{-1}$)	Mass DOC utilised (from nominal input) ($mg\,d^{-1}$)	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	2.35	31.32	24.40	0.00	2.95	24.40	6.76	48.02	48.02	0.00	10.8	0.0
05-02-02	3	2.18	31.32	27.43	0.00	+ 0.29	27.43	6.32	48.02	48.08	+ 0.06	0.0	0.0
06-02-02	4	2.93	31.32	26.50	0.00	1.41	26.50	6.52	48.02	49.90	+ 1.88	5.1	0.0
07-02-02	5	2.49	31.32	34.22	0.00	1.27	34.22	6.39	48.02	46.14	1.88	3.6	3.9
08-02-02	6	2.50	31.32	30.97	0.00	+ 0.06	30.97	6.83	48.02	49.85	+ 1.83	0.0	0.0
09-02-02	7	2.58	31.32	32.77	0.00	1.35	32.77	6.47	48.02	53.89	+ 5.87	4.0	0.0
10-02-02	8	2.46	31.32	40.52	0.00	+ 4.05	40.52	7.45	48.02	56.79	+ 8.77	0.0	0.0
19-02-02	15	2.48	31.32	36.48	0.00	+ 0.01	36.48	7.10	48.02	56.35	+ 8.33	0.0	0.0
22-02-02	18	2.30	31.32	34.17	0.00	1.02	34.17	7.02	48.02	-	-	2.9	

Trial I			MC 2		Autotrophic control							
Date	Exp day.	Measured influent NO_3^--N ($mg\,l^{-1}$)	Nominal influent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_2^--N ($mg\,d^{-1}$)	Mass TON denitrified (from nominal input) ($mg\,d^{-1}$)	Mass TON in effluent ($mg\,d^{-1}$)	Measured effluent DOC ($mg\,l^{-1}$)	Nominal influent DOC ($mg\,d^{-1}$)	Effluent DOC ($mg\,d^{-1}$)	Mass DOC utilised (from measured input) ($mg\,d^{-1}$)	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	2.30	31.32	24.15	0.00	7.17	24.15	2.91	N/A	10.23	- 1.91	22.9	0.0
05-02-02	3	2.16	31.32	27.24	0.00	4.08	27.24	2.84	N/A	6.06	0.06	13.0	0.0
06-02-02	4	2.25	31.32	34.47	0.00	+ 3.15	34.47	2.97	N/A	10.65	+ 2.25	0.0	0.0
07-02-02	5	2.05	31.32	35.39	0.00	+ 4.07	35.39	3.63	N/A	7.60	- 0.80	0.0	0.0
08-02-02	6	2.28	31.32	31.98	0.00	+ 0.66	31.98	3.87	N/A	4.80	+ 0.40	0.0	0.0
09-02-02	7	2.37	31.32	29.50	0.00	1.82	29.50	-	N/A	7.39	-	0.0	
10-02-02	8	2.36	31.32	35.73	0.00	+ 4.41	35.73	3.08	N/A	8.14	+ 1.60	0.0	0.0
19-02-02	15	2.40	31.32	26.08	0.00	5.24	26.08	-	N/A	8.39	-	16.7	
22-02-02	18	2.28	31.32	36.50	0.00	+ 5.18	36.50	2.86	N/A	5.85	+ 0.93	0.0	0.0

Trial I			MC 3		Glucose; stoichiometric C/N = 1.53							
Date	Exp day.	Measured influent NO_3^--N ($mg\,l^{-1}$)	Nominal influent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_3^--N ($mg\,d^{-1}$)	Effluent NO_2^--N ($mg\,d^{-1}$)	Mass TON denitrified (from nominal input) ($mg\,d^{-1}$)	Mass TON in effluent ($mg\,d^{-1}$)	Measured effluent DOC ($mg\,l^{-1}$)	Nominal influent DOC ($mg\,d^{-1}$)	Effluent DOC ($mg\,d^{-1}$)	Mass DOC utilised (from nominal input) ($mg\,d^{-1}$)	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	2.09	31.32	23.91	0.00	7.41	23.91	4.06	48.02	46.77	1.25	23.7	2.6
05-02-02	3	2.16	31.32	26.67	0.00	4.65	26.67	4.01	48.02	33.83	14.20	14.9	29.6
06-02-02	4	2.09	31.32	31.63	0.00	+ 0.31	31.63	3.75	48.02	36.54	11.48	0.0	23.9
07-02-02	5	2.51	31.32	30.92	0.00	0.40	30.92	-	48.02	-	-	1.3	
08-02-02	6	2.48	31.32	32.21	0.00	+ 0.89	32.21	4.07	48.02	20.88	27.14	0.0	56.5
09-02-02	7	2.43	31.32	23.88	7.79	+ 0.35	31.67	-	48.02	-	-	0.0	
10-02-02	8	2.50	31.32	24.98	12.42	+ 4.08	37.40	4.22	48.02	9.81	38.21	0.0	79.6
19-02-02	15	2.31	31.32	4.97	10.38	15.97	15.35	-	48.02	-	-	51.0	
22-02-02	18	2.42	31.32	4.72	17.06	9.54	21.78	3.29	48.02	7.73	40.30	30.5	83.9

Trial I			MC 4	Glucose: stoichiometric C/N = 1.53								
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	34.00	31.32	22.85	0.00	**8.47**	**22.85**	64.09	48.02	45.31	**2.71**	27.0	5.7
05-02-02	3	30.36	31.32	27.49	0.00	**3.83**	**27.49**	58.51	48.02	33.83	**14.20**	12.2	29.6
06-02-02	4	31.68	31.32	33.37	0.00	+ 2.05	**33.37**	56.73	48.02	38.63	**9.40**	0.0	19.6
07-02-02	5	31.61	31.32	30.03	0.00	**1.29**	**30.03**	48.02				4.1	
08-02-02	6	31.68	31.32	24.58	7.07	+ 0.23	**31.65**	58.02	48.02	6.89	**41.13**	0.0	85.7
09-02-02	7	31.84	31.32	12.76	15.60	**2.97**	**28.35**		48.02	-	-	9.5	
10-02-02	8	34.50	31.32	17.04	17.49	+ 3.00	**34.53**	48.26	48.02	8.98	**39.05**	0.0	81.3
19-02-02	15	31.13	31.32	2.92	4.36	**24.03**	**7.29**		48.02	-	-	76.7	
22-02-02	18	31.97	31.32	8.00	15.53	**7.79**	**23.53**	58.21	48.02	5.64	**42.39**	24.9	88.3

Trial I			MC 5	Glucose: stoichiometric C/N = 0.73								
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	32.30	31.32	21.22	0.00	**10.10**	**21.22**	53.41	22.97	29.44	+ 6.47	32.2	0.0
05-02-02	3	31.20	31.32	25.97	0.00	**5.35**	**25.97**	37.64	22.97	14.82	**8.14**	17.1	35.5
06-02-02	4	31.80	31.32	31.02	0.00	**0.30**	**31.02**	29.14	22.97	6.68	**16.29**	0.9	70.9
07-02-02	5	32.26	31.32	33.93	0.00	+ 2.61	**33.93**		22.97	-	-	0.0	
08-02-02	6	34.85	31.32	35.91	0.00	+ 3.59	**35.91**	28.48	22.97	5.64	**17.33**	0.0	75.5
09-02-02	7	34.13	31.32	35.59	0.00	+ 3.87	**35.59**		22.97	-	-	0.0	
10-02-02	8	32.75	31.32	35.35	0.00	+ 3.03	**35.35**	22.97	22.97	10.02	**12.95**	0.0	56.4
19-02-02	15	31.75	31.32	23.74	8.39	+ 0.81	**32.13**		22.97	-	-	0.0	
22-02-02	18	32.97	31.32	20.92	9.60	**0.79**	**30.53**	33.40	22.97	5.22	**17.75**	2.5	77.3

Trial I			MC 6	Glucose: stoichiometric C/N = 0.73								
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	31.30	31.32	21.98	0.00	**9.34**	**21.98**	53.85	22.97	26.31	+ 3.34	29.8	0.0
05-02-02	3	31.66	31.32	26.78	0.00	**4.54**	**26.78**	41.53	22.97	15.66	**7.31**	14.5	31.8
06-02-02	4	31.75	31.32	30.89	0.00	**0.43**	**30.89**	32.13	22.97	9.40	**13.57**	1.4	59.1
07-02-02	5	33.82	31.32	30.50	0.00	**0.82**	**30.50**		22.97	-	-	2.6	
08-02-02	6	32.69	31.32	36.11	0.00	+ 3.79	**36.11**	30.05	22.97	6.89	**16.08**	0.0	70.0
09-02-02	7	35.17	31.32	28.76	6.96	+ 4.40	**35.72**		22.97	-	-	0.0	
10-02-02	8	33.65	31.32	32.26	0.00	+ 0.94	**32.26**	32.97	22.97	14.20	**8.77**	0.0	38.2
19-02-02	15	31.70	31.32	23.97	7.45	+ 0.09	**31.42**		22.97	-	-	0.0	
22-02-02	18	33.51	31.32	24.28	8.15	+ 0.95	**32.42**	39.73	22.97	7.73	**15.24**	0.0	66.4

Trial I			MC 7	Glucose: stoichiometric C/N = 2.60 ? 3.00								
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	34.07	31.32	23.79	0.00	**7.53**	**23.79**	75.48	81.43	76.00	**5.43**	24.0	6.7
05-02-02	3	29.36	31.32	25.98	0.00	**5.34**	**25.98**	81.51	81.43	73.08	**8.35**	17.0	10.3
06-02-02	4	30.88	31.32	30.51	0.00	**0.81**	**30.51**	68.07	81.43	54.50	**26.94**	2.6	33.1
07-02-02	5	31.02	31.32	29.87	0.52	**0.92**	**30.40**		81.43	-		2.9	
08-02-02	6	31.02	31.32	23.02	8.16	**0.14**	**31.18**	81.43	81.43	34.87	**46.56**	0.4	57.2
09-02-02	7	30.45	31.32	12.49	16.67	**2.16**	**29.16**		81.43	-	-	6.9	
10-02-02	8	31.00	31.32	16.01	15.87	+ 0.56	**31.88**	71.76	81.43	14.20	**67.23**	0.0	82.6
19-02-02	15	31.64	31.32	1.88	10.31	**19.13**	**12.19**		93.96	-		61.1	
22-02-02	18	31.98	31.32	0.54	9.29	**21.49**	**9.83**	21.23	93.96	9.81	**84.15**	68.6	89.6

Trial I			MC 8	Glucose: stoichiometric C/N = 2.60 ? 3.00								
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
04-02-02	2	34.00	31.32	21.32	0.00	**10.00**	**21.32**	72.08	81.43	72.35	**9.08**	31.9	11.15
05-02-02	3	30.31	31.32	25.99	0.00	**5.33**	**25.99**	81.51	81.43	65.98	**15.45**	17.0	18.97
06-02-02	4	31.69	31.32	30.71	2.09	+ 1.48	**32.80**	56.20	81.43	36.12	**45.31**	0.0	55.64
07-02-02	5	31.09	31.32	30.17	0.98	**0.17**	**31.15**		81.43	-	-	0.5	
08-02-02	6	31.32	31.32	26.18	5.08	**0.05**	**31.27**	77.43	81.43	22.72	**58.71**	0.2	72.10
09-02-02	7	31.88	31.32	14.45	14.22	**2.65**	**28.67**		81.43	-	-	8.5	
10-02-02	8	31.91	31.32	10.37	19.50	**1.45**	**29.87**	68.01	81.43	13.57	**67.86**	4.6	83.33
19-02-02	15	31.54	31.32	1.94	9.06	**20.32**	**11.00**		93.96	-		64.9	
22-02-02	18	31.76	31.32	2.74	3.61	**24.97**	**6.35**	72.05	93.96	5.85	**88.11**	79.7	93.78

Trial II			MC 1		Sterile control							
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	[illegible]	31.32	32.64	0.00	[illegible]	32.64	[illegible]	45.94	52.20	[illegible]	0.0	0.0
30-05-02	4	[illegible]	31.32	28.08	0.00	3.24	28.08	[illegible]	45.94	53.62	[illegible]	10.3	0.0
31-05-02	5	[illegible]	31.32	25.58	0.00	5.74	25.58	[illegible]	45.94	53.60	[illegible]	18.3	0.0
01-06-02	6	[illegible]	31.32	31.32	0.00	0.00	31.32	[illegible]	45.94	43.22	2.71	0.0	5.9
02-06-02	7	[illegible]	31.32	33.47	0.00	[illegible]	33.47	[illegible]	45.94	41.23	4.71	0.0	10.3
03-06-02	8	[illegible]	31.32	34.47	0.00	[illegible]	34.47	[illegible]	45.94	45.41	0.52	0.0	1.1
05-06-02	10	[illegible]	31.32	30.07	0.00	1.25	30.07	[illegible]	45.94	49.01	[illegible]	4.0	0.0
08-06-02	13	[illegible]	31.32	26.08	0.00	5.24	26.08	[illegible]	45.94	38.63	7.31	16.7	15.9
11-06-02	16	[illegible]	31.32	28.86	0.00	2.46	28.86	[illegible]	45.94	45.37	0.56	7.9	1.2
14-06-02	19	[illegible]	31.32	31.61	0.00	[illegible]	31.61	[illegible]	45.94	36.00	9.94	0.0	21.6
16-06-02	21	[illegible]	31.32	33.62	0.00	[illegible]	33.62	[illegible]	45.94	41.99	3.95	0.0	8.6
18-06-02	23	[illegible]	31.32	32.87	0.00	[illegible]	32.87	[illegible]	45.94	45.67	[illegible]	0.0	0.0
21-06-02	26	[illegible]	31.32	32.93	0.00	[illegible]	32.93	[illegible]	45.94	43.53	2.40	0.0	5.2
23-06-02	28	[illegible]	31.32	33.09	0.00	[illegible]	33.09	[illegible]	45.94	43.08	2.86	0.0	6.2
26-06-02	31	[illegible]	31.32	32.22	0.00	[illegible]	32.22	[illegible]	45.94	42.93	3.01	0.0	6.5
28-06-02	33	[illegible]	31.32	34.35	0.00	[illegible]	34.35	[illegible]	45.94	38.75	7.19	0.0	15.6
16-07-02	36	[illegible]	31.32	35.02	0.00	[illegible]	35.02	[illegible]	45.94	42.32	3.61	0.0	7.9
20-07-02	40	[illegible]	31.32	31.24	0.00	0.08	31.24	[illegible]	45.94	43.91	2.03	0.3	4.4
22-07-02	42	[illegible]	31.32	30.09	0.00	1.23	30.09	[illegible]	45.94	38.73	7.20	3.9	15.7
25-07-02	45	[illegible]	31.32	31.05	0.00	0.27	31.05	[illegible]	45.94	40.16	5.78	0.9	12.6
27-07-02	47	[illegible]	31.32	31.19	0.00	0.13	31.19	[illegible]	45.94	41.26	4.68	0.4	10.2
29-07-02	49	[illegible]	31.32	24.58	0.00	6.74	24.58	[illegible]	45.94	36.54	9.40	21.5	20.5
31-07-02	51	[illegible]	31.32	34.58	0.00	[illegible]	34.58	[illegible]	45.94	38.94	6.99	0.0	15.2
03-08-02	54	[illegible]	31.32	28.02	0.00	3.30	28.02	[illegible]	45.94	42.62	3.31	10.5	7.2
06-08-02	56	[illegible]	31.32	35.06	0.00	[illegible]	35.06	[illegible]	45.94	41.70	4.24	0.0	9.2
10-08-02	58	[illegible]	31.32	24.18	0.00	7.14	24.18	[illegible]	45.94	43.85	2.09	22.8	4.5
12-08-02	60	[illegible]	31.32	29.82	0.00	1.50	29.82	[illegible]	45.94	39.59	6.35	4.8	13.8
25-08-02	64	[illegible]	31.32	32.43	0.00	[illegible]	32.43	[illegible]	45.94	47.23	[illegible]	0.0	0.0
27-08-02	66	[illegible]	31.32	31.56	0.00	[illegible]	31.55	[illegible]	45.94	44.22	1.71	0.0	3.7
31-08-02	70	[illegible]	31.32	29.97	0.00	1.35	29.97	[illegible]	45.94	41.72	4.22	4.3	9.2
02-09-02	72	[illegible]	31.32	32.47	0.00	[illegible]	32.47	[illegible]	45.94	42.20	3.74	0.0	8.1
04-09-02	74	[illegible]	31.32	33.39	0.00	[illegible]	33.39	[illegible]	45.94	50.59	[illegible]	0.0	0.0
10-09-02	76	[illegible]	31.32	32.26	0.00	[illegible]	32.26	[illegible]	45.94	43.22	2.71	0.0	5.9
12-09-02	78	[illegible]	31.32	30.23	0.00	1.09	30.23	[illegible]	45.94	43.28	2.65	3.5	5.8
13-09-02	79	[illegible]	31.32	30.69	0.00	0.63	30.69	[illegible]	45.94	46.28	[illegible]	2.0	0.0
14-09-02	80	[illegible]	31.32	32.11	0.00	[illegible]	32.11	[illegible]	45.94	44.22	1.71	0.0	3.7
16-09-02	81	[illegible]	31.32	31.57	0.00	[illegible]	31.57	[illegible]	45.94	47.84	[illegible]	0.0	0.0
20-09-02	84	[illegible]	31.32	31.65	0.00	[illegible]	31.65	[illegible]	45.94	41.91	4.03	0.0	8.8

Trial II			MC 2		Autotrophic control							
Date	Exp day.	Measured influent NO_3^-N (mg l^{-1})	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Measured influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from measured input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	[illegible]	31.32	22.32	0.00	9.00	22.32	[illegible]	N/A	4.50	[illegible]	28.7	0.0
30-05-02	4	[illegible]	31.32	29.80	0.00	1.52	29.80	[illegible]	N/A	3.59	[illegible]	4.9	0.0
31-05-02	5	[illegible]	31.32	25.01	0.00	6.31	25.01	[illegible]	N/A	4.06	[illegible]	20.1	0.0
01-06-02	6	[illegible]	31.32	24.45	0.00	6.87	24.45	[illegible]	N/A	3.57	[illegible]	21.9	0.0
02-06-02	7	[illegible]	31.32	32.16	0.00	[illegible]	32.16	[illegible]	N/A	3.14	[illegible]	0.0	0.0
03-06-02	8	[illegible]	31.32	35.04	0.00	[illegible]	35.04	[illegible]	N/A	4.02	[illegible]	0.0	0.0
05-06-02	10	[illegible]	31.32	33.28	0.00	[illegible]	33.28	[illegible]	N/A	4.18	[illegible]	0.0	0.0
08-06-02	13	[illegible]	31.32	32.61	0.00	[illegible]	32.61	[illegible]	N/A	2.46	[illegible]	0.0	0.0
11-06-02	16	[illegible]	31.32	30.68	0.00	0.64	30.68	[illegible]	N/A	3.16	[illegible]	0.0	0.0
14-06-02	19	[illegible]	31.32	32.95	0.00	[illegible]	32.95	[illegible]	N/A	5.32	[illegible]	0.0	0.0
16-06-02	21	[illegible]	31.32	33.55	0.00	[illegible]	33.55	[illegible]	N/A	6.28	[illegible]	0.0	0.0
18-06-02	23	[illegible]	31.32	33.45	0.00	[illegible]	33.45	[illegible]	N/A	5.71	[illegible]	0.0	0.0
21-06-02	26	[illegible]	31.32	32.87	0.00	[illegible]	32.87	[illegible]	N/A	4.08	[illegible]	0.0	0.0
23-06-02	28	[illegible]	31.32	33.26	0.00	[illegible]	33.26	[illegible]	N/A	3.97	[illegible]	0.0	0.0
26-06-02	31	[illegible]	31.32	33.62	0.00	[illegible]	33.62	[illegible]	N/A	5.37	[illegible]	0.0	0.0
28-06-02	33	[illegible]	31.32	29.61	0.00	1.71	29.61	[illegible]	N/A	6.76	[illegible]	0.0	0.0
16-07-02	36	[illegible]	31.32	33.70	0.00	[illegible]	33.70	[illegible]	N/A	4.55	[illegible]	0.0	0.0
20-07-02	40	[illegible]	31.32	32.05	0.00	[illegible]	32.05	[illegible]	N/A	5.11	[illegible]	0.0	0.0
22-07-02	42	[illegible]	31.32	28.92	0.00	2.40	28.92	[illegible]	N/A	2.17	[illegible]	0.0	0.0
25-07-02	45	[illegible]	31.32	29.57	0.00	1.75	29.57	[illegible]	N/A	3.73	[illegible]	0.0	0.0
27-07-02	47	[illegible]	31.32	24.33	0.00	6.99	24.33	[illegible]	N/A	4.06	[illegible]	0.0	0.0
29-07-02	49	[illegible]	31.32	29.04	0.00	2.28	29.04	[illegible]	N/A	2.71	[illegible]	0.0	0.0
31-07-02	51	[illegible]	31.32	32.05	0.00	[illegible]	32.05	[illegible]	N/A	3.93	[illegible]	0.0	0.0
03-08-02	54	[illegible]	31.32	31.36	0.00	[illegible]	31.36	[illegible]	N/A	3.14	[illegible]	0.0	0.0
06-08-02	56	[illegible]	31.32	32.39	0.00	[illegible]	32.39	[illegible]	N/A	2.70	[illegible]	0.0	0.0
10-08-02	58	[illegible]	31.32	31.42	0.00	[illegible]	31.42	[illegible]	N/A	1.90	[illegible]	0.0	0.0
12-08-02	60	[illegible]	31.32	31.32	0.00	0.00	31.32	[illegible]	N/A	3.72	[illegible]	0.0	0.0
25-08-02	64	[illegible]	31.32	30.23	0.00	1.09	30.23	[illegible]	N/A	2.92	[illegible]	3.5	0.0
27-08-02	66	[illegible]	31.32	32.72	0.00	[illegible]	32.72	[illegible]	N/A	3.23	[illegible]	0.0	0.0
31-08-02	70	[illegible]	31.32	31.26	0.00	0.06	31.26	[illegible]	N/A	3.30	[illegible]	0.2	0.0
02-09-02	72	[illegible]	31.32	31.07	0.00	0.25	31.07	[illegible]	N/A	2.00	0.02	0.8	0.0
04-09-02	74	[illegible]	31.32	24.82	0.00	6.50	24.82	[illegible]	N/A	2.00	0.06	20.7	0.0
10-09-02	76	[illegible]	31.32	32.18	0.00	[illegible]	32.18	[illegible]	N/A	6.51	[illegible]	0.0	0.0
12-09-02	78	[illegible]	31.32	33.47	0.00	[illegible]	33.47	[illegible]	N/A	3.21	[illegible]	0.0	0.0
13-09-02	79	[illegible]	31.32	32.85	0.00	[illegible]	32.85	[illegible]	N/A	3.06	[illegible]	0.0	0.0
14-09-02	80	[illegible]	31.32	31.34	0.00	[illegible]	31.34	[illegible]	N/A	4.43	[illegible]	0.0	0.0
16-09-02	81	[illegible]	31.32	31.74	0.00	[illegible]	31.74	[illegible]	N/A	3.26	[illegible]	0.0	0.0
20-09-02	84	[illegible]	31.32	31.51	0.00	[illegible]	31.51	[illegible]	N/A	3.09	[illegible]	0.0	0.0

Trial II		MC 3	Glucose; stoichiometric C/N = 1.46 ? 1.80 ? 2.20 ? 2.53									
Date	Exp day.	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	31.32	23.20	0.00	8.12	0.00	[illegible]	45.94	42.74	3.19	25.9	7.0
30-05-02	4	31.32	27.10	0.00	4.22	27.10	[illegible]	45.94	29.40	16.54	13.5	36.0
31-05-02	5	[illegible]	31.32	33.28	0.51	[illegible]	33.79	[illegible]	45.94	9.68	36.25	0.0	78.9
01-06-02	5	[illegible]	31.32	30.30	0.51	30.81	[illegible]	45.94	32.03	13.91	1.6	30.3
02-06-02	7	[illegible]	31.32	27.83	4.09	[illegible]	31.93	[illegible]	45.94	20.06	25.88	0.0	56.3
03-06-02	8	[illegible]	31.32	12.19	5.39	13.74	17.58	[illegible]	45.94	12.58	33.36	43.9	72.6
05-06-02	10	[illegible]	31.32	24.81	5.41	1.11	30.21	[illegible]	45.94	21.34	24.60	3.5	53.5
08-06-02	13	[illegible]	31.32	3.26	19.50	8.56	22.76	[illegible]	45.94	10.75	35.18	27.3	76.6
11-06-02	16	[illegible]	31.32	8.37	11.69	11.25	20.07	[illegible]	45.94	19.84	26.10	35.9	56.8
14-06-02	19	[illegible]	31.32	2.80	23.99	4.53	26.79	[illegible]	45.94	11.61	34.33	14.5	74.7
16-06-02	21	[illegible]	31.32	14.89	5.15	11.28	20.04	[illegible]	45.94	17.73	28.21	36.0	61.4
18-06-02	23	[illegible]	31.32	5.49	5.85	19.98	11.34	[illegible]	45.94	10.82	35.12	63.8	76.5
21-06-02	26	[illegible]	31.32	15.47	5.83	10.02	21.30	[illegible]	45.94	3.48	42.45	32.0	92.4
23-06-02	28	[illegible]	31.32	22.43	6.15	2.74	28.58	[illegible]	45.94	4.32	41.62	8.7	90.6
26-06-02	31	[illegible]	31.32	7.08	21.57	2.67	28.65	[illegible]	45.94	-	-	8.5	
28-06-02	33	[illegible]	31.32	6.08	23.11	2.13	29.19	[illegible]	45.94	4.21	41.73	6.8	90.8
16-07-02	36	[illegible]	31.32	18.96	11.13	1.23	30.09	[illegible]	45.94	18.88	27.06	3.9	58.9
20-07-02	40	[illegible]	31.32	9.40	16.54	5.39	25.93	[illegible]	45.94	6.76	39.18	17.2	85.3
22-07-02	42	[illegible]	31.32	12.74	6.93	11.65	19.67	[illegible]	45.94	13.07	32.87	37.2	71.6
25-07-02	45	[illegible]	31.32	3.30	19.56	8.46	22.86	[illegible]	45.94	5.56	40.38	27.0	87.9
27-07-02	47	[illegible]	31.32	23.33	7.54	0.45	30.87	[illegible]	45.94	7.42	38.52	1.4	83.8
29-07-02	49	[illegible]	31.32	9.77	28.84	1.71	29.61	[illegible]	45.94	3.49	42.44	5.5	92.4
31-07-02	51	[illegible]	31.32	15.18	18.21	[illegible]	33.39	[illegible]	45.94	4.17	41.77	0.0	90.9
03-08-02	54	[illegible]	31.32	1.27	21.88	8.16	23.16	[illegible]	45.94	7.36	38.58	26.1	84.0
08-08-02	56	[illegible]	31.32	21.97	7.64	1.71	29.61	[illegible]	45.94	35.93	10.00	5.5	21.8
10-08-02	58	[illegible]	31.32	20.59	4.36	6.37	24.95	[illegible]	45.94	3.51	42.42	20.3	92.4
12-08-02	60	[illegible]	31.32	22.03	8.27	1.02	30.30	[illegible]	45.94	11.13	34.80	3.3	75.8
25-08-02	64	[illegible]	31.32	15.46	12.67	3.19	28.13	[illegible]	56.38	22.15	34.22	10.2	60.7
27-08-02	66	[illegible]	31.32	17.93	11.61	1.78	29.54	[illegible]	56.38	33.53	22.84	5.7	40.5
31-08-02	70	[illegible]	31.32	4.67	16.78	9.67	21.65	[illegible]	56.38	13.93	42.44	30.9	75.3
02-09-02	72	[illegible]	31.32	3.73	16.56	10.93	20.39	[illegible]	58.90	9.84	59.07	34.9	85.7
04-09-02	74	[illegible]	31.32	0.35	2.51	28.46	2.86	[illegible]	58.90	6.32	62.58	90.9	90.8
10-09-02	76	[illegible]	31.32	0.19	20.62	10.51	20.81	[illegible]	58.90	25.91	42.99	33.6	62.4
12-09-02	78	[illegible]	31.32	5.09	16.17	10.06	21.26	[illegible]	58.90	17.34	51.57	32.1	74.8
13-09-02	79	[illegible]	31.32	0.13	13.39	17.81	13.51	[illegible]	79.34	15.78	63.56	56.9	80.1
14-09-02	80	[illegible]	31.32	0.02	7.50	23.80	7.52	[illegible]	79.34	8.08	71.26	76.0	89.8
16-09-02	81	[illegible]	31.32	0.08	7.12	24.12	7.20	[illegible]	79.34	7.20	72.14	77.0	90.9
20-09-02	84	[illegible]	31.32	1.09	4.84	25.39	5.93	[illegible]	79.34	6.31	73.04	81.1	92.1

Trial II		MC 4	Glucose; stoichiometric C/N = 1.46 ? 1.80 ? 2.20 ? 2.53									
Date	Exp day.	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	[illegible]	31.32	30.15	0.00	1.17	30.15	[illegible]	45.94	50.61	[illegible]	3.7	0.0
30-05-02	4	[illegible]	31.32	27.25	0.00	4.07	27.25	[illegible]	45.94	42.63	3.31	13.0	7.2
31-05-02	5	[illegible]	31.32	30.43	2.67	[illegible]	33.10	[illegible]	45.94	9.88	36.05	0.0	78.5
01-06-02	6	[illegible]	31.32	25.52	0.00	5.80	25.52	[illegible]	45.94	10.78	35.15	18.5	76.5
02-06-02	7	[illegible]	31.32	9.17	4.68	17.48	13.84	[illegible]	45.94	6.25	39.68	55.8	86.4
03-06-02	8	[illegible]	31.32	26.50	4.99	[illegible]	31.49	[illegible]	45.94	6.99	38.94	0.0	84.8
05-06-02	10	[illegible]	31.32	17.83	2.44	11.05	20.27	[illegible]	45.94	3.75	42.19	35.3	91.8
08-06-02	13	[illegible]	31.32	14.97	8.56	7.79	23.53	[illegible]	45.94	9.06	36.87	24.9	80.3
11-06-02	16	[illegible]	31.32	14.89	9.67	6.77	24.55	[illegible]	45.94	5.55	40.38	21.6	87.9
14-06-02	19	[illegible]	31.32	10.36	9.42	11.55	19.77	[illegible]	45.94	9.05	36.89	36.9	80.3
16-06-02	21	[illegible]	31.32	7.98	4.74	18.60	12.72	[illegible]	45.94	10.94	34.99	59.4	76.2
18-06-02	23	[illegible]	31.32	14.41	4.97	11.94	19.38	[illegible]	45.94	14.76	31.17	38.1	67.9
21-06-02	26	[illegible]	31.32	18.19	6.49	6.64	24.68	[illegible]	45.94	2.57	43.37	21.2	94.4
23-06-02	28	[illegible]	31.32	28.21	4.97	[illegible]	33.18	[illegible]	45.94	3.15	42.78	0.0	93.1
26-06-02	31	[illegible]	31.32	10.94	10.75	9.63	21.69	[illegible]	45.94	2.86	43.08	30.7	93.8
28-06-02	33	[illegible]	31.32	5.62	15.64	10.06	21.26	[illegible]	45.94	3.74	42.19	32.1	91.9
16-07-02	36	[illegible]	31.32	16.20	5.05	10.06	21.26	[illegible]	45.94	27.08	18.85	32.1	41.0
20-07-02	40	[illegible]	31.32	8.06	9.76	13.51	17.81	[illegible]	45.94	8.84	37.10	43.1	80.8
22-07-02	42	[illegible]	31.32	15.64	5.22	10.46	20.86	[illegible]	45.94	16.99	28.94	33.4	63.0
25-07-02	45	[illegible]	31.32	10.40	16.45	4.47	26.85	[illegible]	45.94	14.24	31.69	14.3	69.0
27-07-02	47	[illegible]	31.32	4.30	25.20	1.82	29.50	[illegible]	45.94	9.26	36.68	5.8	79.8
29-07-02	49	[illegible]	31.32	0.35	31.32	[illegible]	31.67	[illegible]	45.94	6.67	39.26	0.0	85.5
31-07-02	51	[illegible]	31.32	6.26	26.08	[illegible]	32.34	[illegible]	45.94	5.01	40.93	0.0	89.1
03-08-02	54	[illegible]	31.32	7.04	18.12	6.16	25.16	[illegible]	45.94	6.06	39.88	19.7	86.8
08-08-02	56	[illegible]	31.32	20.55	1.52	9.25	22.07	[illegible]	45.94	36.50	9.44	29.5	20.5
10-08-02	58	[illegible]	31.32	15.76	8.08	7.48	23.84	[illegible]	45.94	4.82	41.12	23.9	89.5
12-08-02	60	[illegible]	31.32	23.30	6.79	1.23	30.09	[illegible]	45.94	13.19	32.75	3.9	71.3
25-08-02	64	[illegible]	31.32	16.36	11.88	3.06	28.24	[illegible]	56.38	25.04	31.34	9.8	55.6
27-08-02	66	[illegible]	31.32	28.07	1.24	2.01	29.31	[illegible]	56.38	26.58	29.80	6.4	52.9
31-08-02	70	[illegible]	31.32	1.72	15.60	14.01	17.31	[illegible]	56.38	4.56	51.81	44.7	91.9
02-09-02	72	[illegible]	31.32	2.15	10.32	18.85	12.47	[illegible]	68.90	2.12	66.78	60.2	96.9
04-09-02	74	[illegible]	31.32	0.00	1.67	29.65	1.67	[illegible]	68.90	5.05	63.85	94.7	92.7
10-09-02	76	[illegible]	31.32	0.38	14.47	16.48	14.84	[illegible]	68.90	21.99	46.92	52.6	68.1
12-09-02	78	[illegible]	31.32	8.94	2.11	20.28	11.04	[illegible]	68.90	4.21	64.70	64.7	93.9
13-09-02	79	[illegible]	31.32	0.19	13.40	17.73	13.59	[illegible]	79.34	16.07	63.27	56.6	79.7
14-09-02	80	[illegible]	31.32	0.00	9.75	21.57	9.75	[illegible]	79.34	12.28	67.07	68.9	84.5
16-09-02	81	[illegible]	31.32	0.00	7.50	22.91	8.41	[illegible]	79.34	9.32	70.03	73.1	88.3
20-09-02	84	[illegible]	31.32	0.00	6.70	24.62	6.70	[illegible]	79.34	7.59	71.75	78.6	90.4

Trial II			MC 5	Sodium acetate; stoichiometric C/N = 1.26 ? 1.60 ? 1.93 ? 2.26								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N ($mg\ d^{-1}$)	Effluent NO_3^-N ($mg\ d^{-1}$)	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) ($mg\ d^{-1}$)	Mass TON in effluent ($mg\ d^{-1}$)	[illegible]	Nominal influent DOC ($mg\ d^{-1}$)	Effluent DOC ($mg\ d^{-1}$)	Mass DOC utilised (from nominal input) ($mg\ d^{-1}$)	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	[illegible]	31.32	28.46	0.00	**2.36**	**28.96**	[illegible]	39.67	35.27	**4.40**	7.5	11.1
30-05-02	4	[illegible]	31.32	28.80	2.93	**2.59**	**28.73**	[illegible]	39.67	29.48	**10.19**	8.3	25.7
31-05-02	5	[illegible]	31.32	27.87	1.15	**2.30**	**29.02**	[illegible]	39.67	23.59	**16.08**	7.3	40.5
01-06-02	6	[illegible]	31.32	23.57	4.93	**2.82**	**28.50**	[illegible]	39.67	15.94	**23.73**	9.0	59.8
02-06-02	7		31.32	-	-			[illegible]	39.67	14.47	**25.20**		63.5
03-06-02	8	[illegible]	31.32	26.94	1.21	**3.17**	**28.15**	[illegible]	39.67	4.88	**34.79**	10.1	87.7
05-06-02	10	[illegible]	31.32	30.82	0.00	**0.50**	**30.82**	[illegible]	39.67	8.07	**31.60**	1.6	79.7
08-06-02	13	[illegible]	31.32	15.05	2.36	**13.91**	**17.41**	[illegible]	39.67	7.54	**32.13**	44.4	81.0
11-06-02	16	[illegible]	31.32	17.37	3.61	**10.34**	**20.98**	[illegible]	39.67	2.49	**37.18**	33.0	93.7
14-06-02	19	[illegible]	31.32	18.44	4.80	**8.08**	**23.24**	[illegible]	39.67	5.93	**33.74**	25.8	85.1
16-06-02	21	[illegible]	31.32	15.08	4.59	**11.66**	**19.67**	[illegible]	39.67	11.38	**28.29**	37.2	71.3
18-06-02	23	[illegible]	31.32	15.95	5.09	**10.27**	**21.05**	[illegible]	39.67	9.08	**30.59**	32.8	77.1
21-06-02	26	[illegible]	31.32	14.14	5.22	**11.96**	**19.36**		39.67	3.98	**35.69**	38.2	90.0
23-06-02	28	[illegible]	31.32	6.72	18.08	**6.51**	**24.81**	[illegible]	39.67	10.82	**28.85**	20.8	72.7
26-06-02	31	[illegible]	31.32	13.57	14.66	**3.09**	**28.23**	[illegible]	39.67	10.38	**29.29**	9.9	73.8
28-06-02	33	[illegible]	31.32	8.50	11.13	**11.69**	**19.63**	[illegible]	39.67	12.63	**27.04**	37.3	68.2
16-07-02	36	[illegible]	31.32	6.79	16.18	**8.35**	**22.97**	[illegible]	39.67	7.63	**32.04**	26.7	80.8
20-07-02	40	[illegible]	31.32	12.49	9.44	**9.40**	**21.92**	[illegible]	39.67	4.22	**35.45**	30.0	89.4
22-07-02	42	[illegible]	31.32	9.06	21.21	**1.04**	**30.28**	[illegible]	39.67	5.26	**34.41**	3.3	86.7
25-07-02	46	[illegible]	31.32	7.20	24.12	**0.00**	**31.32**	[illegible]	39.67	2.91	**36.76**	0.0	92.7
27-07-02	47	[illegible]	31.32	4.07	22.78	**4.47**	**26.85**	[illegible]	39.67	2.33	**37.34**	14.3	94.1
29-07-02	49	[illegible]	31.32	1.90	24.66	**4.76**	**26.56**	[illegible]	39.67	3.08	**36.59**	15.2	92.2
31-07-02	51	[illegible]	31.32	5.93	23.43	**1.96**	**29.36**	[illegible]	39.67	3.21	**36.46**	6.3	91.9
03-08-02	54	[illegible]	31.32	12.65	3.82	**14.85**	**16.47**	[illegible]	39.67	3.95	**35.72**	47.4	90.0
08-08-02	56	[illegible]	31.32	14.74	3.19	**13.38**	**17.94**	[illegible]	39.67	23.28	**16.39**	42.7	41.3
10-08-02	58	[illegible]	31.32	13.72	5.97	**11.63**	**19.69**	[illegible]	39.67	5.58	**34.09**	37.1	85.9
12-08-02	60	[illegible]	31.32	1.41	14.40	**15.51**	**15.81**	[illegible]	39.67	5.40	**34.27**	49.5	86.4
25-08-02	64	[illegible]	31.32	1.45	13.97	**15.89**	**15.43**	[illegible]	50.11	4.95	**45.16**	50.7	90.1
27-08-02	66	[illegible]	31.32	2.20	13.40	**15.62**	**15.70**	[illegible]	50.11	7.46	**42.65**	49.9	85.1
31-08-02	70	[illegible]	31.32	1.78	13.83	**15.71**	**15.61**	[illegible]	50.11	4.32	**45.79**	50.2	91.4
02-09-02	72	[illegible]	31.32	1.47	12.21	**17.65**	**13.67**	[illegible]	60.55	5.68	**54.87**	56.3	90.6
04-09-02	74	[illegible]	31.32	0.00	8.71	**22.61**	**8.71**	[illegible]	60.55	4.50	**56.05**	72.2	92.6
10-09-02	76	[illegible]	31.32	0.38	14.47	**16.48**	**14.84**	[illegible]	60.55	20.65	**39.90**	52.6	65.9
12-09-02	78	[illegible]	31.32	8.14	0.00	**23.18**	**8.14**	[illegible]	60.55	6.04	**54.51**	74.0	90.0
13-09-02	79	[illegible]	31.32	0.24	4.63	**26.45**	**4.87**	[illegible]	70.99	12.46	**58.53**	84.5	82.5
14-09-02	80	[illegible]	31.32	1.17	0.90	**29.25**	**2.07**	[illegible]	70.99	13.54	**57.45**	93.4	80.9
16-09-02	81	[illegible]	31.32	0.00	1.61	**29.71**	**1.61**	[illegible]	70.99	12.51	**58.38**	94.9	82.2
20-09-02	84	[illegible]	31.32	0.00	0.63	**30.69**	**0.63**	[illegible]	70.99	12.44	**58.55**	98.0	82.5

Trial II			MC 6	Sodium acetate; stoichiometric C/N = 1.26 ? 1.60 ? 1.93 ? 2.26								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N ($mg\ d^{-1}$)	Effluent NO_3^-N ($mg\ d^{-1}$)	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) ($mg\ d^{-1}$)	Mass TON in effluent ($mg\ d^{-1}$)	[illegible]	Nominal influent DOC ($mg\ d^{-1}$)	Effluent DOC ($mg\ d^{-1}$)	Mass DOC utilised (from nominal input) ($mg\ d^{-1}$)	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	[illegible]	31.32	30.49	0.00	**0.83**	**30.49**	[illegible]	39.67	35.60	**4.07**	2.7	10.3
30-05-02	4	[illegible]	31.32	26.10	0.00	**5.22**	**26.10**	[illegible]	39.67	32.65	**7.02**	16.7	17.7
31-05-02	5	[illegible]	31.32	28.77	1.00	**1.54**	**29.78**	[illegible]	39.67	24.10	**15.57**	4.9	39.3
01-06-02	6	[illegible]	31.32	27.79	1.48	**2.05**	**29.27**	[illegible]	39.67	17.33	**22.34**	6.5	56.3
02-06-02	7		31.32	27.87	3.36	**0.08**	**31.24**	[illegible]	39.67	17.85	**21.82**	0.3	55.0
03-06-02	8	[illegible]	31.32	13.51	14.57	**3.24**	**28.08**	[illegible]	39.67	8.05	**31.62**	10.3	79.7
05-06-02	10	[illegible]	31.32	29.19	1.73	**0.40**	**30.92**	[illegible]	39.67	3.94	**35.73**	1.3	90.1
08-06-02	13	[illegible]	31.32	31.05	0.00	**0.27**	**31.05**	[illegible]	39.67	2.21	**37.46**	0.9	94.4
11-06-02	16	[illegible]	31.32	30.67	1.63	[illegible]	**32.30**	[illegible]	39.67	2.82	**36.85**	0.0	92.9
14-06-02	19	[illegible]	31.32	10.13	16.04	**5.16**	**26.16**	[illegible]	39.67	6.91	**32.76**	16.5	82.6
16-06-02	21	[illegible]	31.32	6.72	8.67	**15.93**	**15.39**	[illegible]	39.67	13.55	**26.12**	50.9	65.8
18-06-02	23	[illegible]	31.32	20.32	5.78	**5.22**	**26.10**	[illegible]	39.67	10.48	**29.19**	16.7	73.6
21-06-02	26	[illegible]	31.32	17.81	5.93	**7.58**	**23.74**		39.67	9.50	**30.17**	24.2	76.1
23-06-02	28	[illegible]	31.32	20.27	6.26	**4.78**	**26.54**	[illegible]	39.67	6.51	**33.16**	15.3	83.6
26-06-02	31	[illegible]	31.32	6.08	22.01	**3.24**	**28.08**	[illegible]	39.67	8.90	**30.77**	10.3	77.6
28-06-02	33	[illegible]	31.32	10.69	19.54	**1.09**	**30.23**	[illegible]	39.67	11.00	**28.67**	3.5	72.3
16-07-02	36	[illegible]	31.32	8.25	24.05	[illegible]	**32.30**	[illegible]	39.67	9.97	**29.70**	0.0	74.9
20-07-02	40	[illegible]	31.32	3.88	20.59	**6.85**	**24.47**	[illegible]	39.67	5.50	**34.17**	21.9	86.1
22-07-02	42	[illegible]	31.32	15.26	7.68	**8.37**	**22.95**	[illegible]	39.67	4.54	**35.13**	26.7	88.5
25-07-02	45	[illegible]	31.32	3.90	24.07	**3.34**	**27.98**	[illegible]	39.67	5.17	**34.50**	10.7	87.0
27-07-02	47	[illegible]	31.32	5.60	25.72	**0.00**	**31.32**	[illegible]	39.67	4.03	**35.64**	0.0	89.8
29-07-02	49	[illegible]	31.32	2.88	27.37	**1.06**	**30.26**	[illegible]	39.67	4.44	**35.23**	3.4	88.8
31-07-02	51	[illegible]	31.32	1.88	25.64	**3.80**	**27.52**	[illegible]	39.67	4.00	**35.67**	12.1	89.9
03-08-02	54	[illegible]	31.32	3.11	25.35	**2.86**	**28.46**	[illegible]	39.67	4.90	**34.77**	9.1	87.7
08-08-02	56	[illegible]	31.32	15.39	5.12	**10.82**	**20.50**	[illegible]	39.67	11.19	**28.48**	34.8	71.8
10-08-02	58	[illegible]	31.32	17.06	9.98	**4.28**	**27.04**	[illegible]	39.67	9.36	**30.31**	13.7	76.4
12-08-02	60	[illegible]	31.32	15.37	9.40	**6.56**	**24.76**	[illegible]	39.67	6.33	**33.34**	20.9	84.0
25-08-02	64	[illegible]	31.32	3.04	17.86	**10.42**	**20.90**	[illegible]	50.11	4.72	**45.39**	33.3	90.6
27-08-02	66	[illegible]	31.32	2.89	18.06	**10.37**	**20.95**	[illegible]	50.11	5.85	**44.26**	33.1	88.3
31-08-02	70	[illegible]	31.32	0.62	15.79	**14.91**	**16.41**	[illegible]	50.11	4.62	**45.49**	47.6	90.8
02-09-02	72	[illegible]	31.32	0.00	11.51	**19.81**	**11.51**	[illegible]	60.55	6.03	**54.52**	63.3	90.0
04-09-02	74	[illegible]	31.32	4.23	5.43	**21.67**	**9.65**	[illegible]	60.55	6.58	**53.97**	69.2	89.1
10-09-02	76	[illegible]	31.32	0.68	10.14	**20.51**	**10.81**	[illegible]	60.55	20.11	**40.44**	65.5	66.8
12-09-02	78	[illegible]	31.32	10.38	0.00	**20.94**	**10.38**	[illegible]	60.55	5.22	**55.33**	66.9	91.4
13-09-02	79	[illegible]	31.32	0.09	5.66	**25.57**	**5.75**	[illegible]	70.99	6.95	**64.04**	81.7	90.2
14-09-02	80	[illegible]	31.32	0.27	1.88	**29.17**	**2.15**	[illegible]	70.99	10.19	**60.80**	93.1	85.7
16-09-02	81	[illegible]	31.32	0.13	3.09	**28.10**	**3.22**	[illegible]	70.99	10.52	**60.47**	89.7	85.2
20-09-02	84	[illegible]	31.32	0.00	1.82	**29.50**	**1.82**	[illegible]	70.99	10.67	**60.32**	94.2	85.0

Trial II			MC 7		Sodium acetate; stoichiometric C/N = 0.73							
Date	Exp day.	Mixed liquid influent NO_3 N (mg l^{-1})	Nominal influent NO_3 N (mg d^{-1})	Effluent NO_3 N (mg d^{-1})	Effluent NO_2 N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Mixed liquid influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	31.40	31.32	30.87	0.00	**0.45**	**30.87**	1,440	22.96	22.30	**0.66**	1.4	2.9
30-05-02	4	31.40	31.32	26.54	0.00	**4.78**	**26.54**	1,440	22.96	7.49	**15.47**	15.3	67.4
31-05-02	5	31.20	31.32	29.41	1.37	**0.54**	**30.78**	1,440	22.96	5.72	**17.24**	1.7	75.1
01-06-02	6	31.83	31.32	28.73	2.88	**0.08**	**31.61**	1,440	22.96	4.86	**18.07**	0.3	78.7
02-06-02	7	31.02	31.32	29.38	1.85	**0.08**	**31.24**	1,440	22.96	4.09	**18.87**	0.3	82.2
03-06-02	8	34.53	31.32	26.76	4.74	**0.02**	**31.49**	1,440	22.96	4.69	**18.27**	0.0	79.6
05-06-02	10	32.67	31.32	32.13	0.00	**+0.81**	**32.13**	1,440	22.96	3.35	**19.61**	0.0	85.4
08-06-02	13	31.67	31.32	29.75	0.00	**1.57**	**29.75**	1,440	22.96	4.87	**18.09**	5.0	78.8
11-06-02	16	33.80	31.32	28.96	3.63	**+1.27**	**32.59**	1,440	22.96	3.74	**19.22**	0.0	83.7
14-06-02	19	31.50	31.32	29.92	0.00	**1.40**	**29.92**	1,440	22.96	8.75	**14.21**	4.5	61.9
16-06-02	21	35.62	31.32	27.97	4.13	**+0.16**	**32.01**	1,440	22.96	11.36	**11.60**	0.0	50.5
18-06-02	23	40.85	31.32	13.57	4.38	**13.36**	**17.96**	1,440	22.96	8.31	**14.65**	42.7	63.8
21-06-02	26	32.11	31.32	24.93	4.43	**1.96**	**29.36**	1,440	22.96	14.45	**8.51**	6.3	37.1
23-06-02	28	34.08	31.32	25.41	4.32	**1.59**	**29.73**	1,440	22.96	10.71	**12.25**	5.1	53.3
26-06-02	31	33.65	31.32	28.08	2.23	**1.00**	**30.32**	1,440	22.96	9.08	**13.88**	3.2	60.4
28-06-02	33	33.40	31.32	30.05	0.73	**0.54**	**30.78**	1,440	22.96	6.75	**16.21**	1.7	70.6
30-06-02	36	34.06	31.32	29.64	4.19	**+2.51**	**33.83**	1,440	22.96	7.39	**15.57**	0.0	67.8
20-07-02	40	29.52	31.32	26.00	1.69	**3.63**	**27.69**	1,440	22.96	3.71	**19.25**	11.6	83.8
22-07-02	42	29.35	31.32	27.42	1.00	**2.90**	**28.42**	1,440	22.96	3.41	**19.55**	9.3	85.1
25-07-02	45	31.15	31.32	24.66	5.83	**0.84**	**30.48**	1,440	22.96	4.33	**18.63**	2.7	81.1
27-07-02	47	31.94	31.32	24.10	7.22	**0.00**	**31.32**	1,440	22.96	3.31	**19.65**	0.0	85.6
29-07-02	49	30.28	31.32	27.92	3.42	**+0.02**	**31.34**	1,440	22.96	2.97	**19.99**	0.0	87.1
31-07-02	51	31.98	31.32	31.94	0.00	**+0.62**	**31.94**	1,440	22.96	2.50	**20.46**	0.0	89.1

Trial II			MC 8		Sodium acetate; stoichiometric C/N = 0.73							
Date	Exp day.	Mixed liquid influent NO_3 N (mg l^{-1})	Nominal influent NO_3 N (mg d^{-1})	Effluent NO_3 N (mg d^{-1})	Effluent NO_2 N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Mixed liquid influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
29-05-02	3	33.42	31.32	30.14	0.00	**1.18**	**30.14**	1,440	22.97	20.04	**2.92**	3.8	12.7
30-05-02	4	31.92	31.32	25.56	0.00	**5.76**	**25.56**	1,440	22.97	8.86	**14.11**	18.4	61.4
31-05-02	5	31.20	31.32	28.29	2.57	**0.46**	**30.86**	1,440	22.97	8.77	**14.19**	1.5	61.8
01-06-02	6	31.80	31.32	31.05	0.65	**+0.38**	**31.70**	1,440	22.97	4.63	**18.34**	0.0	79.8
02-06-02	7	31.98	31.32	27.06	3.82	**0.44**	**30.88**	1,440	22.97	3.64	**19.33**	1.4	84.2
03-06-02	8	32.32	31.32	28.63	2.11	**0.58**	**30.74**	1,440	22.97	4.93	**18.04**	1.9	78.5
05-06-02	10	32.87	31.32	31.95	0.00	**+0.63**	**31.95**	1,440	22.97	3.74	**19.23**	0.0	83.7
08-06-02	13	31.95	31.32	31.53	0.00	**+0.20**	**31.54**	1,440	22.97	7.10	**15.87**	0.0	69.1
11-06-02	16	32.06	31.32	30.76	0.52	**0.04**	**31.28**	1,440	22.97	4.99	**17.98**	0.1	78.3
14-06-02	19	31.50	31.32	27.73	0.52	**3.07**	**28.25**	1,440	22.97	9.50	**13.47**	9.8	58.6
16-06-02	21	32.32	31.32	30.46	2.76	**+0.90**	**33.22**	1,440	22.97	12.19	**10.77**	0.0	46.9
18-06-02	23	30.97	31.32	26.14	4.45	**0.73**	**30.59**	1,440	22.97	9.59	**13.28**	2.3	57.8
21-06-02	26	32.12	31.32	25.16	4.53	**1.63**	**29.69**	1,440	22.97	12.97	**10.00**	5.2	43.5
23-06-02	28	34.36	31.32	26.50	4.47	**0.35**	**30.97**	1,440	22.97	11.59	**11.38**	1.1	49.5
26-06-02	31	33.90	31.32	29.38	1.29	**0.65**	**30.67**	1,440	22.97	9.38	**13.59**	2.1	59.2
28-06-02	33	33.46	31.32	28.69	0.73	**1.90**	**29.42**	1,440	22.97	6.31	**16.66**	6.1	72.5
16-07-02	36	36.60	31.32	23.51	3.19	**4.61**	**26.71**	1,440	22.97	5.63	**17.34**	14.7	75.5
20-07-02	40	29.32	31.32	27.96	1.48	**1.88**	**29.44**	1,440	22.97	4.28	**18.69**	6.0	81.4
22-07-02	42	29.37	31.32	29.11	0.00	**2.21**	**29.11**	1,440	22.97	3.82	**19.15**	7.1	83.4
25-07-02	45	30.15	31.32	21.23	4.49	**5.60**	**25.72**	1,440	22.97	4.40	**18.57**	17.9	80.8
27-07-02	47	31.54	31.32	20.96	5.64	**3.72**	**27.60**	1,440	22.97	4.27	**18.70**	11.9	81.4
29-07-02	49	32.29	31.32	28.86	2.11	**0.35**	**30.97**	1,440	22.97	5.08	**17.89**	1.1	77.9
31-07-02	51	31.98	31.32	30.54	0.52	**0.26**	**31.06**	1,440	22.97	3.58	**19.39**	0.8	84.4

Trial III			MC 1	Sterile control								
Date	Exp day.	Mixed liquid influent NO_3 N (mg l^{-1})	Nominal influent NO_3 N (mg d^{-1})	Effluent NO_3 N (mg d^{-1})	Effluent NO_2 N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	Mixed liquid influent DOC (mg l^{-1})	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	32.86	31.32	31.82	0.00	**+0.50**	**31.82**	70.27	73.08	59.02	**14.05**	0.0	19.2
03-02-03	3	32.42	31.32	31.94	0.00	**+0.62**	**31.94**	70.27	73.08	70.72	**2.36**	0.0	3.2
04-02-03	4	32.40	31.32	31.53	0.00	**+0.20**	**31.53**	72.10	73.08	71.72	**1.36**	0.0	1.9
05-02-03	5	32.15	31.32	32.61	0.00	**+1.29**	**32.61**	71.99	73.08	63.83	**9.25**	0.0	12.7
06-02-03	6	31.70	31.32	31.35	0.00	**+0.03**	**31.35**	73.75	73.08	65.69	**7.39**	0.0	10.1
10-02-03	9	32.65	31.32	29.60	0.00	**1.72**	**29.60**	**+1.24**	73.08	69.22	**3.86**	5.5	5.3
11-02-03	10	28.54	31.32	30.92	0.00	**0.40**	**30.92**	**+0.91**	73.08	73.44	**+0.36**	1.3	0.0
13-02-03	12	31.17	31.32	33.45	0.00	**+2.13**	**33.45**	68.10	73.08	65.29	**7.79**	0.0	10.7
15-02-03	14	28.78	31.32	26.64	0.00	**4.68**	**26.64**	66.21	73.08	64.02	**9.06**	14.9	12.4
17-02-03	16	30.56	31.32	26.06	0.00	**5.26**	**26.06**	63.21	73.08	63.68	**9.40**	16.8	12.9
24-02-03	21	31.47	31.32	31.09	0.00	**0.23**	**31.09**	71.37	73.08	71.31	**1.77**	0.7	2.4
26-02-03	23	32.60	31.32	32.37	0.00	**+1.05**	**32.37**	67.07	73.08	64.56	**8.52**	0.0	11.7
28-02-03	25	30.90	31.32	33.70	0.00	**+2.38**	**33.70**	**+1.20**	73.08	69.01	**4.07**	0.0	5.6
02-03-03	27	27.61	31.32	29.48	0.00	**1.84**	**29.48**	66.81	73.08	65.77	**7.31**	5.9	10.0
04-03-03	29	27.32	31.32	29.19	0.00	**2.13**	**29.19**	65.00	73.08	66.13	**6.95**	6.8	9.5
06-03-03	31	30.01	31.32	29.71	0.00	**1.61**	**29.71**	65.88	73.08	70.03	**3.05**	5.1	4.2
08-03-03	33	30.20	31.32	31.18	0.00	**0.14**	**31.18**	72.65	73.08	80.47	**−7.39**	0.5	0.0
10-03-03	35	33.31	31.32	30.07	0.00	**1.25**	**30.07**	83.95	73.08	80.22	**−7.14**	4.0	0.0
12-03-03	37	31.19	31.32	31.38	0.00	**+0.05**	**31.38**	68.35	73.08	64.48	**8.60**	0.0	11.8
13-03-03	38	29.60	31.32	27.57	0.00	**3.75**	**27.57**	104.96	73.08	76.40	**−3.32**	12.0	0.0
15-03-03	40	30.11	31.32	31.27	0.00	**0.05**	**31.27**	103.10	104.40	99.45	**4.95**	0.2	4.7
19-03-03	44	29.23	31.32	30.19	0.00	**1.13**	**30.19**	103.23	104.40	98.97	**5.43**	3.6	5.2
21-03-03	46	32.44	31.32	29.29	0.00	**2.03**	**29.29**	102.36	104.40	96.68	**7.72**	6.5	7.4
24-03-03	49	34.86	31.32	33.48	0.00	**+2.15**	**33.48**		104.40	95.90	**8.50**	0.0	8.1
27-03-03	52	31.68	31.32	30.96	0.00	**0.36**	**30.96**		104.40	96.13	**8.27**	1.2	7.9
30-03-03	55	32.52	31.32	30.23	0.00	**1.09**	**30.23**		104.40	98.47	**5.93**	3.5	5.7

Trial III			MC 2	Sterile control							
Date	Exp day.	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	31.56	0.00		31.56		73.08	64.83	8.25	0.0	11.3
03-02-03	3	31.32	31.44	0.00		31.44		73.08	71.28	1.80	0.0	2.5
04-02-03	4	31.32	31.03	0.00	0.29	31.03		73.08	68.72	4.36	0.9	6.0
05-02-03	5	31.32	31.29	0.00	0.03	31.29		73.08	69.62	3.46	0.1	4.7
06-02-03	6	31.32	32.04	0.00		32.04		73.08	67.19	5.89	0.0	8.1
10-02-03	9	31.32	32.55	0.00		32.55		73.08	74.48		0.0	0.0
11-02-03	10	31.32	28.54	0.00	2.78	28.54		73.08	70.24	2.84	8.9	3.9
13-02-03	12	31.32	32.80	0.00		32.80		73.08	69.17	3.91	0.0	5.4
15-02-03	14	31.32	31.68	0.00		31.68		73.08	65.42	7.66	0.0	10.5
17-02-03	16	31.32	28.45	0.00	2.87	28.45		73.08	70.45	2.63	9.2	3.6
24-02-03	21	31.32	30.78	0.00	0.54	30.78		73.08	71.52	1.56	1.7	2.1
26-02-03	23	31.32	32.86	0.00		32.86		73.08	66.36	6.72	0.0	9.2
28-02-03	25	31.32	36.22	0.00		36.22		73.08	61.97	11.11	0.0	15.2
02-03-03	27	31.32	29.62	0.00	1.70	29.62		73.08	66.86	6.22	5.4	8.5
04-03-03	29	31.32	28.70	0.00	2.62	28.70		73.08	67.34	5.74	8.4	7.9
06-03-03	31	31.32	31.82	0.00		31.82		73.08	68.34	4.74	0.0	6.5
08-03-03	33	31.32	31.71	0.00		31.71		73.08	71.31	1.77	0.0	2.4
10-03-03	35	31.32	30.89	0.00	0.43	30.89		73.08	69.83	3.25	1.4	4.4
12-03-03	37	31.32	30.21	0.00	1.11	30.21		73.08	68.78	4.30	3.5	5.9
13-03-03	38	31.32	30.76	0.00	0.56	30.76		73.08	78.84		1.8	0.0
15-03-03	40	31.32	31.36	0.00		31.36		104.40	100.64	3.76	0.0	3.6
19-03-03	44	31.32	30.60	0.00	0.72	30.60		104.40	92.20	12.20	2.3	11.7
21-03-03	46	31.32	32.00	0.00		32.00		104.40	102.81	1.59	0.0	1.5
24-03-03	49	31.32	33.13	0.00		33.13		104.40	95.13	9.27	0.0	8.9
27-03-03	52	31.32	31.65	0.00		31.65		104.40	103.39	1.01	0.0	1.0
30-03-03	55	31.32	30.74	0.00	0.58	30.74		104.40	98.70	5.70	1.9	5.5

Trial III			MC 3	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33							
Date	Exp day.	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	23.79	0.00	7.53	23.79	75.09	73.08	75.42		24.0	0.0
03-02-03	3	31.32	30.16	0.00	1.16	30.16	64.39	73.08	56.07	16.01	3.7	20.5
04-02-03	4	31.32	28.46	0.00	2.86	28.46	69.72	73.08	52.33	20.75	9.1	28.4
05-02-03	5	31.32	23.61	0.00	7.71	23.61	64.44	73.08	40.49	32.59	24.6	44.6
06-02-03	6	31.32	15.37	0.00	15.95	15.37	62.64	73.08	29.06	44.02	50.9	60.2
10-02-03	9	31.32	15.89	4.17	11.56	19.76	61.18	73.08	32.49	40.59	36.9	55.5
11-02-03	10	31.32	13.93	3.64	13.75	17.57	65.29	73.08	23.87	49.21	43.9	67.3
13-02-03	12	31.32	10.49	6.99	13.84	17.48	-	73.08	17.45	55.63	44.2	76.1
15-02-03	14	31.32	17.40	4.26	9.65	21.67	57.00	73.08	27.35	45.73	30.8	62.6
17-02-03	16	31.32	11.53	5.87	13.92	17.40	65.63	73.08	24.99	48.09	44.4	65.8
24-02-03	21	31.32	0.00	0.00	31.32	0.00	-	73.08	14.64	58.44	100.0	80.0
26-02-03	23	31.32	0.00	0.00	31.32	0.00	46.14	73.08	20.61	52.47	100.0	71.8
28-02-03	25	31.32	4.86	0.00	26.46	4.86	-	73.08	9.28	63.80	84.5	87.3
02-03-03	27	31.32	2.51	4.06	24.73	6.59	61.70	73.08	5.18	67.90	79.0	92.9
04-03-03	29	31.32	0.00	0.00	31.32	0.00	66.73	73.08	16.23	56.85	100.0	77.8
06-03-03	31	31.32	2.99	0.00	28.33	2.99	57.00	73.08	17.07	56.01	90.5	76.6
08-03-03	33	31.32	2.09	0.00	29.23	2.09	56.17	73.08	5.57	67.51	93.3	92.4
10-03-03	35	31.32	3.55	1.18	26.59	4.73	65.35	73.08	4.51	68.57	84.9	93.8
12-03-03	37	31.32	3.11	0.00	28.21	3.11	59.65	73.08	12.62	60.26	90.1	82.5
13-03-03	38	31.32	8.16	0.00	23.16	8.16	80.20	104.40	38.02	66.38	73.9	63.6
15-03-03	40	31.32	2.77	0.00	28.55	2.77	-	104.40	46.27	58.13	91.2	55.7
19-03-03	44	31.32	0.00	0.00	31.32	0.00	-	104.40	41.61	62.79	100.0	60.1
21-03-03	46	31.32	0.00	0.00	31.32	0.00	-	104.40	50.01	54.39	100.0	52.1
24-03-03	49	31.32	0.00	0.00	31.32	0.00	-	104.40	41.93	62.47	100.0	59.8
27-03-03	52	31.32	0.00	0.00	31.32	0.00	-	104.40	42.62	61.78	100.0	59.2
30-03-03	55	31.32	0.00	0.00	31.32	0.00	-	104.40	36.67	67.73	100.0	64.9

Trial III			MC 4	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33							
Date	Exp day.	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	30.76	0.00	0.56	30.76	75.09	73.08	75.06		1.8	0.0
03-02-03	3	31.32	33.24	0.00		33.24	64.39	73.08	63.16	9.92	0.0	13.6
04-02-03	4	31.32	28.73	0.00	2.59	28.73	69.72	73.08	61.83	11.25	8.3	15.4
05-02-03	5	31.32	29.20	0.00	2.12	29.20	64.44	73.08	52.49	20.59	6.8	28.2
06-02-03	6	31.32	26.97	0.00	4.35	26.97	62.64	73.08	50.47	22.61	13.9	30.9
10-02-03	9	31.32	20.58	0.00	10.74	20.58	61.18	73.08	39.80	33.28	34.3	45.5
11-02-03	10	31.32	18.33	0.70	12.29	19.03	65.29	73.08	28.96	44.12	39.2	60.4
13-02-03	12	31.32	7.79	2.33	21.20	10.12	-	73.08	8.15	64.93	67.7	88.8
15-02-03	14	31.32	7.68	3.86	19.77	11.55	57.00	73.08	5.88	67.20	63.1	92.0
17-02-03	16	31.32	19.26	2.09	9.97	21.35	65.63	73.08	46.50	26.58	31.8	36.4
24-02-03	21	31.32	7.11	0.86	23.35	7.97	-	73.08	12.70	60.38	74.5	82.6
26-02-03	23	31.32	10.47	7.10	13.75	17.57	46.14	73.08	31.17	41.91	43.9	57.3
28-02-03	25	31.32	1.92	1.87	27.53	3.79	-	73.08	27.54	45.54	87.9	62.3
02-03-03	27	31.32	1.85	4.22	25.27	6.05	61.70	73.08	8.56	64.52	80.7	88.3
04-03-03	29	31.32	0.00	0.00	31.32	0.00	66.73	73.08	5.66	67.42	100.0	92.3
06-03-03	31	31.32	0.00	0.00	31.32	0.00	57.00	73.08	13.29	59.79	100.0	81.8
08-03-03	33	31.32	0.00	0.00	31.32	0.00	56.17	73.08	14.61	58.47	100.0	80.0
10-03-03	35	31.32	3.90	2.98	24.45	6.87	65.35	73.08	6.42	66.66	78.1	91.2
12-03-03	37	31.32	0.00	0.00	31.32	0.00	59.65	73.08	4.31	68.77	100.0	94.1
13-03-03	38	31.32	0.00	0.00	31.32	0.00	80.20	104.40	9.44	94.96	100.0	91.0
15-03-03	40	31.32	0.00	0.00	31.32	0.00	-	104.40	30.03	74.37	100.0	71.2
19-03-03	44	31.32	0.00	0.00	31.32	0.00	-	104.40	41.80	62.60	100.0	60.0
21-03-03	46	31.32	0.00	0.00	31.32	0.00	-	104.40	43.14	61.26	100.0	58.7
24-03-03	49	31.32	0.00	0.00	31.32	0.00	-	104.40	41.18	63.22	100.0	60.6
27-03-03	52	31.32	0.00	0.00	31.32	0.00	-	104.40	36.58	67.82	100.0	65.0
30-03-03	55	31.32	0.00	0.00	31.32	0.00	-	104.40	40.42	63.98	100.0	61.3

Trial III			MC 5	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33								
Date	Exp day.	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	14.39	0.00	16.93	14.39	75.09	73.08	39.86	33.22	54.1	45.6
03-02-03	3	31.32	30.54	0.00	0.78	30.54	64.39	73.08	57.40	15.68	2.5	21.6
04-02-03	4	31.32	27.81	0.00	3.51	27.81	69.72	73.08	32.68	40.40	11.2	55.3
05-02-03	5	31.32	22.15	3.20	5.97	25.35	64.44	73.08	35.35	37.73	19.1	51.6
06-02-03	6	31.32	17.59	3.90	9.83	21.49	62.64	73.08	26.41	46.67	31.4	63.9
10-02-03	9	31.32	4.75	2.15	24.41	6.91	61.18	73.08	16.82	56.26	77.9	77.0
11-02-03	10	31.32	12.69	6.08	12.56	18.77	65.29	73.08	14.69	58.39	40.1	79.9
13-02-03	12	31.32	12.13	8.99	10.20	21.12	-	73.08	7.81	65.27	32.6	89.3
15-02-03	14	31.32	11.12	11.11	9.09	22.23	57.00	73.08	13.16	59.92	29.0	82.0
17-02-03	16	31.32	9.27	12.54	10.51	20.81	65.63	73.08	24.55	48.53	33.6	66.4
24-02-03	21	31.32	3.70	10.70	16.91	14.41	-	73.08	8.63	64.45	54.0	88.2
26-02-03	23	31.32	2.30	7.31	21.72	9.60	46.14	73.08	11.02	62.06	69.3	84.9
28-02-03	25	31.32	1.75	5.30	24.27	7.05	-	73.08	7.82	65.26	77.5	89.3
02-03-03	27	31.32	2.88	10.88	17.56	13.77	61.70	73.08	6.45	66.63	56.0	91.2
04-03-03	29	31.32	1.88	4.68	24.76	6.56	66.73	73.08	12.02	61.06	79.1	83.5
06-03-03	31	31.32	1.01	3.82	26.49	4.83	57.00	73.08	11.67	61.41	84.6	84.0
08-03-03	33	31.32	1.39	2.54	27.39	3.93	56.17	73.08	3.63	69.45	87.4	95.0
10-03-03	35	31.32	1.53	6.90	22.89	8.43	65.35	73.08	3.51	69.57	73.1	95.2
12-03-03	37	31.32	2.05	3.58	25.69	5.63	59.65	73.08	3.72	69.36	82.0	94.9
13-03-03	38	31.32	0.00	0.00	31.32	0.00	80.20	104.40	18.57	85.83	100.0	82.2
15-03-03	40	31.32	0.00	0.00	31.32	0.00	-	104.40	34.33	70.07	100.0	67.1
19-03-03	44	31.32	0.00	0.00	31.32	0.00	-	104.40	20.68	83.72	100.0	80.2
21-03-03	46	31.32	0.00	0.00	31.32	0.00	-	104.40	53.49	50.91	100.0	48.8
24-03-03	49	31.32	0.00	0.00	31.32	0.00	-	104.40	38.36	66.04	100.0	63.3
27-03-03	52	31.32	0.00	0.00	31.32	0.00	-	104.40	32.13	72.27	100.0	69.2
30-03-03	55	31.32	0.00	0.00	31.32	0.00	-	104.40	33.45	70.95	100.0	68.0

Trial III			MC 6	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33								
Date	Exp day.	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	31.20	0.00	0.12	31.20	75.09	73.08	75.24	-2.16	0.4	0.0
03-02-03	3	31.32	29.28	0.00	2.04	29.28	64.39	73.08	55.65	17.43	6.5	23.9
04-02-03	4	31.32	29.15	0.00	2.17	29.15	69.72	73.08	47.90	25.18	6.9	34.5
05-02-03	5	31.32	25.06	1.66	4.60	26.72	64.44	73.08	38.63	34.45	14.7	47.1
06-02-03	6	31.32	21.96	2.10	7.26	24.06	62.64	73.08	32.13	40.95	23.2	56.0
10-02-03	9	31.32	14.73	3.70	12.89	18.43	61.18	73.08	43.68	29.40	41.2	40.2
11-02-03	10	31.32	18.42	5.35	7.56	23.77	65.29	73.08	34.60	38.48	24.1	52.7
13-02-03	12	31.32	9.07	10.20	12.05	19.27	-	73.08	11.50	61.58	38.5	84.3
15-02-03	14	31.32	13.47	5.20	12.64	18.68	57.00	73.08	17.69	55.39	40.4	75.8
17-02-03	16	31.32	5.33	9.25	16.73	14.59	65.63	73.08	9.33	63.75	53.4	87.2
24-02-03	21	31.32	3.86	11.45	16.01	15.31	-	73.08	14.43	58.65	51.1	80.3
26-02-03	23	31.32	2.77	8.68	19.86	11.46	46.14	73.08	15.67	57.41	63.4	78.6
28-02-03	25	31.32	3.22	8.50	19.60	11.72	-	73.08	12.22	60.86	62.6	83.3
02-03-03	27	31.32	2.00	6.75	22.56	8.76	61.70	73.08	7.16	65.92	72.0	90.2
04-03-03	29	31.32	1.91	6.24	23.17	8.15	66.73	73.08	16.63	56.45	74.0	77.2
06-03-03	31	31.32	0.77	7.13	23.42	7.90	57.00	73.08	15.85	57.23	74.8	78.3
08-03-03	33	31.32	2.16	4.84	24.32	7.00	56.17	73.08	10.11	62.97	77.7	86.2
10-03-03	35	31.32	2.82	6.02	22.48	8.84	65.35	73.08	4.88	68.20	71.8	93.3
12-03-03	37	31.32	0.00	0.00	31.32	0.00	59.65	73.08	7.70	65.38	100.0	89.5
13-03-03	38	31.32	0.00	0.00	31.32	0.00	80.20	104.40	23.05	81.35	100.0	77.9
15-03-03	40	31.32	0.00	0.00	31.32	0.00	-	104.40	35.08	69.32	100.0	66.4
19-03-03	44	31.32	0.00	0.00	31.32	0.00	83.65	104.40	40.21	64.19	100.0	61.5
21-03-03	46	31.32	0.00	0.00	31.32	0.00	-	104.40	52.78	51.62	100.0	49.4
24-03-03	49	31.32	0.00	0.00	31.32	0.00	60.66	104.40	39.38	65.02	100.0	62.3
27-03-03	52	31.32	0.00	0.00	31.32	0.00	82.87	104.40	35.31	69.09	100.0	66.2
30-03-03	55	31.32	0.00	0.00	31.32	0.00	86.34	104.40	37.65	66.75	100.0	63.9

Trial III			MC 7	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33								
Date	Exp day.	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})		Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	31.32	31.57	0.00	0.00	31.57	75.09	73.08	72.06	1.02	0.0	1.4
03-02-03	3	31.32	30.16	0.00	1.16	30.16	64.39	73.08	64.06	9.02	3.7	12.3
04-02-03	4	31.32	29.69	0.00	1.63	29.69	69.72	73.08	56.79	16.29	5.2	22.3
05-02-03	5	31.32	27.22	2.34	1.76	29.56	64.44	73.08	39.02	34.06	5.6	46.6
06-02-03	6	31.32	25.20	4.04	2.07	29.25	62.64	73.08	39.13	33.95	6.6	46.5
10-02-03	9	31.32	8.64	3.31	19.37	11.95	61.18	73.08	24.20	48.88	61.9	66.9
11-02-03	10	31.32	20.93	3.93	6.52	24.80	65.29	73.08	41.38	31.70	20.8	43.4
13-02-03	12	31.32	9.53	8.07	13.71	17.61	-	73.08	12.16	60.92	43.8	83.4
15-02-03	14	31.32	8.91	5.27	17.14	14.18	57.00	73.08	11.62	61.46	54.7	84.1
17-02-03	16	31.32	4.18	6.84	20.30	11.02	65.63	73.08	6.41	66.67	64.8	91.2
24-02-03	21	31.32	1.63	3.61	26.09	5.23	-	73.08	9.66	63.42	83.3	86.8
26-02-03	23	31.32	0.00	0.00	31.32	0.00	46.14	73.08	12.01	61.07	100.0	83.6
28-02-03	25	31.32	0.00	0.00	31.32	0.00	-	73.08	7.83	65.25	100.0	89.3
02-03-03	27	31.32	1.26	1.48	28.58	2.74	61.70	73.08	7.86	65.22	91.3	89.2
04-03-03	29	31.32	0.00	0.00	31.32	0.00	66.73	73.08	11.77	61.31	100.0	83.9
06-03-03	31	31.32	0.00	0.00	31.32	0.00	57.00	73.08	11.34	61.74	100.0	84.5
08-03-03	33	31.32	0.00	0.00	31.32	0.00	56.17	73.08	5.47	67.61	100.0	92.5
10-03-03	35	31.32	5.61	0.72	24.98	6.34	65.35	73.08	5.27	67.81	79.8	92.8
12-03-03	37	31.32	2.21	1.19	27.92	3.40	59.65	73.08	5.56	67.52	89.1	92.4
13-03-03	38	31.32	0.00	0.00	31.32	0.00	80.20	104.40	22.72	81.68	100.0	78.2
15-03-03	40	31.32	0.00	0.00	31.32	0.00	-	104.40	17.31	87.09	100.0	83.4
19-03-03	44	31.32	0.00	0.00	31.32	0.00	-	104.40	36.89	67.51	100.0	64.7
21-03-03	46	31.32	0.00	0.00	31.32	0.00	-	104.40	51.55	52.85	100.0	50.6
24-03-03	49	31.32	0.00	0.00	31.32	0.00	-	104.40	36.48	67.92	100.0	65.1
27-03-03	52	31.32	0.00	0.00	31.32	0.00	-	104.40	28.44	75.96	100.0	72.8
30-03-03	55	31.32	0.00	0.00	31.32	0.00	-	104.40	33.80	70.60	100.0	67.6

Trial III			MC 8	Sodium acetate; stoichiometric C/N = 2.33 ? 3.33								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
02-02-03	2	[illegible]	31.32	23.14	0.00	8.18	23.14	75.09	73.08	63.20	9.88	26.1	13.5
03-02-03	3	[illegible]	31.32	29.83	0.00	1.50	29.82	64.39	73.08	63.40	9.68	4.8	13.3
04-02-03	4	[illegible]	31.32	29.09	0.00	2.23	29.09	69.72	73.08	51.18	21.90	7.1	30.0
05-02-03	5	[illegible]	31.32	31.65	2.59	7.08	24.24	64.44	73.08	26.89	46.19	22.6	63.2
06-02-03	6	[illegible]	31.32	19.67	4.71	6.94	24.38	62.64	73.08	31.19	41.89	22.2	57.3
10-02-03	9	[illegible]	31.32	1.31	0.74	29.27	2.05	61.18	73.08	11.86	61.22	93.5	83.8
11-02-03	10	[illegible]	31.32	3.84	5.72	21.76	9.56	65.29	73.08	10.47	62.61	69.5	85.7
13-02-03	12	[illegible]	31.32	6.42	7.30	17.61	13.71	-	73.08	7.90	65.18	56.2	89.2
15-02-03	14	[illegible]	31.32	6.02	7.36	17.94	13.38	57.00	73.08	7.92	65.16	57.3	89.2
17-02-03	16	[illegible]	31.32	2.10	7.93	21.29	10.03	65.63	73.08	6.47	66.61	68.0	91.1
24-02-03	21	[illegible]	31.32	2.26	3.86	25.20	6.12	-	73.08	10.10	62.98	80.5	86.2
26-02-03	23	[illegible]	31.32	0.00	0.00	31.32	0.00	46.14	73.08	7.84	65.24	100.0	89.3
28-02-03	25	[illegible]	31.32	1.82	2.58	26.92	4.40	-	73.08	5.47	67.61	85.9	92.5
02-03-03	27	[illegible]	31.32	1.29	0.77	29.26	2.06	61.70	73.08	7.85	65.23	93.4	89.3
04-03-03	29	[illegible]	31.32	0.00	0.00	31.32	0.00	66.73	73.08	7.62	65.46	100.0	89.6
06-03-03	31	[illegible]	31.32	0.00	0.00	31.32	0.00	57.00	73.08	15.41	57.67	100.0	78.9
08-03-03	33	[illegible]	31.32	0.00	0.00	31.32	0.00	56.17	73.08	10.79	62.29	100.0	85.2
10-03-03	35	[illegible]	31.32	1.52	0.98	28.82	2.50	55.35	73.08	6.24	66.84	92.0	91.5
12-03-03	37	[illegible]	31.32	0.00	0.59	30.73	0.59	59.65	73.08	5.01	68.07	98.1	93.1
13-03-03	38	[illegible]	31.32	0.00	0.00	31.32	0.00	80.20	104.40	25.60	78.80	100.0	75.5
15-03-03	40	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	29.52	74.88	100.0	71.7
19-03-03	44	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	34.56	69.84	100.0	66.9
21-03-03	46	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	64.10	40.30	100.0	38.6
24-03-03	49	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	35.96	68.44	100.0	65.6
27-03-03	52	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	21.19	83.21	100.0	79.7
30-03-03	55	[illegible]	31.32	0.00	0.00	31.32	0.00	-	104.40	34.33	70.07	100.0	67.1

Trial IV			MC 1	No OC added								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from measured input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	32.12	0.00	[illegible]	32.12	[illegible]	N/A	6.77	2.07	0.0	23.4
23-02-04	4	[illegible]	31.32	32.26	0.00	[illegible]	32.26	[illegible]	N/A	9.88	[illegible]	0.0	0.0
24-02-04	5	[illegible]	31.32	33.69	0.00	[illegible]	33.69	[illegible]	N/A	7.53	0.99	0.0	11.6
25-02-04	6	[illegible]	31.32	32.41	0.00	[illegible]	32.41	[illegible]	N/A	6.28	1.79	0.0	22.3
26-02-04	7	[illegible]	31.32	30.03	0.00	1.29	30.03	[illegible]	N/A	2.32	3.01	4.1	56.4
27-02-04	8	[illegible]	31.32	29.67	0.00	1.65	29.67	[illegible]	N/A	5.19	0.29	5.3	5.4
28-02-04	9	[illegible]	31.32	30.86	0.00	0.46	30.86	[illegible]	N/A	8.50	[illegible]	1.5	0.0
01-03-04	11	[illegible]	31.32	29.98	0.00	1.34	29.98	[illegible]	N/A	5.42	0.76	4.3	10.6
03-03-04	13	[illegible]	31.32	34.74	0.00	[illegible]	34.74	[illegible]	N/A	6.17	[illegible]	0.0	0.0
11-03-04	21	[illegible]	31.32	27.55	0.00	3.77	27.55	[illegible]	N/A	5.20	[illegible]	12.0	0.0
12-03-04	22	[illegible]	31.32	31.54	0.00	[illegible]	31.54	[illegible]	N/A	5.81	[illegible]	0.0	0.0
15-03-04	25	[illegible]	31.32	33.96	0.00	[illegible]	33.96	[illegible]	N/A	6.62	[illegible]	0.0	0.0
16-03-04	26	[illegible]	31.32	33.43	0.00	[illegible]	33.43	[illegible]	N/A	9.87	0.23	0.0	2.3
18-03-04	28	[illegible]	31.32	36.70	0.00	[illegible]	36.70	[illegible]	N/A	6.26	[illegible]	0.0	0.0
20-03-04	30	[illegible]	31.32	36.78	0.00	[illegible]	36.78	[illegible]	N/A	10.38	[illegible]	0.0	0.0
22-03-04	32	[illegible]	31.32	33.07	0.00	[illegible]	33.07	[illegible]	N/A	6.65	[illegible]	0.0	0.0

Trial IV			MC 2	No OC added								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from measured input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	32.90	0.00	[illegible]	32.90	[illegible]	N/A	8.74	0.10	0.0	1.2
23-02-04	4	[illegible]	31.32	32.22	0.00	[illegible]	32.22	[illegible]	N/A	8.21	1.41	0.0	14.6
24-02-04	5	[illegible]	31.32	32.96	0.00	[illegible]	32.96	[illegible]	N/A	5.56	2.84	0.0	33.3
25-02-04	6	[illegible]	31.32	32.04	0.00	[illegible]	32.04	[illegible]	N/A	-	-	0.0	
26-02-04	7	[illegible]	31.32	29.39	0.00	1.93	29.39	[illegible]	N/A	4.86	0.44	8.2	8.2
27-02-04	8	[illegible]	31.32	28.14	0.00	3.18	28.14	[illegible]	N/A	4.76	0.72	10.2	13.2
28-02-04	9	[illegible]	31.32	30.77	0.00	0.55	30.77	[illegible]	N/A	4.48	[illegible]	1.7	0.0
01-03-04	11	[illegible]	31.32	31.05	0.00	0.27	31.05	[illegible]	N/A	4.14	3.04	0.8	42.4
03-03-04	13	[illegible]	31.32	32.39	0.00	[illegible]	32.39	[illegible]	N/A	3.61	2.25	0.0	38.4
11-03-04	21	[illegible]	31.32	26.75	0.00	4.57	26.75	[illegible]	N/A	3.61	1.11	14.6	23.5
12-03-04	22	[illegible]	31.32	28.63	0.00	2.49	28.83	[illegible]	N/A	3.85	0.91	8.0	19.1
15-03-04	25	[illegible]	31.32	32.74	0.00	[illegible]	32.74	[illegible]	N/A	7.29	[illegible]	0.0	0.0
16-03-04	26	[illegible]	31.32	33.25	0.00	[illegible]	33.25	[illegible]	N/A	7.50	0.52	0.0	6.5
18-03-04	28	[illegible]	31.32	36.56	0.00	[illegible]	36.56	[illegible]	N/A	7.06	[illegible]	0.0	0.0
20-03-04	30	[illegible]	31.32	35.70	0.00	[illegible]	35.70	[illegible]	N/A	9.82	[illegible]	0.0	0.0
22-03-04	32	[illegible]	31.32	31.53	0.00	[illegible]	31.53	[illegible]	N/A	7.88	[illegible]	0.0	0.0

Trial IV			MC 3	Glucose; stoichiometric C/N = 2.33								
Date	Exp day.	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	31.09	0.00	0.23	31.09	[illegible]	73.08	60.70	12.38	0.7	16.9
23-02-04	4	[illegible]	31.32	25.64	1.85	3.83	27.49	[illegible]	73.08	42.68	30.40	12.2	41.6
24-02-04	5	[illegible]	31.32	23.43	6.65	1.24	30.08	[illegible]	73.08	42.16	30.92	4.0	42.3
25-02-04	6	[illegible]	31.32	13.14	10.37	7.80	23.52	[illegible]	73.08	28.90	44.18	24.9	60.5
26-02-04	7	[illegible]	31.32	7.91	4.22	19.19	12.13	[illegible]	73.08	7.58	65.50	61.3	89.6
27-02-04	8	[illegible]	31.32	2.30	5.20	23.82	7.50	[illegible]	73.08	6.83	66.25	76.0	90.6
28-02-04	9	[illegible]	31.32	0.01	3.11	28.20	3.12	[illegible]	73.08	4.64	68.44	90.0	93.6
01-03-04	11	[illegible]	31.32	0.46	1.60	29.36	1.96	[illegible]	73.08	5.06	68.02	93.8	93.1
03-03-04	13	[illegible]	31.32	1.77	1.16	28.39	2.93	[illegible]	73.08	7.13	65.95	90.6	90.2
05-03-04	15	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	9.26	63.82	100.0	87.3
07-03-04	17	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	15.02	58.06	100.0	79.4
09-03-04	19	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	16.49	56.59	100.0	77.4
11-03-04	21	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	8.27	64.81	100.0	88.7
12-03-04	22	[illegible]	31.32	0.00	3.83	27.49	3.83	[illegible]	73.08	6.59	66.49	87.8	91.0
15-03-04	25	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	16.80	56.28	100.0	77.0
16-03-04	26	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	7.56	65.52	100.0	89.7
18-03-04	28	[illegible]	31.32	7.15	2.98	21.21	10.11	[illegible]	73.08	6.94	66.14	67.7	90.5
20-03-04	30	[illegible]	31.32	15.72	9.40	6.20	25.12	[illegible]	73.08	4.76	68.32	19.8	93.5
22-03-04	32	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	11.14	61.94	100.0	84.8

Trial IV		MC 4	Glucose: stoichiometric C/N = 2.33								
Date	Exp day	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	30.79	0.00	**0.53**	**30.79**	[illegible]	73.08	50.17	**22.91**	1.7	31.3
23-02-04	4	[illegible]	31.32	10.95	5.99	**14.39**	**16.93**	[illegible]	73.08	16.27	**56.81**	45.9	77.7
24-02-04	5	[illegible]	31.32	6.84	9.28	**15.19**	**16.13**	[illegible]	73.08	4.91	**68.17**	48.5	93.3
25-02-04	6	[illegible]	31.32	11.46	6.65	**13.21**	**18.11**	[illegible]	73.08	6.11	**66.97**	42.2	91.6
26-02-04	7	[illegible]	31.32	8.99	9.50	**16.73**	**14.59**	[illegible]	73.08	5.61	**67.47**	53.4	92.3
27-02-04	8	[illegible]	31.32	2.67	9.63	**19.02**	**12.30**	[illegible]	73.08	7.22	**65.86**	60.7	90.1
28-02-04	9	[illegible]	31.32	0.34	8.01	**22.97**	**8.35**	[illegible]	73.08	5.85	**67.23**	73.3	92.0
01-03-04	11	[illegible]	31.32	1.46	7.56	**22.30**	**9.02**	[illegible]	73.08	6.08	**67.03**	71.2	91.7
03-03-04	13	[illegible]	31.32	2.17	8.23	**20.92**	**10.40**	[illegible]	73.08	7.52	**65.46**	66.8	89.6
05-03-04	15	[illegible]	31.32	1.80	7.59	**21.94**	**9.38**	[illegible]	73.08	5.60	**67.48**	70.0	92.3
07-03-04	17	[illegible]	31.32	0.23	3.60	**27.49**	**3.83**	[illegible]	73.08	6.68	**66.40**	87.8	90.9
09-03-04	19	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	9.01	**64.07**	100.0	87.7
11-03-04	21	[illegible]	31.32	0.00	8.58	**22.74**	**8.58**	[illegible]	73.08	5.47	**67.61**	72.6	92.5
12-03-04	22	[illegible]	31.32	4.82	13.18	**13.32**	**18.00**	[illegible]	73.08	4.94	**68.14**	42.5	93.2
15-03-04	25	[illegible]	31.32	0.40	7.91	**23.01**	**8.31**	[illegible]	73.08	13.30	**59.78**	73.5	81.8
16-03-04	26	[illegible]	31.32	0.27	7.67	**23.38**	**7.94**	[illegible]	73.08	6.53	**66.55**	74.6	91.1
18-03-04	28	[illegible]	31.32	6.00	3.90	**21.42**	**9.90**	[illegible]	73.08	7.37	**65.71**	68.4	89.9
20-03-04	30	[illegible]	31.32	20.33	15.06	**-4.07**	**35.39**	[illegible]	73.08	5.06	**68.02**	13.0	93.1
22-03-04	32	[illegible]	31.32	14.22	5.85	**11.25**	**20.07**	[illegible]	73.08	7.34	**65.74**	35.9	90.0

Trial IV		MC 5	Glucose: stoichiometric C/N = 1.86								
Date	Exp day	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	31.04	0.00	**0.28**	**31.04**	[illegible]	58.46	46.09	**12.37**	0.9	21.2
23-02-04	4	[illegible]	31.32	10.08	10.93	**10.31**	**21.01**	[illegible]	58.46	11.61	**46.85**	32.9	80.1
24-02-04	5	[illegible]	31.32	7.85	13.13	**10.33**	**20.99**	[illegible]	58.46	5.37	**53.09**	33.0	90.8
25-02-04	6	[illegible]	31.32	6.11	15.67	**9.34**	**21.98**	[illegible]	58.46	5.19	**53.27**	29.8	91.1
26-02-04	7	[illegible]	31.32	-	-	[illegible]	[illegible]	[illegible]	58.46	4.65	**53.81**	[illegible]	92.1
27-02-04	8	[illegible]	31.32	5.14	17.55	**8.63**	**22.69**	[illegible]	58.46	5.32	**53.14**	27.5	90.9
28-02-04	9	[illegible]	31.32	8.54	12.31	**10.37**	**20.95**	[illegible]	58.46	4.30	**54.16**	33.1	92.6
01-03-04	11	[illegible]	31.32	0.01	19.14	**12.18**	**19.14**	[illegible]	58.46	4.01	**54.45**	38.9	93.1
03-03-04	13	[illegible]	31.32	6.51	15.72	**9.08**	**22.24**	[illegible]	58.46	5.96	**51.50**	29.0	89.8
05-03-04	15	[illegible]	31.32	3.32	10.37	**17.62**	**13.70**	[illegible]	58.46	4.67	**53.79**	56.3	92.0
07-03-04	17	[illegible]	31.32	5.56	14.73	**11.83**	**20.29**	[illegible]	58.46	4.81	**53.65**	35.2	91.8
09-03-04	19	[illegible]	31.32	11.23	7.36	**12.72**	**18.60**	[illegible]	58.46	5.03	**53.43**	40.6	91.4
11-03-04	21	[illegible]	31.32	4.51	13.77	**13.04**	**18.28**	[illegible]	58.46	5.55	**52.91**	41.6	90.5
12-03-04	22	[illegible]	31.32	-	-	-	-	[illegible]	58.46	4.70	**53.76**	-	92.0
15-03-04	25	[illegible]	31.32	7.22	14.02	**10.08**	**21.24**	[illegible]	58.46	7.29	**51.17**	32.2	87.5
16-03-04	26	[illegible]	31.32	0.00	3.95	**27.37**	**3.95**	[illegible]	58.46	6.93	**51.53**	87.4	88.1
18-03-04	28	[illegible]	31.32	19.85	11.68	**0.12**	**31.20**	[illegible]	58.46	5.61	**52.85**	0.4	90.4
20-03-04	30	[illegible]	31.32	21.22	10.02	**0.09**	**31.23**	[illegible]	58.46	4.31	**54.15**	0.3	92.6
22-03-04	32	[illegible]	31.32	21.05	10.12	**0.15**	**31.17**	[illegible]	58.46	4.84	**53.62**	0.5	91.7

Trial IV		MC 6	Glucose: stoichiometric C/N = 2.93								
Date	Exp day	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	31.12	0.00	**0.20**	**31.12**	[illegible]	91.87	68.00	**23.87**	0.6	26.0
23-02-04	4	[illegible]	31.32	10.80	4.98	**15.54**	**15.78**	[illegible]	91.87	32.45	**59.42**	49.6	64.7
24-02-04	5	[illegible]	31.32	3.26	4.59	**23.46**	**7.86**	[illegible]	91.87	12.71	**79.16**	74.9	86.2
25-02-04	6	[illegible]	31.32	1.26	4.45	**25.61**	**5.71**	[illegible]	91.87	4.98	**86.89**	81.8	94.6
26-02-04	7	[illegible]	31.32	-	-	-	-	[illegible]	91.87	6.28	**85.59**	-	93.2
27-02-04	8	[illegible]	31.32	0.75	2.56	**28.01**	**3.31**	[illegible]	91.87	6.04	**85.83**	89.4	93.4
28-02-04	9	[illegible]	31.32	0.00	3.66	**27.66**	**3.66**	[illegible]	91.87	10.67	**81.20**	88.3	88.4
01-03-04	11	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	91.87	7.89	**83.98**	100.0	91.4
03-03-04	13	[illegible]	31.32	4.22	4.45	**22.65**	**8.67**	[illegible]	91.87	19.54	**72.33**	72.3	78.7
05-03-04	15	[illegible]	31.32	0.00	0.81	**30.51**	**0.81**	[illegible]	91.87	27.93	**63.94**	97.4	69.6
07-03-04	17	[illegible]	31.32	6.42	3.71	**21.19**	**10.13**	[illegible]	91.87	24.56	**67.31**	67.7	73.3
09-03-04	19	[illegible]	31.32	8.24	2.06	**21.02**	**10.30**	[illegible]	91.87	35.78	**56.09**	67.1	61.1
11-03-04	21	[illegible]	31.32	5.87	4.26	**21.19**	**10.13**	[illegible]	91.87	39.12	**52.75**	67.7	57.4
12-03-04	22	[illegible]	31.32	-	-	-	-	[illegible]	91.87	39.90	**51.97**	-	56.6
15-03-04	25	[illegible]	31.32	8.45	4.26	**18.62**	**12.70**	[illegible]	91.87	42.49	**49.38**	59.4	53.7
16-03-04	26	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	91.87	17.46	**74.41**	100.0	81.0
18-03-04	28	[illegible]	31.32	-	-	-	-	[illegible]	91.87	-	-	-	-
20-03-04	30	[illegible]	31.32	12.48	3.83	**15.01**	**16.31**	[illegible]	91.87	23.82	**68.05**	47.9	74.1
22-03-04	32	[illegible]	31.32	16.81	4.16	**10.35**	**20.97**	[illegible]	91.87	35.98	**55.89**	33.1	60.8

Trial IV		MC 7	Acetic acid: stoichiometric C/N = 2.33								
Date	Exp day	[illegible]	Nominal influent NO_3^-N (mg d^{-1})	Effluent NO_3^-N (mg d^{-1})	Effluent NO_2^-N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-04	1	[illegible]	31.32	31.34	0.00	**-0.02**	**31.34**	[illegible]	73.08	57.45	**15.63**	0.0	21.4
23-02-04	4	[illegible]	31.32	11.22	6.33	**13.77**	**17.55**	[illegible]	73.08	37.02	**36.06**	44.0	49.3
24-02-04	5	[illegible]	31.32	3.21	10.11	**18.00**	**13.32**	[illegible]	73.08	21.96	**51.12**	57.5	69.9
25-02-04	6	[illegible]	31.32	0.00	1.12	**30.20**	**1.12**	[illegible]	73.08	7.80	**65.28**	96.4	89.3
26-02-04	7	[illegible]	31.32	0.00	0.82	**30.50**	**0.82**	[illegible]	73.08	6.51	**66.57**	97.4	91.1
27-02-04	8	[illegible]	31.32	0.00	0.80	**30.52**	**0.80**	[illegible]	73.08	9.57	**63.51**	97.4	86.9
28-02-04	9	[illegible]	31.32	0.01	0.87	**30.45**	**0.87**	[illegible]	73.08	8.97	**64.11**	97.2	87.7
01-03-04	11	[illegible]	31.32	2.95	1.41	**26.96**	**4.36**	[illegible]	73.08	7.80	**65.28**	86.1	89.3
03-03-04	13	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	9.01	**64.07**	100.0	87.7
05-03-04	15	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	19.24	**53.84**	100.0	73.7
07-03-04	17	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	24.02	**49.06**	100.0	67.1
09-03-04	19	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	31.08	**42.00**	100.0	57.5
11-03-04	21	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	15.53	**57.55**	100.0	78.7
12-03-04	22	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	20.54	**52.54**	100.0	71.9
15-03-04	25	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	31.64	**41.44**	100.0	56.7
16-03-04	26	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	12.21	**60.87**	100.0	83.3
18-03-04	28	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	73.08	11.35	**61.73**	100.0	84.5
20-03-04	30	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	60.55	5.00	**55.55**	100.0	91.7
22-03-04	32	[illegible]	31.32	0.00	0.00	**31.32**	**0.00**	[illegible]	60.55	10.23	**50.32**	100.0	83.1

Trial IV			MC 8	Acetic acid; stoichiometric C/N = 2.33 ? 1.93 (D30, D32)							
Date	Exp day.	[illegible]	Nominal influent NO_3^- N (mg d^{-1})	Effluent NO_3^- N (mg d^{-1})	Effluent NO_2^- N (mg d^{-1})	Mass TON denitrified (from nominal input) (mg d^{-1})	Mass TON in effluent (mg d^{-1})	[illegible]	Nominal influent DOC (mg d^{-1})	Effluent DOC (mg d^{-1})	Mass DOC utilised (from nominal input) (mg d^{-1})	% Denitrification Efficiency	% DOC Utilisation
20-02-02	1	[illegible]	31.32	31.54	0.00	[illegible]	31.54	[illegible]	73.08	58.20	14.88	0.0	20.4
23-02-02	4	[illegible]	31.32	10.02	6.62	14.68	16.64	[illegible]	73.08	36.57	36.51	46.9	50.0
24-02-02	5	[illegible]	31.32	1.15	6.84	23.33	7.99	[illegible]	73.08	19.66	53.42	74.5	73.1
25-02-02	6	[illegible]	31.32	0.00	0.83	30.49	0.83	[illegible]	73.08	11.97	61.11	97.3	83.6
26-02-02	7	[illegible]	31.32	0.01	0.78	30.53	0.79	[illegible]	73.08	10.60	62.48	97.5	85.5
27-02-02	8	[illegible]	31.32	0.00	0.79	30.53	0.79	[illegible]	73.08	11.77	61.31	97.5	83.9
28-02-02	9	[illegible]	31.32	0.00	0.84	30.48	0.84	[illegible]	73.08	11.17	61.91	97.3	84.7
01-03-04	11	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	4.38	68.70	100.0	94.0
03-03-04	13	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	19.72	53.36	100.0	73.0
05-03-04	15	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	20.22	52.86	100.0	72.3
07-03-04	17	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	24.32	48.76	100.0	66.7
09-03-04	19	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	30.91	42.17	100.0	57.7
11-03-04	21	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	22.19	50.89	100.0	69.6
12-03-04	22	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	20.57	52.51	100.0	71.9
15-03-04	25	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	32.93	40.15	100.0	54.9
16-03-04	26	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	19.00	54.08	100.0	74.0
18-03-04	28	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	73.08	9.92	63.16	100.0	86.4
20-03-04	30	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	60.55	4.91	55.64	100.0	91.9
22-03-04	32	[illegible]	31.32	0.00	0.00	31.32	0.00	[illegible]	60.55	8.02	52.53	100.0	86.7

2.3 DOC MASS BALANCES (For Pie Charts)

Trial I		MC 3								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	7.93	0.00	11.68	1.25	46.77	0.19	-	-
05-02-02	3	3.75	4.98	0.00	8.73	14.20	33.83	2.13	5.47	3.34
06-02-02	4	3.75	0.00	0.00	3.75	11.48	36.54	1.72	7.73	6.01
08-02-02	6	3.75	0.00	0.00	3.75	27.14	20.88	4.07	23.39	19.32
10-02-02	8	3.75	0.00	5.34	9.09	38.21	9.81	5.73	29.12	23.39
22-02-02	21	3.75	10.21	7.34	21.30	40.30	7.73	6.04	19.00	12.96
cumulative total (d 4 ? d 21)		15.00	10.21	12.68	37.89	117.14	74.96	17.57	79.25	61.68

Trial I		MC 4								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	9.07	0.00	12.82	2.71	45.31	0.41	-	-
05-02-02	3	3.75	4.10	0.00	7.85	14.20	33.83	2.13	6.35	4.22
06-02-02	4	3.75	0.00	0.00	3.75	9.40	38.63	1.41	5.65	4.24
08-02-02	6	3.75	0.00	3.04	6.79	41.13	6.89	6.17	34.34	28.17
10-02-02	8	3.75	0.00	7.52	11.27	39.05	8.98	5.86	27.78	21.92
22-02-02	21	3.75	8.33	6.68	18.76	42.39	5.64	6.36	23.62	17.27
cumulative total (d 4 ? d 21)		15.00	8.33	17.24	40.57	131.96	60.13	19.79	91.39	71.59

Trial I		MC 5								
Date	Exp day	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	10.81	0.00	14.56	0.00	29.44	0.00	-	-
05-02-02	3	3.75	5.73	0.00	9.48	8.14	14.82	1.22	-	-
06-02-02	4	3.75	0.32	0.00	4.07	16.29	6.68	2.44	12.22	9.78
08-02-02	6	3.75	0.00	0.00	3.75	17.33	5.64	2.60	13.58	10.98
10-02-02	8	3.75	0.00	0.00	3.75	12.95	10.02	1.94	9.20	7.25
22-02-02	18	3.75	0.85	4.13	8.73	17.75	5.22	2.66	9.02	6.36
cumulative total (d 4 ? d 21)		15.00	1.17	4.13	20.30	64.31	27.56	9.65	44.02	34.37

Trial I		MC 6								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	10.00	0.00	13.75	0.00	26.31	0.00	-	-
05-02-02	3	3.75	4.85	0.00	8.60	7.31	15.66	1.10	-	-
06-02-02	4	3.75	0.46	0.00	4.21	13.57	9.40	2.04	9.37	7.33
08-02-02	6	3.75	0.00	0.00	3.75	16.08	6.89	2.41	12.33	9.92
10-02-02	8	3.75	0.00	0.00	3.75	8.77	14.20	1.32	5.02	3.70
22-02-02	21	3.75	0.00	3.50	7.25	15.24	7.73	2.29	7.99	5.70
cumulative total (d 4 ? d 21)		15.00	0.46	3.50	18.96	53.66	38.21	6.05	34.70	26.65

Trial I		MC 7								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	8.05	0.00	11.80	5.43	76.00	0.81	-	-
05-02-02	3	3.75	5.71	0.00	9.46	8.35	73.08	1.25	-	-
06-02-02	4	3.75	0.87	0.00	4.62	26.94	54.50	4.04	22.32	18.28
08-02-02	6	3.75	0.15	3.51	7.41	46.56	34.87	6.98	39.16	32.17
10-02-02	8	3.75	0.00	6.82	10.57	67.23	14.20	10.09	56.66	46.57
22-02-02	21	3.75	22.99	4.00	30.73	84.15	9.81	12.62	53.41	40.79
cumulative total	(d 4 ? d 21)	15.00	24.01	14.33	53.33	224.86	113.38	33.73	171.55	137.81

Trial I		MC 8								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
04-02-02	2	3.75	10.70	0.00	14.45	9.08	72.35	1.36	-	-
05-02-02	3	3.75	5.71	0.00	9.46	15.45	65.98	2.32	6.00	3.68
06-02-02	4	3.75	0.00	0.90	4.65	45.31	36.12	6.80	40.66	33.87
08-02-02	6	3.75	0.06	2.19	5.99	58.71	22.72	8.81	52.72	43.91
10-02-02	8	3.75	1.55	8.38	13.68	67.85	13.57	10.18	54.18	44.00
22-02-02	21	3.75	26.72	1.55	32.02	88.11	5.85	13.22	56.09	42.87
cumulative total	(d 4 ? d 21)	15.00	28.33	13.02	56.35	260.00	78.26	39.00	203.65	164.65

Trial II		MC 3								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	8.69	0.00	12.44	3.19	42.74	0.48	-	-
30-05-02	4	3.75	4.51	0.00	8.26	16.54	29.40	2.48	8.27	5.79
31-05-02	5	3.75	0.00	0.22	3.97	36.25	9.68	5.44	32.28	26.84
01-06-02	6	3.75	0.55	0.22	4.52	13.91	32.03	2.09	9.39	7.30
02-06-02	7	3.75	0.00	1.76	5.51	25.88	20.06	3.88	20.37	16.49
03-06-02	8	3.75	14.70	2.32	20.77	33.36	12.58	5.00	12.59	7.59
05-06-02	10	3.75	1.18	2.33	7.26	24.60	21.34	3.69	17.34	13.65
08-06-02	13	3.75	9.16	8.39	21.30	35.18	10.75	5.28	13.89	8.61
11-06-02	16	3.75	12.04	5.03	20.82	26.10	19.84	3.92	5.28	1.36
14-06-02	19	3.75	4.85	10.32	18.91	34.33	11.61	5.15	15.41	10.26
16-06-02	21	3.75	12.06	2.22	18.03	28.21	17.73	4.23	10.18	5.95
18-06-02	23	3.75	21.38	2.51	27.64	35.12	10.82	5.27	7.48	2.21
21-06-02	26	3.75	10.72	2.50	16.98	42.45	3.48	6.37	25.47	19.10
23-06-02	28	3.75	2.93	2.65	9.33	41.62	4.32	6.24	32.29	26.05
26-06-02	31	3.75	2.86	9.27	15.88	-	-	-	-	-
28-06-02	33	3.75	2.28	9.94	15.97	41.73	4.21	6.26	25.76	19.50
16-07-02	36	3.75	1.32	4.79	9.85	27.06	18.88	4.06	17.21	13.15
20-07-02	40	3.76	5.76	7.11	16.63	39.18	6.76	5.88	22.55	16.68
22-07-02	42	3.75	12.47	2.98	19.20	32.87	13.07	4.93	13.67	8.74
25-07-02	45	3.75	9.05	8.41	21.21	40.38	5.56	6.06	19.16	13.11
27-07-02	47	3.75	0.48	3.24	7.48	38.52	7.42	5.78	31.04	25.26
29-07-02	49	3.75	1.83	12.40	17.98	42.44	3.49	6.37	24.46	18.10
31-07-02	51	3.75	0.00	7.83	11.58	41.77	4.17	6.26	30.19	23.92
03-08-02	54	3.75	8.74	9.41	21.89	38.58	7.36	5.79	16.68	10.90
08-08-02	56	3.75	1.83	3.29	8.87	10.00	35.93	1.13	1.13	0.00
10-08-02	58	3.75	6.81	1.88	12.44	42.42	3.51	6.36	29.98	23.62
12-08-02	60	3.75	1.09	3.56	8.40	34.80	11.13	5.22	26.40	21.18
25-08-02	64	3.75	3.41	5.45	12.61	34.22	22.15	5.13	21.61	16.48
27-08-02	66	3.75	1.90	4.99	10.64	22.84	33.53	3.43	12.20	8.77
31-08-02	70	3.75	10.35	7.22	21.31	42.44	13.93	6.37	21.13	14.76
02-09-02	72	3.75	11.70	7.16	22.61	59.07	9.84	8.86	36.46	27.60
04-09-02	74	3.75	30.45	1.08	35.28	62.58	6.32	9.39	27.30	17.92
10-09-02	76	3.75	11.26	8.87	23.86	42.99	25.91	6.45	19.13	12.68
12-09-02	78	3.75	10.76	6.95	21.46	51.57	17.34	7.73	30.10	22.37
13-09-02	79	3.75	19.05	5.76	28.56	63.56	15.78	9.53	35.00	25.47
14-09-02	80	3.75	25.46	3.23	32.44	71.26	8.08	10.69	38.82	28.13
16-09-02	81	3.75	25.80	3.06	32.62	72.14	7.20	10.82	39.52	28.70
20-09-02	84	3.75	27.17	2.08	33.00	73.04	6.31	10.96	40.04	29.08
cumulative total	(d 8 ? d 60)	82.50	143.56	122.36	348.42	730.70	233.95	109.24	398.17	288.94
	(d 799 d 84)	15.00	97.48	14.13	126.61	280.00	37.38	42.00	153.39	111.39
	(d 8 ? d 84)	123.75	320.86	178.21	622.82	1326.42	400.36	198.59	719.48	520.89

Trial II		MC 4								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	1.25	0.00	5.00	0.00	50.61	0.00	-	-
30-05-02	4	3.75	4.36	0.00	8.11	3.31	42.63	0.50	-	-
31-05-02	5	3.75	0.00	1.15	4.90	36.05	9.88	5.41	31.15	25.75
01-06-02	6	3.75	6.21	0.00	9.96	35.15	10.78	5.27	25.19	19.92
02-06-02	7	3.75	18.70	2.01	24.46	39.68	6.25	5.95	15.22	9.27
03-06-02	8	3.75	0.00	2.15	5.90	38.94	6.99	5.84	33.05	27.20
05-06-02	10	3.75	11.82	1.05	16.62	42.19	3.75	6.33	25.57	19.24
08-06-02	13	3.75	8.33	3.68	15.76	36.87	9.06	5.53	21.11	15.58
11-06-02	16	3.75	7.24	4.16	15.16	40.38	5.55	6.06	25.24	19.18
14-06-02	19	3.75	12.35	4.05	20.15	36.89	9.05	5.53	16.74	11.20
16-06-02	21	3.75	19.91	2.04	25.69	34.99	10.94	5.25	9.30	4.05
18-06-02	23	3.75	12.78	2.14	18.67	31.17	14.76	4.68	12.51	7.83
21-06-02	26	3.75	7.10	2.79	13.65	43.37	2.57	6.50	29.72	23.21
23-06-02	28	3.75	0.00	2.14	5.89	42.78	3.16	6.42	36.90	30.48
26-06-02	31	3.75	10.30	4.62	18.67	43.08	2.86	6.46	24.40	17.94
28-06-02	33	3.75	10.77	6.72	21.24	42.19	3.74	6.33	20.95	14.62
16-07-02	36	3.75	10.77	2.17	16.69	18.85	27.08	2.16	0.00	0.00
20-07-02	40	3.75	14.46	4.19	22.40	37.10	8.84	5.56	14.70	9.13
22-07-02	42	3.75	11.19	2.24	17.19	28.94	16.99	4.34	11.76	7.41
25-07-02	45	3.75	4.78	7.07	15.61	31.69	14.24	4.75	16.09	11.33
27-07-02	47	3.75	1.94	10.84	16.53	36.68	9.26	5.50	20.15	14.65
29-07-02	49	3.75	0.00	13.47	17.22	39.26	6.67	5.89	22.05	16.16
31-07-02	51	3.75	0.00	11.21	14.96	40.93	5.01	6.14	25.96	19.82
03-08-02	54	3.75	6.60	7.79	18.14	39.88	6.06	5.98	21.74	15.76
08-08-02	56	3.75	9.90	0.66	14.30	9.44	36.50	1.42	0.00	0.00
10-08-02	58	3.75	8.00	3.47	15.22	41.12	4.82	6.17	25.89	19.73
12-08-02	60	3.75	1.32	2.92	7.99	32.75	13.19	4.91	24.76	19.85
25-08-02	64	3.75	3.30	5.11	12.16	31.34	25.04	4.70	19.18	14.48
27-08-02	66	3.75	2.15	0.53	6.44	29.80	26.58	4.47	23.36	18.89
31-08-02	70	3.75	14.99	6.71	25.44	51.81	4.56	7.77	26.37	18.60
02-09-02	72	3.75	20.17	4.44	28.36	66.78	2.12	10.02	38.43	28.41
04-09-02	74	3.75	31.73	0.72	36.19	63.85	5.05	9.58	27.66	18.08
10-09-02	76	3.75	17.63	6.22	27.60	46.92	21.99	7.04	19.32	12.28
12-09-02	78	3.75	21.70	0.91	26.35	64.70	4.21	9.70	38.35	28.64
13-09-02	79	3.75	18.97	5.76	28.48	63.27	16.07	9.49	34.79	25.30
14-09-02	80	3.75	23.08	4.19	31.02	67.07	12.28	10.06	36.04	25.98
16-09-02	81	3.75	24.51	3.23	31.49	70.03	9.32	10.50	38.53	28.03
20-09-02	84	3.75	26.34	2.68	32.97	71.75	7.59	10.76	38.78	28.02
cumulative total	(d 8 ? d 60)	82.50	169.56	101.58	353.63	789.51	221.09	117.76	440.74	324.40
	(d 797 d 84)	15.00	92.90	16.07	123.97	272.12	45.26	40.82	148.15	107.33
	(d 8 ? d 84)	123.75	374.11	142.28	640.14	1416.82	355.89	211.86	781.55	571.11

Trial II		MC 5								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	2.54	0.00	6.29	4.40	35.27	0.66	-	-
30-05-02	4	3.75	2.79	1.26	7.80	10.19	29.48	1.53	2.39	0.86
31-05-02	5	3.75	2.48	0.49	6.72	16.08	23.59	2.41	9.36	6.94
01-06-02	6	3.75	3.03	2.12	8.90	23.73	15.94	3.56	14.83	11.27
03-06-02	8	3.75	3.41	0.52	7.68	34.79	4.88	5.22	27.11	21.89
05-06-02	10	3.75	0.54	0.00	4.29	31.60	8.07	4.74	27.31	22.57
08-06-02	13	3.75	14.95	1.01	19.72	32.13	7.54	4.82	12.42	7.60
11-06-02	16	3.75	11.11	1.55	16.42	37.18	2.49	5.58	20.77	15.19
14-06-02	19	3.75	8.69	2.07	14.50	33.74	5.93	5.06	19.24	14.18
16-06-02	21	3.75	12.53	1.98	18.25	28.29	11.38	4.24	10.04	5.79
18-06-02	23	3.75	11.05	2.19	16.99	30.59	9.08	4.59	13.60	9.01
21-06-02	26	3.75	12.86	2.24	18.86	35.69	3.98	5.35	16.83	11.48
23-06-02	28	3.75	7.00	7.78	18.53	28.85	10.82	4.33	10.32	6.00
26-06-02	31	3.75	3.32	6.30	13.38	29.29	10.38	4.39	15.92	11.52
28-06-02	33	3.75	12.57	4.79	21.11	27.04	12.63	4.06	5.93	1.87
16-07-02	36	3.75	8.98	6.96	19.69	32.04	7.63	4.81	12.35	7.55
20-07-02	40	3.75	10.10	4.06	17.91	35.45	4.22	5.32	17.54	12.22
22-07-02	42	3.75	1.12	9.12	13.99	34.41	5.26	5.16	20.41	15.25
25-07-02	45	3.75	0.00	10.37	14.12	36.76	2.91	5.51	22.64	17.13
27-07-02	47	3.75	4.80	9.80	18.35	37.34	2.33	5.60	18.99	13.39
29-07-02	49	3.75	5.12	10.60	19.47	36.59	3.08	5.49	17.12	11.63
31-07-02	51	3.75	2.11	10.07	15.93	36.46	3.21	5.47	20.53	15.06
03-08-02	54	3.75	15.96	1.64	21.36	35.72	3.95	5.36	14.36	9.01
08-08-02	56	3.75	14.39	1.37	19.51	16.39	23.28	0.00	0.00	0.00
10-08-02	58	3.75	12.50	2.57	18.82	34.09	5.58	5.11	15.27	10.16
12-08-02	60	3.75	16.67	6.19	26.62	34.27	5.40	5.14	7.66	2.52
25-08-02	64	3.75	17.08	6.01	26.84	45.16	4.95	6.77	18.32	11.55
27-08-02	66	3.75	16.80	5.76	26.31	42.65	7.46	6.40	16.34	9.94
31-08-02	70	3.75	16.89	5.95	26.59	45.79	4.32	6.87	19.21	12.34
02-09-02	72	3.75	18.97	5.25	27.97	54.87	5.68	8.23	26.90	18.67
04-09-02	74	3.75	24.31	3.74	31.81	56.05	4.50	8.41	24.24	15.83
10-09-02	76	3.75	17.71	6.22	27.69	39.90	20.65	5.98	12.21	6.23
12-09-02	78	3.75	24.93	0.00	28.68	54.51	6.04	8.18	25.83	17.66
13-09-02	79	3.75	28.44	1.99	34.18	58.53	12.46	8.78	24.36	15.58
14-09-02	80	3.75	31.45	0.39	35.58	57.45	13.54	8.62	21.87	13.25
16-09-02	81	3.75	31.95	0.69	36.39	58.38	12.61	8.76	21.99	13.23
20-09-02	84	3.75	33.00	0.27	37.02	58.55	12.44	8.78	21.52	12.74
cumulative total	(d 8 ? d 60)	82.50	189.81	103.19	375.49	718.72	154.02	125.35	346.36	241.01
	(d 797 d 84)	15.00	124.84	3.34	143.17	232.91	51.05	34.94	69.74	54.80
	(d 8 ? d 84)	123.75	451.34	139.45	714.54	1290.57	258.66	191.13	579.15	388.03

Trial II				MC 6						
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	0.89	0.00	4.64	4.07	35.60	0.61	-	-
30-05-02	4	3.75	5.61	0.00	9.36	7.02	32.65	1.05	-	-
31-05-02	5	3.75	1.65	0.43	5.84	15.57	24.10	2.34	9.74	7.40
01-06-02	6	3.75	2.20	2.12	8.07	22.34	17.33	3.35	14.27	10.92
02-06-02	7	3.75	0.09	1.46	5.29	21.82	17.65	3.27	16.53	13.26
03-06-02	8	3.75	3.48	6.27	13.50	31.62	8.05	4.74	18.13	13.38
05-06-02	10	3.75	0.43	0.75	4.92	35.73	3.94	5.36	30.81	25.45
08-06-02	13	3.75	0.29	0.00	4.04	37.46	2.21	5.62	33.41	27.80
11-06-02	16	3.75	0.00	0.70	4.45	36.85	2.82	5.53	32.40	26.87
14-06-02	19	3.75	5.55	6.90	16.19	32.76	6.91	4.91	16.57	11.65
16-06-02	21	3.75	17.13	3.73	24.61	26.12	13.55	1.51	1.51	0.00
18-06-02	23	3.75	5.61	2.49	11.85	29.19	10.48	4.38	17.34	12.96
21-06-02	26	3.75	8.16	2.65	14.45	30.17	9.50	4.53	15.72	11.19
23-06-02	28	3.75	6.14	2.69	11.56	33.16	6.51	4.97	21.57	16.60
25-06-02	31	3.75	3.48	9.46	16.69	30.77	8.90	4.62	14.08	9.46
28-06-02	33	3.75	1.17	8.40	13.32	28.67	11.00	4.30	15.34	11.04
16-07-02	36	3.75	0.00	10.34	14.09	29.70	9.97	4.46	15.61	11.15
20-07-02	40	3.75	7.36	8.85	19.97	34.17	5.50	5.13	14.20	9.08
22-07-02	42	3.75	9.00	3.30	16.06	35.18	4.54	5.27	19.07	13.80
25-07-02	45	3.75	3.59	10.85	17.69	34.50	5.17	5.18	16.81	11.63
27-07-02	47	3.75	0.00	11.06	14.81	35.64	4.03	5.35	20.82	15.48
29-07-02	49	3.75	1.14	11.77	16.67	35.23	4.44	5.28	18.57	13.28
31-07-02	51	3.75	4.09	11.03	18.86	35.67	4.00	5.35	16.81	11.46
03-08-02	54	3.75	3.08	10.90	17.73	34.77	4.90	5.22	17.05	11.83
08-08-02	56	3.75	11.63	2.20	17.58	28.48	11.19	4.27	10.91	6.63
10-08-02	58	3.75	4.60	4.29	12.64	30.31	9.36	4.55	17.66	13.12
12-08-02	60	3.75	7.05	4.04	14.84	33.34	6.33	5.00	18.50	13.50
25-08-02	64	3.75	11.20	7.68	22.63	45.39	4.72	6.81	22.76	15.95
27-08-02	66	3.75	11.15	7.77	22.67	44.26	5.85	6.64	21.59	14.95
31-08-02	70	3.75	16.03	6.79	26.57	45.49	4.62	6.82	18.91	12.09
02-09-02	72	3.75	21.30	4.95	30.00	54.62	6.03	8.18	24.62	16.44
04-09-02	74	3.75	23.29	2.33	29.38	53.97	6.58	8.10	24.60	16.50
10-09-02	76	3.75	22.05	4.36	30.16	40.44	20.11	6.07	10.28	4.22
12-09-02	78	3.75	22.51	0.00	26.26	55.33	5.22	8.30	29.06	20.77
13-09-02	79	3.75	27.50	2.43	33.68	64.04	6.95	9.61	30.36	20.75
14-09-02	80	3.75	31.36	0.81	35.92	60.80	10.19	9.12	24.88	15.76
16-09-02	81	3.75	30.22	1.33	35.30	60.47	10.52	9.07	25.17	16.10
20-09-02	84	3.75	31.72	0.78	36.25	60.32	10.67	9.05	24.07	15.02
cumulative total	(d 8 ? d 60)	82.50	101.97	132.07	316.55	719.43	163.31	105.51	402.89	297.38
	(d 79? d 84)	16.00	120.80	5.35	141.16	245.63	38.33	36.85	104.48	67.64
	(d 8 ? d 84)	123.75	350.32	171.30	645.37	1304.46	244.77	193.26	659.09	465.83

Trial II				MC 7						
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	0.48	0.00	4.23	0.66	22.30	0.10	-	-
30-05-02	4	3.75	5.14	0.00	8.89	15.47	7.49	2.32	6.58	4.26
31-05-02	5	3.75	0.58	0.59	4.92	17.24	5.72	2.59	12.32	9.73
01-06-02	6	3.75	0.00	1.24	4.99	18.07	4.89	2.71	13.08	10.37
02-06-02	7	3.75	0.09	0.80	4.64	18.87	4.09	2.83	14.23	11.40
03-06-02	8	3.75	0.00	2.04	5.79	18.27	4.69	2.74	12.49	9.75
05-06-02	10	3.75	0.00	0.00	3.75	19.61	3.35	2.94	15.86	12.92
08-06-02	13	3.75	1.68	0.00	5.43	18.09	4.87	2.71	12.66	9.95
11-06-02	16	3.75	0.00	1.56	5.31	19.22	3.74	2.88	13.91	11.03
14-06-02	19	3.75	1.50	0.00	5.25	14.21	8.75	2.13	8.96	6.83
16-06-02	21	3.75	0.00	1.78	5.53	11.69	11.36	1.74	6.07	4.33
18-06-02	23	3.75	14.37	1.89	20.00	14.65	8.31	0.00	0.00	0.00
21-06-02	26	3.75	2.11	1.90	7.76	8.51	14.45	0.75	0.75	0.00
23-06-02	28	3.75	1.71	1.86	7.31	12.25	10.71	1.84	4.93	3.10
26-06-02	31	3.75	1.08	0.96	5.79	13.88	9.08	2.08	8.09	6.01
28-06-02	33	3.75	0.58	0.31	4.65	16.21	6.75	2.43	11.56	9.13
16-07-02	36	3.75	0.00	1.80	5.55	15.57	7.39	2.34	10.02	7.69
20-07-02	40	3.75	3.91	0.73	8.38	19.25	3.71	2.89	10.87	7.98
22-07-02	42	3.75	3.12	0.43	7.30	19.55	3.41	2.93	12.25	9.31
25-07-02	45	3.75	0.90	2.50	7.15	18.63	4.33	2.79	11.47	8.68
27-07-02	47	3.75	0.00	3.11	6.86	19.65	3.31	2.95	12.79	9.84
29-07-02	49	3.75	0.00	1.47	5.22	19.99	2.97	3.00	14.77	11.77
31-07-02	51	3.75	0.00	0.00	3.75	20.46	2.60	3.07	16.71	13.64
cumulative total	(d 8 ? d 51)	67.50	32.96	22.34	120.80	299.61	113.67	42.22	184.16	141.94

Trial II				MC 8						
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
29-05-02	3	3.75	1.27	0.00	5.02	2.92	20.04	0.44	-	-
30-05-02	4	3.75	6.20	0.00	9.95	14.11	8.86	2.12	4.16	2.04
31-05-02	5	3.75	0.49	1.11	5.35	14.19	8.77	2.13	8.84	6.71
01-06-02	6	3.75	0.00	0.28	4.03	18.34	4.63	2.75	14.31	11.56
02-06-02	7	3.75	0.47	1.64	5.86	19.33	3.64	2.90	13.46	10.56
03-06-02	8	3.75	0.63	0.91	5.29	18.04	4.93	2.71	12.75	10.05
05-06-02	10	3.75	0.00	0.00	3.75	19.23	3.74	2.88	15.48	12.60
08-06-02	13	3.75	0.00	0.00	3.75	15.87	7.10	2.38	12.12	9.74
11-06-02	16	3.75	0.04	0.22	4.02	17.98	4.99	2.70	13.96	11.26
14-06-02	19	3.75	3.30	0.22	7.27	13.47	9.60	2.02	6.19	4.17
16-06-02	21	3.75	0.00	1.20	4.95	10.77	12.19	1.62	5.83	4.21
18-06-02	23	3.75	0.79	1.91	6.45	13.28	9.69	1.99	6.83	4.84
21-06-02	26	3.75	1.75	1.95	7.45	10.00	12.97	1.50	2.55	1.05
23-06-02	28	3.75	0.38	1.92	6.05	11.38	11.59	1.71	5.33	3.62
26-06-02	31	3.75	0.70	0.56	5.00	13.59	9.38	2.04	8.59	6.55
28-06-02	33	3.75	2.04	0.31	6.11	16.65	6.31	2.50	10.56	8.06
14-07-02	36	3.75	4.96	1.37	10.09	17.34	5.63	2.60	7.26	4.45
20-07-02	40	3.75	2.02	0.64	6.41	18.69	4.28	2.80	12.28	9.48
22-07-02	42	3.75	2.38	0.00	6.13	19.15	3.82	2.87	13.02	10.15
25-07-02	45	3.75	6.02	1.93	11.70	18.57	4.40	2.79	6.87	4.09
27-07-02	47	3.75	4.00	2.86	10.60	18.70	4.27	2.80	8.10	5.29
29-07-02	49	3.75	0.38	0.91	5.04	17.89	5.08	2.68	12.85	10.17
31-07-02	51	3.75	0.27	0.22	4.25	19.39	3.58	2.91	15.14	12.23
cumulative total	(d 8 ? d 51)	67.50	29.66	17.13	114.29	290.00	125.43	43.50	175.70	132.21

Trial III		MC 3								
		Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N:	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_3^- reduction (after DO removal), based on theoretical C/N:	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
Date	Exp day.		1.07	0.43						
02-02-03	2	3.75	8.05	0.00	11.80	0.00	75.42	0.00	-	-
03-02-03	3	3.75	1.24	0.00	4.99	15.01	68.07	2.25	10.02	7.77
04-02-03	4	3.75	3.06	0.00	6.81	20.75	62.33	3.11	13.95	10.83
06-02-03	5	3.75	8.25	0.00	12.00	32.59	40.49	4.89	20.59	15.70
06-02-03	6	3.75	17.07	0.00	20.82	44.02	29.06	6.60	23.19	16.59
10-02-03	9	3.75	12.37	1.79	17.91	40.59	32.49	6.09	22.68	16.59
11-02-03	10	3.75	14.71	1.57	20.02	49.21	23.87	7.38	29.19	21.81
13-02-03	12	3.75	14.81	3.01	21.57	55.63	17.45	8.34	34.07	26.72
15-02-03	14	3.75	10.33	1.83	15.91	45.73	27.35	6.86	29.82	22.96
17-02-03	16	3.75	14.89	2.52	21.17	48.09	24.99	7.21	26.92	19.71
24-02-03	21	3.75	33.51	0.00	37.26	58.44	14.64	8.77	21.18	12.41
26-02-03	23	3.75	33.51	0.00	37.26	52.47	20.61	7.87	15.21	7.34
28-02-03	25	3.75	28.32	0.00	32.07	63.80	9.28	9.57	31.73	22.16
02-03-03	27	3.75	26.46	1.75	31.97	67.90	5.18	10.18	35.93	25.75
04-03-03	29	3.75	33.51	0.00	37.26	56.85	16.23	8.53	19.59	11.06
06-03-03	31	3.75	30.31	0.00	34.06	56.01	17.07	8.40	21.95	13.55
08-03-03	33	3.75	31.27	0.00	35.02	67.51	5.57	10.13	32.49	22.36
10-03-03	35	3.75	28.46	0.51	32.70	68.57	4.51	10.29	35.87	25.58
12-03-03	37	3.75	30.18	0.00	33.93	60.26	12.82	9.04	26.33	17.29
13-03-03	38	3.75	24.78	0.00	28.53	66.38	38.02	9.96	37.85	27.89
15-03-03	40	3.75	30.55	0.00	34.30	58.13	46.27	8.72	23.83	15.11
19-03-03	44	3.75	33.51	0.00	37.26	62.79	41.61	9.42	25.52	16.11
21-03-03	46	3.75	33.51	0.00	37.26	54.39	50.01	8.16	17.13	8.97
24-03-03	49	3.75	33.51	0.00	37.26	62.47	41.93	9.37	25.21	15.84
27-03-03	52	3.75	33.51	0.00	37.26	41.78	42.62	9.27	24.52	15.25
30-03-03	55	3.75	33.51	0.00	37.26	67.73	36.67	10.16	30.47	20.31
cumulative total	(d 99 d 37)	52.50	342.65	12.98	408.13	791.08	232.04	118.66	382.95	264.29
	(d 387 d 55)	26.25	222.89	0.00	249.14	433.68	297.12	65.06	184.54	119.48
	(d 97 d 55)	78.75	565.54	12.98	657.27	1224.76	529.16	183.71	567.49	383.77

Trial III		MC 4								
		Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N:	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_3^- reduction (after DO removal), based on theoretical C/N:	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
Date	Exp day.		1.07	0.43						
02-02-03	2	3.75	0.61	0.00	4.36	0.00	75.06	0.00	-	-
03-02-03	3	3.75	0.00	0.00	3.75	9.92	63.16	1.49	6.17	4.68
04-02-03	4	3.75	2.78	0.00	6.53	11.25	61.83	1.69	4.72	3.03
05-02-03	5	3.75	2.28	0.00	6.03	20.59	52.49	3.09	14.56	11.47
06-02-03	6	3.75	4.67	0.00	8.42	22.61	50.47	3.39	14.19	10.80
10-02-03	9	3.75	11.55	0.00	15.30	33.28	39.80	4.99	17.98	12.99
11-02-03	10	3.75	13.21	0.30	17.26	44.12	28.96	6.62	26.85	20.24
13-02-03	12	3.75	22.80	1.00	27.55	64.93	8.15	9.74	37.38	27.64
15-02-03	14	3.75	21.26	1.66	26.67	67.20	5.88	10.08	40.53	30.45
17-02-03	16	3.75	10.71	0.90	15.36	26.58	46.50	3.99	11.22	7.23
24-02-03	21	3.75	26.10	0.37	29.22	60.38	12.70	9.06	31.16	22.10
26-02-03	23	3.75	14.76	3.05	21.59	41.91	31.17	6.29	20.32	14.03
28-02-03	25	3.75	29.60	0.80	34.15	45.54	27.54	6.83	11.39	4.56
02-03-03	27	3.75	27.17	1.82	32.74	64.52	8.56	9.68	31.78	22.10
04-03-03	29	3.75	33.68	0.00	37.43	67.42	5.66	10.11	29.99	19.88
06-03-03	31	3.75	33.68	0.00	37.43	59.79	13.29	8.97	22.37	13.40
08-03-03	33	3.75	33.68	0.00	37.43	58.47	14.61	8.77	21.04	12.27
10-03-03	35	3.75	26.29	1.28	31.32	66.66	6.42	10.00	35.35	25.35
12-03-03	37	3.75	33.68	0.00	37.43	68.77	4.31	10.31	31.34	21.03
13-03-03	38	3.75	33.68	0.00	37.43	94.96	9.44	14.24	57.53	43.29
15-03-03	40	3.75	33.68	0.00	37.43	74.37	30.03	11.16	36.95	25.79
19-03-03	44	3.75	33.68	0.00	37.43	62.60	41.80	9.39	25.17	15.78
21-03-03	46	3.75	33.68	0.00	37.43	61.26	43.14	9.19	23.84	14.65
24-03-03	49	3.75	33.68	0.00	37.43	63.22	41.18	9.48	25.80	16.32
27-03-03	52	3.75	33.68	0.00	37.43	67.82	36.58	10.17	30.39	20.22
30-03-03	55	3.75	33.68	0.00	37.43	63.98	40.42	9.60	26.55	16.95
cumulative total	(d 99 d 37)	52.50	337.18	11.19	400.87	769.56	253.56	115.43	368.69	253.26
	(d 387 d 55)	26.25	235.73	0.00	261.98	468.21	242.59	73.23	226.24	153.01
	(d 99 d 55)	78.75	572.91	11.19	662.85	1237.77	496.15	188.67	594.93	406.26

Trial III		MC 5								
		Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N:	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_3^- reduction (after DO removal), based on theoretical C/N:	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DDC consumption (mg)	Effluent) DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
Date	Exp day.		1.07	0.43						
02-02-03	2	3.75	18.20	0.00	21.95	33.22	39.86	4.98	11.27	6.29
03-02-03	3	3.75	0.83	0.00	4.58	15.68	57.40	2.35	11.10	8.74
04-02-03	4	3.75	3.77	0.00	7.52	40.40	32.68	6.06	32.88	26.82
05-02-03	5	3.75	6.42	1.38	11.54	37.73	35.35	5.66	26.19	20.53
06-02-03	6	3.75	10.57	1.68	15.99	46.67	26.41	7.00	30.67	23.67
10-02-03	9	3.75	26.25	0.93	30.92	56.26	16.82	8.44	25.33	16.90
11-02-03	10	3.75	18.50	2.61	19.86	58.39	14.69	8.76	38.53	29.78
13-02-03	12	3.75	10.96	3.87	18.58	65.27	7.81	9.79	46.69	36.90
15-02-03	14	3.75	9.77	4.78	18.30	59.92	13.16	8.99	41.63	32.64
17-02-03	16	3.75	11.30	5.39	20.44	48.53	24.55	7.28	28.08	20.80
24-02-03	21	3.75	18.19	4.60	26.54	64.45	8.63	9.67	37.91	28.24
26-02-03	23	3.75	23.35	3.14	30.24	62.06	11.02	9.31	31.82	22.51
28-02-03	25	3.75	26.10	2.28	32.13	65.26	7.82	9.79	33.13	23.34
02-03-03	27	3.75	18.87	4.68	27.30	66.63	6.45	9.99	39.32	29.33
04-03-03	29	3.75	26.62	2.01	32.39	61.06	12.02	9.16	28.67	19.51
06-03-03	31	3.75	28.48	1.64	33.88	61.41	11.67	9.21	27.54	18.33
08-03-03	33	3.75	29.45	1.09	34.29	69.46	3.63	10.42	35.16	24.75
10-03-03	35	3.75	24.61	2.97	31.33	69.57	3.51	10.43	38.24	27.80
12-03-08	37	3.75	27.62	1.64	32.91	69.36	3.72	10.40	36.44	26.04
13-03-03	38	3.75	33.68	0.00	37.43	85.63	18.57	12.87	48.40	35.53
15-03-03	40	3.75	33.68	0.00	37.43	70.07	34.33	10.51	32.65	22.14
19-03-03	44	3.75	33.68	0.00	37.43	83.72	20.68	12.56	46.30	33.74
21-03-03	46	3.75	33.68	0.00	37.43	50.91	53.49	7.64	13.48	5.84
24-03-03	49	3.75	33.68	0.00	37.43	66.04	38.36	9.91	28.62	18.71
27-03-03	52	3.75	33.68	0.00	37.43	72.27	32.13	10.84	34.84	24.00
30-03-03	55	3.75	33.68	0.00	37.43	70.95	33.45	10.64	33.52	22.88
cumulative total	(d 97 d 37)	52.50	295.08	41.53	389.11	877.60	146.52	131.64	488.60	356.86
	(d 387 d 55)	26.25	235.73	0.00	261.98	499.79	231.01	74.97	237.81	162.84
	(d 97 d 55)	78.75	530.80	41.53	651.08	1377.39	376.53	206.61	726.31	519.70

Trial III		MC 6								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
02-02-03	2	3.75	0.13	0.00	3.88	0.00	75.24	0.00	-	-
03-02-03	3	3.75	2.19	0.00	5.94	17.43	66.65	2.62	11.49	8.88
04-02-03	4	3.75	2.34	0.00	6.09	25.18	47.90	3.78	19.09	15.32
05-02-03	5	3.75	4.95	0.71	9.41	34.45	38.63	5.17	25.04	19.87
06-02-03	6	3.75	7.80	0.90	12.46	40.95	32.13	6.14	28.49	22.35
10-02-03	9	3.75	13.86	1.59	19.20	29.40	43.68	4.41	10.20	5.79
11-02-03	10	3.75	8.11	2.30	14.17	38.48	34.60	5.77	24.32	18.54
13-02-03	12	3.75	12.95	4.39	21.09	61.58	11.50	9.24	40.49	31.25
15-02-03	14	3.75	13.59	2.24	19.58	55.39	17.69	8.31	35.81	27.50
17-02-03	16	3.75	17.99	3.98	25.72	63.75	9.33	9.56	38.03	28.46
24-02-03	21	3.75	17.21	4.92	25.89	58.65	14.43	8.80	32.77	23.97
26-02-03	23	3.75	21.36	3.73	28.84	57.41	15.67	8.61	28.57	19.96
28-02-03	25	3.75	21.07	3.66	28.48	60.86	12.22	9.13	32.39	23.26
02-03-03	27	3.75	24.26	2.90	30.91	65.92	7.16	9.89	35.00	25.11
04-03-03	29	3.75	24.91	2.68	31.35	56.45	16.63	8.47	25.10	16.64
06-03-03	31	3.75	25.18	3.07	31.99	57.23	15.85	8.59	25.24	16.65
08-03-03	33	3.75	26.16	2.08	31.98	62.97	10.11	9.45	30.99	21.54
10-03-03	35	3.76	24.17	2.59	30.51	68.20	4.88	10.23	37.69	27.46
12-03-03	37	3.75	33.68	0.00	37.43	65.38	7.70	9.81	27.96	18.15
13-03-03	38	3.75	33.68	0.00	37.43	81.35	23.05	12.20	43.92	31.72
15-03-03	40	3.75	33.68	0.00	37.43	69.32	35.08	10.40	31.90	21.50
19-03-03	44	3.76	33.68	0.00	37.43	64.19	43.21	9.63	26.76	17.13
21-03-03	46	3.75	33.68	0.00	37.43	51.62	52.78	7.74	14.19	6.45
24-03-03	49	3.75	33.68	0.00	37.43	65.02	39.38	9.75	27.60	17.84
27-03-03	52	3.75	33.68	0.00	37.43	69.09	35.31	10.36	31.67	21.30
30-03-03	55	3.75	33.68	0.00	37.43	66.75	37.66	10.01	29.33	19.32
cumulative total	(d 99 d 37)	52.50	284.50	40.13	377.13	801.57	221.45	120.25	424.54	304.29
	(d 38? d 55)	26.25	235.73	0.00	261.98	467.34	263.46	70.10	205.36	135.26
	(d 99 d 55)	78.75	520.23	40.13	639.11	1269.01	484.91	190.35	629.90	439.55

Trial III		MC 7								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
02-02-03	2	3.75	0.00	0.00	3.75	1.02	72.06	0.15	-	-
03-02-03	3	3.75	1.24	0.00	4.99	9.02	64.06	1.35	4.03	2.67
04-02-03	4	3.75	1.75	0.00	5.50	16.29	56.79	2.44	10.79	8.34
05-02-03	5	3.75	1.89	1.00	6.65	34.06	39.02	5.11	27.41	22.30
06-02-03	6	3.75	2.23	1.74	7.72	33.95	39.13	5.09	26.23	21.14
10-02-03	9	3.75	20.83	1.42	26.00	48.88	24.20	7.33	22.88	15.55
11-02-03	10	3.75	7.01	1.68	12.44	31.70	41.38	4.75	19.26	14.50
13-02-03	12	3.75	14.75	3.47	21.97	60.92	12.16	9.14	38.95	29.81
15-02-03	14	3.75	18.43	2.27	24.44	61.46	11.62	9.22	37.01	27.79
17-02-03	16	3.75	21.83	2.94	28.52	66.67	6.41	10.00	38.15	28.15
24-02-03	21	3.75	28.05	1.55	33.35	63.42	9.66	9.51	30.07	20.56
26-02-03	23	3.75	33.68	0.00	37.43	61.07	12.01	9.16	23.65	14.49
28-02-03	25	3.75	33.68	0.00	37.43	65.25	7.83	9.79	27.82	18.04
02-03-03	27	3.75	30.73	0.64	35.12	66.22	7.86	9.78	30.10	20.32
04-03-03	29	3.75	33.68	0.00	37.43	61.31	11.77	9.20	23.88	14.69
06-03-03	31	3.75	33.68	0.00	37.43	61.74	11.34	9.26	24.32	15.06
08-03-03	33	3.75	33.48	0.00	37.43	67.61	5.47	10.14	30.18	20.04
10-03-03	35	3.75	26.86	0.31	30.92	67.81	5.27	10.17	36.89	25.72
12-03-03	37	3.75	30.01	0.51	34.28	67.52	5.56	10.13	33.24	23.11
13-03-03	38	3.75	33.68	0.00	37.43	81.68	22.72	12.26	44.26	32.00
15-03-03	40	3.75	33.68	0.00	37.43	87.09	17.31	13.06	49.67	36.61
19-03-03	44	3.75	33.48	0.00	37.43	67.51	36.89	10.13	30.08	19.95
21-03-03	46	3.75	33.68	0.00	37.43	52.85	51.55	7.93	15.42	7.50
24-03-03	49	3.75	33.68	0.00	37.43	67.92	36.48	10.19	30.50	20.31
27-03-03	52	3.75	33.68	0.00	37.43	75.96	28.44	11.39	38.54	27.14
30-03-03	55	3.75	33.68	0.00	37.43	70.60	33.80	10.59	33.17	22.58
cumulative total	(d 99 d 37)	52.50	366.87	14.79	434.16	850.57	172.55	127.59	416.40	288.82
	(d 38? d 55)	26.25	235.73	0.00	261.98	523.61	227.19	75.54	241.53	165.99
	(d 99 d 55)	78.75	602.60	14.79	696.14	1354.18	399.74	203.13	658.04	454.91

Trial III		MC 8								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
02-02-03	2	3.75	8.80	0.00	12.55	9.88	63.20	1.48	-	-
03-02-03	3	3.75	1.61	0.00	5.36	9.68	63.40	1.45	4.33	2.87
04-02-03	4	3.75	2.40	0.00	6.15	21.90	51.18	3.29	15.75	12.47
05-02-03	5	3.75	7.62	1.11	12.48	46.19	26.89	6.93	33.71	26.78
06-02-03	6	3.75	7.47	2.02	13.24	41.89	31.19	6.28	28.65	22.36
10-02-03	9	3.75	31.47	0.32	35.54	61.22	11.86	9.18	25.68	16.50
11-02-03	10	3.75	23.39	2.46	29.60	62.61	10.47	9.39	33.01	23.62
13-02-03	12	3.75	18.93	3.14	25.82	65.18	7.90	9.78	39.36	29.59
15-02-03	14	3.75	19.29	3.16	26.20	65.16	7.92	9.77	38.95	29.18
17-02-03	16	3.75	22.89	3.41	30.06	66.61	6.47	9.99	36.56	26.57
24-02-03	21	3.75	27.10	1.66	32.51	62.98	10.10	9.45	30.48	21.03
26-02-03	23	3.75	33.68	0.00	37.43	65.24	7.84	9.79	27.81	18.03
28-02-03	25	3.75	28.94	1.11	33.80	67.61	5.47	10.14	33.80	23.66
02-03-03	27	3.75	31.46	0.33	35.54	65.23	7.85	9.78	29.68	19.90
04-03-03	29	3.75	33.68	0.00	37.43	65.46	7.62	9.82	28.03	18.21
06-03-03	31	3.75	33.68	0.00	37.43	57.67	15.41	8.65	20.24	11.59
08-03-03	33	3.75	33.68	0.00	37.43	62.29	10.79	9.34	24.87	15.52
10-03-03	35	3.75	30.98	0.42	35.16	66.84	6.24	10.03	31.68	21.66
12-03-03	37	3.75	33.04	0.25	37.04	68.07	5.01	10.21	31.03	20.82
13-03-03	38	3.75	33.68	0.00	37.43	76.80	25.60	11.52	41.38	29.86
15-03-03	40	3.75	33.68	0.00	37.43	74.68	29.52	11.23	37.45	26.22
19-03-03	44	3.75	33.68	0.00	37.43	69.84	34.56	10.48	32.42	21.94
21-03-03	46	3.75	33.68	0.00	37.43	40.30	64.10	2.87	2.87	0.00
24-03-03	49	3.75	33.68	0.00	37.43	68.44	35.96	10.27	31.02	20.75
27-03-03	52	3.75	33.68	0.00	37.43	83.21	21.19	12.48	45.78	33.30
30-03-03	55	3.75	33.68	0.00	37.43	70.07	34.33	10.51	32.65	22.14
cumulative total	(d 99 d 37)	52.50	402.20	16.27	470.97	902.17	120.96	135.33	431.20	295.88
	(d 38? d 55)	26.25	235.73	0.00	261.98	485.55	245.25	69.66	223.57	153.91
	(d 99 d 55)	78.75	637.93	16.27	732.95	1387.72	366.20	204.98	654.77	449.79

Trial IV		MC 3								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.25	0.00	4.00	12.38	60.70	1.86	8.38	6.53
23-02-04	4	3.75	4.10	0.79	8.64	30.40	42.68	4.56	21.76	17.20
24-02-04	5	3.75	1.33	2.83	7.91	30.92	42.16	4.64	23.01	18.37
25-02-04	6	3.75	8.35	4.42	16.52	44.18	28.90	6.63	27.66	21.04
26-02-04	7	3.75	20.53	1.80	26.08	65.50	7.58	9.83	39.42	29.59
27-02-04	8	3.75	25.49	2.21	31.45	66.25	6.83	9.94	34.80	24.86
28-02-04	9	3.75	30.17	1.33	35.25	68.44	4.64	10.27	33.19	22.92
01-03-04	11	3.75	31.42	0.64	35.81	68.02	5.06	10.20	32.21	22.01
03-03-04	13	3.75	30.37	0.50	34.62	65.95	7.13	9.89	31.33	21.44
05-03-04	15	3.75	33.51	0.00	37.26	63.82	9.26	9.57	26.56	16.98
07-03-04	17	3.75	33.51	0.00	37.26	58.06	15.02	8.71	20.80	12.09
09-03-04	19	3.75	33.51	0.00	37.26	56.59	16.49	8.49	19.32	10.84
11-03-04	21	3.75	33.51	0.00	37.26	64.81	8.27	9.72	27.55	17.83
12-03-04	22	3.75	29.42	1.63	34.80	66.49	6.59	9.97	31.70	21.72
15-03-04	25	3.75	33.51	0.00	37.26	56.28	16.80	8.44	19.02	10.57
16-03-04	26	3.75	33.51	0.00	37.26	65.52	7.56	9.83	28.25	18.43
18-03-04	28	3.75	22.69	1.27	27.71	66.14	6.94	9.92	38.42	28.50
20-03-04	30	3.75	6.63	4.00	14.39	68.32	4.76	10.25	53.93	43.68
22-03-04	32	3.75	33.61	0.00	37.26	61.94	11.14	9.29	24.68	15.39
cumulative total	(d 8 ? d 32)	52.50	410.78	1' 58	474.86	896.61	126.61	134.49	421.75	287.26

Trial IV		MC 4								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.56	0.00	4.31	22.91	50.17	3.44	18.59	15.16
23-02-04	4	3.75	15.39	2.57	21.72	66.81	16.27	8.52	35.09	26.57
24-02-04	5	3.75	16.26	3.99	24.00	68.17	4.91	10.23	44.17	33.94
25-02-04	6	3.75	14.13	2.86	20.74	66.97	6.11	10.05	46.23	36.19
26-02-04	7	3.75	17.91	4.08	25.74	67.47	5.61	10.12	41.73	31.61
27-02-04	8	3.75	20.36	4.14	28.25	65.86	7.22	9.88	37.62	27.74
28-02-04	9	3.75	24.58	3.45	31.77	67.23	5.85	10.08	35.45	25.37
01-03-04	11	3.75	23.66	3.25	30.66	67.03	6.05	10.05	36.17	26.12
03-03-04	13	3.75	22.39	3.54	29.67	65.46	7.62	9.82	35.79	25.97
05-03-04	15	3.75	23.47	3.26	30.48	67.48	5.60	10.12	37.00	26.88
07-03-04	17	3.75	29.42	1.55	34.72	66.40	6.68	9.96	31.68	21.72
09-03-04	19	3.75	33.51	0.00	37.26	64.07	9.01	9.61	26.81	17.20
11-03-04	21	3.75	24.33	3.69	31.77	67.61	5.47	10.14	35.84	25.69
12-03-04	22	3.75	14.25	5.67	23.67	68.14	4.94	10.22	44.47	34.25
15-03-04	25	3.75	24.63	3.40	31.78	59.78	13.30	8.97	28.00	19.03
16-03-04	26	3.75	25.01	3.30	32.06	66.55	6.53	9.98	34.49	24.51
18-03-04	28	3.75	22.92	1.68	28.35	65.71	7.37	9.86	37.36	27.51
20-03-04	30	3.75	0.00	6.48	10.23	68.02	5.06	10.20	57.79	47.59
22-03-04	32	3.75	12.04	2.51	18.31	65.74	7.34	9.86	47.43	37.57
cumulative total	(d 8 ? d 32)	52.50	300.77	45.91	399.18	925.07	98.05	138.76	525.89	387.13

Trial IV		MC 5								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.30	0.00	4.05	12.37	46.09	1.86	8.31	6.46
23-02-04	4	3.75	11.03	4.70	19.48	46.85	11.61	7.03	27.37	20.34
24-02-04	5	3.75	11.06	5.65	20.45	53.09	5.37	7.96	32.64	24.67
25-02-04	6	3.75	9.99	6.82	20.57	53.27	5.19	7.99	32.70	24.71
26-02-04	7	-	-	-	-	53.81	4.65	8.07	-	-
27-02-04	8	3.75	9.23	7.55	20.53	53.14	5.32	7.97	32.61	24.64
28-02-04	9	3.75	11.10	5.29	20.14	54.16	4.30	8.12	34.02	25.90
01-03-04	11	3.75	13.03	8.23	25.01	54.45	4.01	8.17	29.44	21.27
03-03-04	13	3.75	9.72	6.76	20.23	51.50	6.96	7.72	31.27	23.54
05-03-04	15	3.75	18.86	4.46	27.07	53.79	4.67	8.07	26.72	18.65
07-03-04	17	3.75	11.80	6.33	21.89	53.65	4.81	8.05	31.77	23.72
09-03-04	19	3.75	13.62	3.17	20.53	53.43	5.03	8.01	32.89	24.88
11-03-04	21	3.75	13.95	5.92	23.62	52.91	5.55	7.94	29.29	21.36
12-03-04	22	-	-	-	-	53.76	4.70	8.06	-	-
15-03-04	25	3.75	10.79	6.03	20.56	51.17	7.29	7.68	30.61	22.93
16-03-04	26	3.75	29.28	1.70	34.73	51.53	6.93	7.73	16.79	9.06
18-03-04	28	3.75	0.13	5.01	8.89	52.85	5.61	7.93	43.96	36.04
20-03-04	30	3.75	0.09	4.31	8.15	54.15	4.31	8.12	46.00	37.88
22-03-04	32	3.75	0.16	4.35	8.26	53.62	4.84	8.04	45.36	37.32
cumulative total	(d 8 ? d 32)	48.75	141.75	69.11	259.61	744.11	74.33	111.62	430.74	327.19

Trial IV		MC 6								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.21	0.00	3.96	23.87	68.00	9.55	19.91	10.36
23-02-04	4	3.75	16.63	2.14	22.52	59.42	32.45	23.77	36.90	13.13
24-02-04	5	3.75	25.11	1.98	30.83	79.16	12.71	31.67	48.33	16.67
25-02-04	6	3.75	27.41	1.91	33.07	86.89	4.98	34.76	53.82	19.06
26-02-04	7	-	-	-	-	85.59	6.28	34.23	-	-
27-02-04	8	3.75	29.07	1.10	34.82	85.83	6.04	12.88	51.01	38.14
28-02-04	9	3.75	29.60	1.57	34.92	81.20	10.67	12.18	46.28	34.10
01-03-04	11	3.75	33.51	0.00	37.26	83.98	7.89	12.60	46.72	34.12
03-03-04	13	3.75	24.23	1.91	29.90	72.33	19.54	10.85	42.43	31.59
05-03-04	15	3.75	32.64	0.35	36.74	63.94	27.93	9.59	27.19	17.60
07-03-04	17	3.75	22.67	1.60	28.02	57.31	24.56	10.10	39.28	29.19
09-03-04	19	3.75	22.50	0.89	27.13	56.09	35.78	8.41	28.96	20.55
11-03-04	21	3.75	22.67	1.83	28.26	52.75	39.12	7.91	24.49	16.58
12-03-04	22	-	-	-	-	51.97	39.90	7.80	-	-
15-03-04	25	3.75	19.92	1.83	25.50	49.38	42.49	7.41	23.87	16.47
16-03-04	26	3.75	33.51	0.00	37.26	74.36	17.51	11.15	37.10	25.94
18-03-04	28	-	-	-	-	-	-	-	-	-
20-03-04	30	3.75	16.06	1.65	21.46	68.05	23.82	10.21	46.59	36.39
22-03-04	32	3.75	11.08	1.79	16.62	55.89	35.98	8.38	39.27	30.89
cumulative total	(d 8 ? d 32)	45.00	298.38	14.52	357.90	863.07	331.24	129.46	453.20	331.54

Trial IV		MC 7								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.00	0.00	3.75	15.63	57.45	2.34	11.88	9.54
23-02-04	4	3.75	14.80	2.72	21.27	36.06	37.02	5.41	14.78	9.37
24-02-04	5	3.75	19.35	4.35	27.45	51.12	21.96	7.67	23.67	16.00
25-02-04	6	3.75	32.47	0.48	36.70	65.28	7.80	9.79	28.57	18.78
26-02-04	7	3.75	32.79	0.35	36.90	66.57	6.51	9.99	29.67	19.69
27-02-04	8	3.75	32.81	0.35	36.91	63.51	9.57	9.53	26.60	17.08
28-02-04	9	3.75	32.74	0.37	36.86	64.11	8.97	9.62	27.25	17.63
01-03-04	11	3.75	28.99	0.61	33.34	65.28	7.80	9.79	31.94	22.14
03-03-04	13	3.75	33.68	0.00	37.43	64.07	9.01	9.61	26.64	17.03
05-03-04	15	3.75	33.68	0.00	37.43	53.84	19.24	8.08	16.41	8.34
07-03-04	17	3.75	33.68	0.00	37.43	49.06	24.02	7.36	11.63	4.27
09-03-04	19	3.75	33.68	0.00	37.43	42.00	31.08	4.58	4.58	0.00
11-03-04	21	3.75	33.68	0.00	37.43	57.55	15.53	8.63	20.12	11.49
12-03-04	22	3.75	33.68	0.00	37.43	52.54	20.54	7.88	15.11	7.23
15-03-04	25	3.75	33.68	0.00	37.43	41.44	31.64	4.02	4.02	0.00
16-03-04	26	3.75	33.68	0.00	37.43	60.87	12.21	9.13	23.44	14.31
18-03-04	28	3.75	33.68	0.00	37.43	61.73	11.35	9.26	24.30	15.04
20-03-04	30	3.75	33.68	0.00	37.43	55.55	5.00	8.33	18.12	9.79
22-03-04	32	3.75	33.68	0.00	37.43	50.32	10.23	7.55	12.90	5.35
cumulative total	(d 8 ? d 32)	52.50	464.96	1.33	518.79	781.86	216.20	113.36	263.07	149.71

Trial IV		MC 8								
Date	Exp day.	Calculated DOC consumption (mg) for removal of 10 mg l^{-1} DO	Calculated DOC consumption (mg) required to satisfy observed denitrification (TON loss), (after DO removal), based on theoretical C/N: 1.07	Calculated DOC consumption (mg) required to satisfy NO_3^- ? NO_2^- reduction (after DO removal), based on theoretical C/N: 0.43	Total calculated DOC consumption (mg) for N-reductions (after DO removal)	Observed DOC consumption (mg)	Effluent DOC (mg)	Estimated DOC assimilated in biomass (mg) (15 % of observed DOC consumption)	DOC unaccounted for (mg) (exc. biomass addition)	DOC unaccounted for (mg) (inc. biomass addition)
20-02-04	1	3.75	0.00	0.00	3.75	14.88	56.20	2.23	11.13	8.90
23-02-04	4	3.75	15.71	2.85	22.30	36.51	36.57	5.48	14.21	8.73
24-02-04	5	3.75	24.96	2.94	31.65	53.42	19.66	8.01	21.76	13.75
25-02-04	6	3.75	32.62	0.36	36.73	61.11	11.97	9.17	24.37	15.21
26-02-04	7	3.75	32.67	0.34	36.75	62.48	10.60	9.37	25.73	16.36
27-02-04	8	3.75	32.67	0.34	36.76	61.31	11.77	9.20	24.55	15.36
28-02-04	9	3.75	32.61	0.36	36.73	61.91	11.17	9.29	25.19	15.90
01-03-04	11	3.75	33.51	0.00	37.26	68.70	4.38	10.31	31.44	21.13
03-03-04	13	3.75	33.51	0.00	37.26	53.36	19.72	8.00	16.09	8.09
05-03-04	15	3.75	33.51	0.00	37.26	52.86	20.22	7.93	15.60	7.67
07-03-04	17	3.75	33.51	0.00	37.26	48.76	24.32	7.31	11.50	4.19
09-03-04	19	3.75	33.51	0.00	37.26	42.17	30.91	4.91	4.91	0.00
11-03-04	21	3.75	33.51	0.00	37.26	50.89	22.19	7.63	13.62	5.99
12-03-04	22	3.75	33.51	0.00	37.26	52.51	20.57	7.88	15.25	7.37
15-03-04	25	3.75	33.51	0.00	37.26	40.15	32.93	2.89	2.89	0.00
16-03-04	26	3.75	33.51	0.00	37.26	54.08	19.00	8.11	16.82	8.71
18-03-04	28	3.75	33.51	0.00	37.26	63.16	9.92	9.47	25.89	16.42
20-03-04	30	3.75	33.51	0.00	37.26	55.64	4.91	8.35	18.38	10.03
22-03-04	32	3.75	33.51	0.00	37.26	52.63	8.02	7.88	15.26	7.38
cumulative total	(d 8 ? d 32)	52.50	457.43	0.70	520.63	758.04	240.02	109.16	237.41	128.25

2.4: MINITAB OUTPUT: GLM ANOVA TABLES, RESIDUAL DESCRIPTIONS AND TUKEY PAIRWAISE COMPARISON DATA

GLMs for Trial I: Denit (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

General Linear Model: denit conc versus Treatment denit, Day denit, ...

Factor	Type	Levels	Values
Treatment denit	fixed	5	1, 2, 3, 4, 5
Day denit	fixed	7	4, 5, 6, 7, 8, 19, 21
MC denit(Treatment denit)	random	8	1, 2, 3, 4, 5, 6, 7, 8

Analysis of Variance for denit conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment denit	4	84.668	84.668	21.167	6.05	0.086
Day denit	6	131.092	131.092	21.849	4.21	0.002
MC denit(Treatment denit)	3	10.500	10.500	3.500	0.67	0.572
Error	42	217.844	217.844	5.187		
Total	55	444.104				

S = 2.27745 R-Sq = 50.95% R-Sq(adj) = 35.76%

NOTE - RESIDUALS DO NOT FIT A NORMAL DISTRUBUTION SO THEREFORE THE DATA WAS TRANSFORMED USING SQUARE ROOT AND GLM WAS REPEATED

General Linear Model: Treatment denit, Day denit, MC denit *(where C6 = square root of denit conc values)*

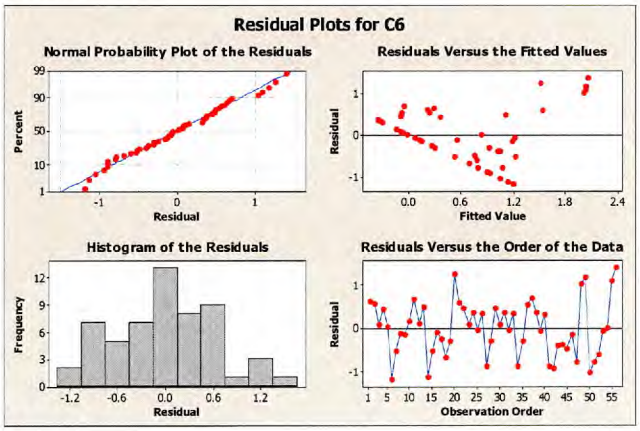
Factor	Type	Levels	Values
Treatment denit	fixed	5	1, 2, 3, 4, 5
Day denit	fixed	7	4, 5, 6, 7, 8, 19, 21
MC denit(Treatment denit)	random	8	1, 2, 3, 4, 5, 6, 7, 8

Analysis of Variance for C6, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment denit	4	10.0007	10.0007	2.5002	5.54	0.096
Day denit	6	13.9441	13.9441	2.3240	4.51	0.001
MC denit(Treatment denit)	3	1.3538	1.3538	0.4513	0.88	0.461
Error	42	21.6218	21.6218	0.5148		
Total	55	46.9204				

S = 0.717499 R-Sq = 53.92% R-Sq(adj) = 39.65%

Residual Plots



GLM for Trial I: Nitrite (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

General Linear Model: nitrite conc versus Treatment ni, Day nitrite, ...

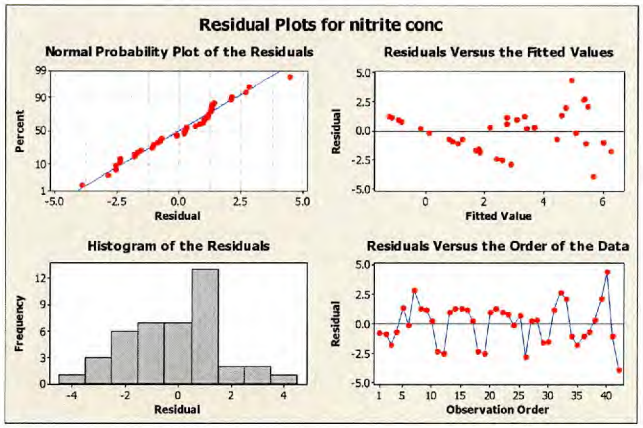
Factor	Type	Levels	Values
Treatment nitrite	fixed	3	3, 4, 5
Day nitrite	fixed	7	4, 5, 6, 7, 8, 19, 21
MC nitrite(Treatment nitrite)	random	6	3, 4, 5, 6, 7, 8

Analysis of Variance for nitrite conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment nitrite	2	44.601	44.601	22.300	4.21	0.135
Day nitrite	6	147.836	147.836	24.639	5.88	0.000
MC nitrite(Treatment nitrite)	3	15.907	15.907	5.302	1.27	0.304
Error	30	125.730	125.730	4.191		
Total	41	334.074				

S = 2.04720 R-Sq = 62.36% R-Sq(adj) = 48.56%

Residual Plots for nitrite conc



GLM for Trial II: Denit (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

General Linear Model: denit Conc versus Treatment Denit, Day Denit, ...

Factor	Type	Levels	Values
Treatment Denit	fixed	5	1, 2, 3, 4, 5
Day Denit	fixed	33	8, 10, 13, 16, 19, 21, 23, 26, 28, 31, 33, 36, 40, 42, 45, 47, 49, 51, 54, 56, 58, 60, 64, 66, 70, 72, 74, 76, 78, 79, 80, 81, 84
MC Denit(Treatment Denit)	random	8	1, 2, 3, 4, 5, 6, 7, 8

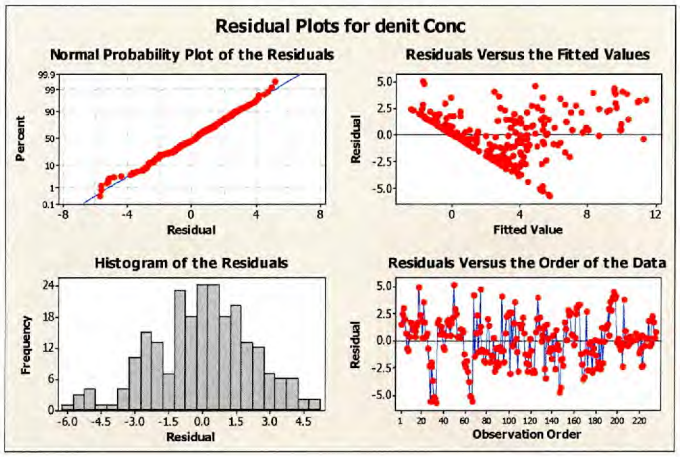
Analysis of Variance for denit Conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P	
Treatment Denit	4	1201.742	1019.627	254.907	18.99	0.020	x
Day Denit	32	1111.952	1111.952	34.749	6.08	0.000	
MC Denit(Treatment Denit)	3	39.274	39.274	13.091	2.29	0.080	
Error	194	1108.599	1108.599	5.714			
Total	233	3461.568					

x Not an exact F-test.

S = 2.39049 R-Sq = 67.97% R-Sq(adj) = 61.54%

Residual Plots for denit Conc




```
Tukey Simultaneous Tests
Response Variable denit Conc
All Pairwise Comparisons among Levels of Treatment Denit
Treatment Denit = 1  subtracted from:

Treatment  Difference       SE of              Adjusted
Denit        of Means  Difference  T-Value    P-Value
2             -0.1270      0.5885  -0.2158     0.9995
3              4.2427      0.5097   8.3247     0.0000
4              4.9398      0.5097   9.6926     0.0000
5              1.6791      0.5966   2.8144     0.0425


Treatment Denit = 2  subtracted from:

Treatment  Difference       SE of              Adjusted
Denit        of Means  Difference  T-Value    P-Value
3               4.370      0.5097    8.574     0.0000
4               5.067      0.5097    9.942     0.0000
5               1.806      0.5966    3.027     0.0232


Treatment Denit = 3  subtracted from:

Treatment  Difference       SE of              Adjusted
Denit        of Means  Difference  T-Value    P-Value
4               0.697      0.4161    1.675     0.4517
5              -2.564      0.5190   -4.939     0.0000


Treatment Denit = 4  subtracted from:

Treatment  Difference       SE of              Adjusted
Denit        of Means  Difference  T-Value    P-Value
5              -3.261      0.5190   -6.283     0.0000
```

GLM for Trial II: Nitrite (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code
(MC 1 and 2 excluded)

General Linear Model: Nitrite conc versus Treatment ni, Day nitrite, ...

```
Factor                          Type    Levels  Values
Treatment nitrite               fixed        3  3, 4, 5
Day nitrite                     fixed       33  8, 10, 13, 16, 19, 21, 23, 26,
                                                28, 31, 33, 36, 40, 42, 45, 47,
                                                49, 51, 54, 56, 58, 60, 64, 66,
                                                70, 72, 74, 76, 78, 79, 80, 81,
                                                84
MC nitrite(Treatment nitrite)   random       6  3, 4, 5, 6, 7, 8
```

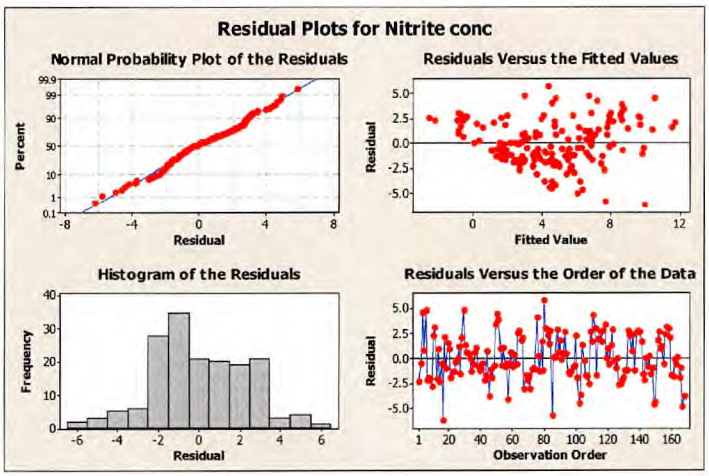
Analysis of Variance for Nitrite conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment nitrite	2	477.530	567.498	283.749	20.22	0.014 x
Day nitrite	32	955.305	955.305	29.853	4.55	0.000
MC nitrite(Treatment nitrite)	3	44.297	44.297	14.766	2.25	0.086
Error	130	853.142	853.142	6.563		
Total	167	2330.275				

x Not an exact F-test.

S = 2.56176 R-Sq = 63.39% R-Sq(adj) = 52.97%

Residual Plots for Nitrite conc



Tukey Simultaneous Tests
Response Variable Nitrite conc
All Pairwise Comparisons among Levels of Treatment nitrite
Treatment nitrite = 3 subtracted from:

Treatment nitrite	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4	-0.164	0.4459	-0.368	0.9283
5	-4.941	0.5685	-8.691	0.0000

Treatment nitrite = 4 subtracted from:

Treatment nitrite	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5	-4.777	0.5685	-8.403	0.0000

GLM for Trial III: Denit (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

General Linear Model: Denit conc versus Treatment de, Day denit te, ...

Factor	Type	Levels
Treatment denit test	fixed	5
Day denit test	fixed	21
MC denit test(Treatment denit test)	random	8

Factor	Values
Treatment denit test	1, 2, 3, 4, 5
Day denit test	9, 10, 12, 14, 16, 21, 23, 25, 27, 29, 31, 33, 35, 37, 38, 40, 44, 46, 49, 52, 55
MC denit test(Treatment denit test)	1, 2, 3, 4, 5, 6, 7, 8

Analysis of Variance for Denit conc, using Adjusted SS for Tests

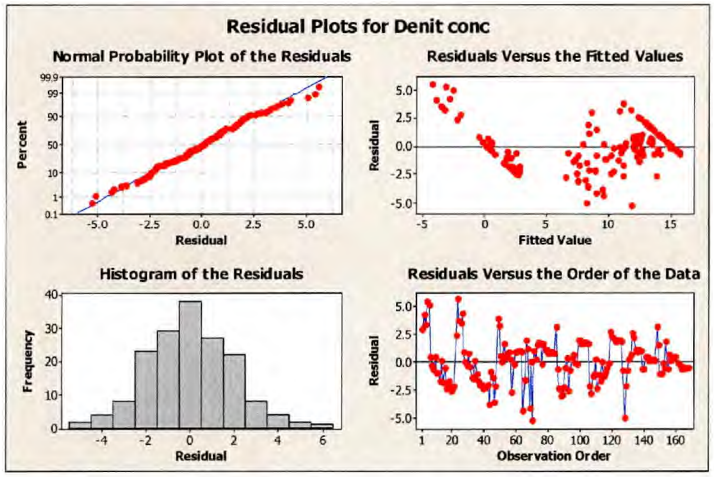
Source	DF	Seq SS	Adj SS	Adj MS	F

Treatment denit test	4	4369.28	4369.28	1092.32	502.58
Day denit test	20	833.02	833.02	41.65	9.34
MC denit test(Treatment denit test)	3	6.52	6.52	2.17	0.49
Error	140	624.19	624.19	4.46	
Total	167	5833.01			

Source	P
Treatment denit test	0.000
Day denit test	0.000
MC denit test(Treatment denit test)	0.692
Error	
Total	

S = 2.11152 R-Sq = 89.30% R-Sq(adj) = 87.24%

Residual Plots for Denit conc



Tukey Simultaneous Tests
Response Variable Denit conc
All Pairwise Comparisons among Levels of Treatment denit test
Treatment denit test = 1 subtracted from:

Treatment denit test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2	-0.3133	0.6516	-0.4808	0.9890
3	11.4719	0.5643	20.3285	0.0000
4	10.5157	0.5643	18.6341	0.0000
5	12.5255	0.5643	22.1954	0.0000

Treatment denit test = 2 subtracted from:

Treatment denit test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
3	11.79	0.5643	20.88	0.0000
4	10.83	0.5643	19.19	0.0000
5	12.84	0.5643	22.75	0.0000

Treatment denit test = 3 subtracted from:

Treatment denit test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4	-0.9562	0.4608	-2.075	0.2367
5	1.0536	0.4608	2.287	0.1554

Treatment denit test = 4 subtracted from:

Treatment denit test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5	2.010	0.4608	4.362	0.0002

GLM for Trial III: Nitrite (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code
(MC 1 and 2 excluded)

General Linear Model: Nitrite conc versus Treatment ni, Day nitrite , ...

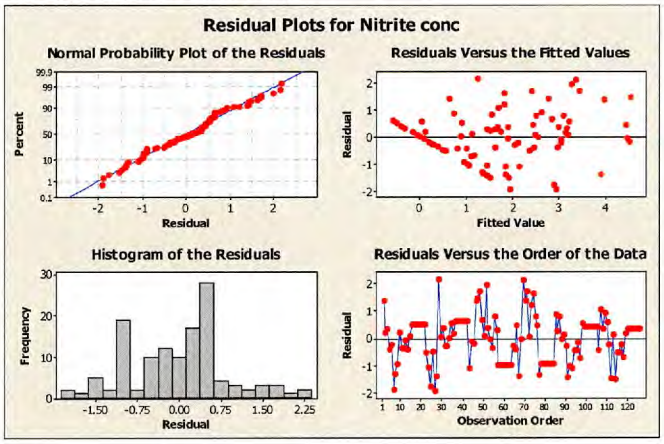
Factor	Type	Levels	Values
Treatment nitrite test	fixed	3	3, 4, 5
Day nitrite test	fixed	21	9, 10, 12, 14, 16, 21, 23, 25, 27, 29, 31, 33, 35, 37, 38, 40, 44, 46, 49, 52, 55
MC nitrite test(Treatment nitrite test)	random	6	3, 4, 5, 6, 7, 8

Analysis of Variance for Nitrite conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment nitrite test	2	58.1807	58.1807	29.0903	403.21	0.000
Day nitrite test	20	174.4891	174.4891	8.7245	9.49	0.000
MC nitrite test(Treatment nitrite test)	3	0.2164	0.2164	0.0721	0.08	0.972
Error	100	91.9434	91.9434	0.9194		
Total	125	324.8297				

S = 0.958871 R-Sq = 71.69% R-Sq(adj) = 64.62%

Residual Plots for Nitrite conc



Tukey Simultaneous Tests
Response Variable Nitrite conc
All Pairwise Comparisons among Levels of Treatment nitrite test
Treatment nitrite test = 3 subtracted from:

Treatment nitrite test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4	1.5243	0.2092	7.2848	0.0000
5	0.1831	0.2092	0.8750	0.6573

Treatment nitrite test = 4 subtracted from:

Treatment nitrite test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5	-1.341	0.2092	-6.410	0.0000

GLM for Trial IV: Denit (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

General Linear Model: denit conc versus Treatment de, Day denit te, ...

Factor	Type	Levels
Treatment denit test	fixed	5
Day denit test	fixed	15
MC denit test(Treatment denit test)	random	8

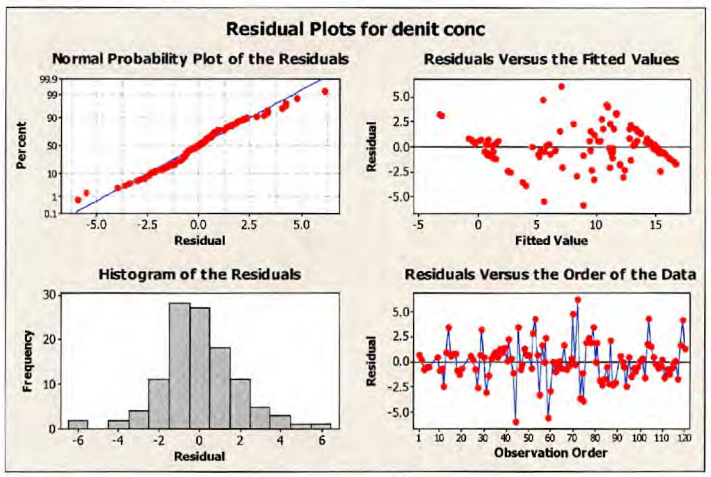
Factor	Values
Treatment denit test	1, 2, 3, 4, 5
Day denit test	7, 8, 9, 11, 13, 15, 17, 19, 21, 22, 25, 26, 28, 30, 32
MC denit test(Treatment denit test)	1, 2, 3, 4, 5, 6, 7, 8

Analysis of Variance for denit conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment denit test	4	3216.58	2970.26	742.56	26.17	0.011
Day denit test	14	223.35	223.35	15.95	3.47	0.000
MC denit test(Treatment denit test)	3	85.11	85.11	28.37	6.17	0.001
Error	91	418.29	418.29	4.60		
Total	112	3943.34				

S = 2.14398 R-Sq = 89.39% R-Sq(adj) = 86.94%

Residual Plots for denit



Tukey Simultaneous Tests
Response Variable denit conc
All Pairwise Comparisons among Levels of Treatment denit test
Treatment denit test = 1 subtracted from:

Treatment denit test	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
2	10.440	0.5980	17.459	0.0000
3	4.455	0.7147	6.234	0.0000
4	10.352	0.7334	14.115	0.0000
5	14.101	0.5980	23.582	0.0000

```
Treatment denit test = 2  subtracted from:

Treatment
denit       Difference       SE of             Adjusted
test          of Means  Difference  T-Value    P-Value
3               -5.985      0.6780   -8.828     0.0000
4               -0.088      0.6962   -0.127     0.9999
5                3.661      0.5536    6.614     0.0000

Treatment denit test = 3  subtracted from:

Treatment
denit       Difference       SE of             Adjusted
test          of Means  Difference  T-Value    P-Value
4                5.897      0.7987    7.383     0.0000
5                9.646      0.6780   14.228     0.0000

Treatment denit test = 4  subtracted from:

Treatment
denit       Difference       SE of             Adjusted
test          of Means  Difference  T-Value    P-Value
5                3.750      0.6962    5.386     0.0000
```

GLM for Trial IV: Nitrite (concentration data) against Treatment Code, Day and Microcosm with Microcosm nested within Treatment Code

(MCs 1 and 2 excluded)

```
General Linear Model: Nitrite conc versus Treatment ni, Day nitrite , ...

Factor                       Type    Levels  Values
Treatment nitrite test       fixed        4  2, 3, 4, 5
Day nitrite test             fixed       15  7, 8, 9, 11, 13, 15, 17, 19, 21, 22,
                                             25, 26, 28, 30, 32
MC nitrite test(             random       6  3, 4, 5, 6, 7, 8
  Treatment nitrite test)

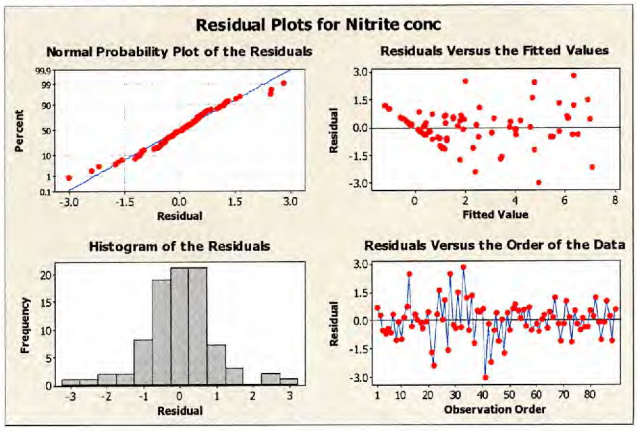
Analysis of Variance for Nitrite conc, using Adjusted SS for Tests

Source                       DF   Seq SS   Adj SS   Adj MS      F      P
Treatment nitrite test        3  359.992  360.178  120.059   4.47  0.188 x
Day nitrite test             14   43.683   41.954    2.997   2.45  0.007
MC nitrite test(              2   54.336   54.336   27.168  22.25  0.000
  Treatment nitrite test)
Error                        68   83.040   83.040    1.221
Total                        87  541.051

x Not an exact F-test.


S = 1.10507   R-Sq = 84.65%   R-Sq(adj) = 80.36%
```

Residual Plots for Nitrite conc



No Comparisons necessary as no signif difference between effluent NO2-

APPENDIX 3: Raw Pressure data from *in situ* pressure transducers (used for calculation of Hydraulic Conductivity in Chapter 5)

Pressure measurements are given in units of cm of pressure (i.e. elevation head)

Trial I	MC 1 - base	MC 1 - mid	MC 1 - top	MC 4 - base	MC 4 - mid	MC 4 - top	MC 8 - base	MC 8 - mid	MC 8 - top
Exp. Day	channel 1	channel 2	channel 3	channel 4	channel 5	channel 6	channel 10	channel 11	channel 12
8	80.80	64.00	48.90	82.00	71.75	45.50	81.60	69.88	51.53
9	83.42	68.29	50.28	83.70	72.48	44.65	83.70	66.48	49.69
10	85.59	68.88	49.83	85.30	72.89	44.56	86.30	68.86	49.92
11	85.30	70.40	51.10	87.88	69.79	46.34	86.13	69.29	48.90
12	86.49	69.71	50.00	95.20	71.76	43.55	88.34	68.73	50.22
13	82.62	68.03	51.35	92.08	71.20	43.72	86.12	68.22	50.22
14	81.81	66.58	50.82	84.66	67.48	42.56	82.51	65.56	49.38
16	82.08	66.69	49.99	86.47	67.33	44.78	84.59	67.48	50.29
20	82.43	67.42	51.61	86.24	68.96	45.70	85.04	69.69	52.60
17	84.57	69.49	52.00	91.81	72.54	50.50	81.10	63.05	51.10
21	85.99	73.63	52.60	94.04	74.08	51.20	87.16	70.43	54.00

Trial II	MC 1 - base	MC 1 - top	MC 3 - base	MC 3 - top	MC 5 - base	MC 5 - top	MC 7 - base	MC 7 - top
Exp.day	channel 1	channel 2	channel 3	channel 4	channel 5	channel 6	channel 10	channel 12
1	82.31	43.89	80.25	49.19	82.70	48.63	75.14	40.31
2	82.12	43.73	80.91	48.91	82.70	50.02	74.90	40.51
3	82.47	43.80	80.86	48.93	82.70	49.96	75.08	40.71
4	82.00	43.64	80.72	48.72	83.20	49.80	75.69	41.74
5	82.00	44.55	80.16	48.69	83.80	50.46	74.25	39.92
6	82.00	44.52	80.83	48.48	84.90	50.56	75.82	40.93
7	82.20	44.87	80.21	47.78	83.98	49.73	75.97	42.00
9	82.88	44.28	80.85	48.23	83.87	49.86	76.38	41.05
10	82.40	44.19	80.05	47.87	84.67	49.44	76.61	41.21
12	82.20	44.17	79.69	48.07	84.75	49.85	75.76	41.65
13	81.98	45.10	80.20	47.98	84.95	50.00	76.10	42.01
14	82.40	44.55	81.19	48.43	84.93	49.86	77.23	41.09
15	80.64	45.94	78.98	48.02	84.70	49.78	75.68	41.32
16	82.73	44.62	81.17	48.57	85.20	50.06	75.75	41.27
17	87.75	52.15	80.60	50.34	85.47	51.42	75.03	41.51
18	82.50	44.90	78.09	48.57	84.98	49.98	76.59	41.51
19	82.38	43.48	82.35	48.49	85.82	51.45	76.36	41.34
20	82.19	43.50	85.04	48.57	84.76	49.70	82.88	48.12
21	81.56	43.97	86.56	48.86	85.32	50.28	82.68	47.46
22	81.60	43.78	86.52	48.72	84.94	50.04	76.65	41.84
23	82.00	44.75	86.30	48.79	85.06	50.25	76.77	41.66
24	82.20	44.39	86.77	48.81	85.53	50.44	76.75	42.01
25	83.00	44.78	86.34	48.55	84.67	49.57	76.98	41.60
26	81.88	43.96	87.34	48.15	84.92	49.67	76.72	41.66
27	82.70	44.00	87.29	48.52	84.57	49.31	76.97	42.12
28	82.79	44.03	87.59	50.33	85.00	50.00	77.97	41.60
29	83.10	45.00	90.42	52.67	84.90	49.70	77.80	41.60
30	82.50	45.22	89.63	52.48	86.00	49.50	77.80	41.40
32	82.00	44.60	90.71	53.23	86.80	50.30	77.30	42.20
34	82.00	44.30	88.32	50.59	86.00	49.70	77.00	42.20
35	82.00	44.00	84.58	48.82	84.81	49.95	76.70	42.54
36	81.60	44.00	88.15	49.11	85.22	50.01	78.00	42.74
37	80.88	44.90	87.24	49.91	85.20	50.30	77.60	42.20
70	81.40	45.50	83.12	47.70	85.52	49.56	Microcosms	terminated
71	81.23	45.20	82.83	47.84	85.35	49.58	-	-
72	81.80	44.50	83.40	48.44	84.84	49.60	-	-
73	81.45	44.50	83.86	48.13	85.74	49.75	-	-
74	81.00	43.60	69.83	35.64	84.93	50.81	-	-
75	81.60	44.50	69.83	35.64	84.30	50.81	-	-
76	81.40	44.20	70.94	36.80	84.73	50.59	-	-
77	81.60	44.50	72.53	38.29	84.57	50.46	-	-
78	81.40	45.80	37.50	3.94	85.40	50.89	-	-
79	81.40	45.80	37.50	3.94	85.06	50.89	-	-
80	81.40	44.20	37.50	3.94	85.06	50.89	-	-
81	81.39	44.90	85.76	51.20	83.92	49.79	-	-
82	81.39	44.90	85.76	51.20	84.60	49.79	-	-
83	81.39	44.90	85.76	51.20	83.92	49.79	-	-
84	81.39	44.50	85.76	51.20	83.92	49.79	-	-

Trial IV	MC 1 - base	MC 1 - top	MC 4 - base	MC 4 - top	MC 6 - base	MC 6 - top	MC 8 - base	MC 8 - top
Exp. Day	Channel 1	Channel 2	Channel 4	Channel 5	Channel 7	Channel 8	Channel 10	Channel 11
1	84.70	49.52	83.25	39.27	81.67	45.20	73.66	38.45
4	85.10	49.17	83.40	39.09	83.60	46.60	74.10	39.00
5	86.11	49.45	84.40	39.10	89.56	47.65	76.83	40.73
7	85.40	49.19	88.53	38.91	95.40	48.45	77.38	39.57
8	85.11	49.14	96.54	39.12	93.44	47.79	78.61	39.55
9	84.50	48.74	101.11	38.80	96.19	49.18	93.98	39.95
10	84.00	48.66	90.00	38.62	95.35	48.32	93.60	39.84
11	83.74	48.71	86.48	39.01	75.53	47.34	86.16	39.35
12	84.27	48.64	87.58	38.81	93.74	48.40	92.06	40.09
13	85.59	48.98	88.62	39.29	94.74	50.41	87.47	40.89
14	-	-	88.50	39.47	90.12	48.69	83.21	39.61
16	-	-	92.91	39.40	94.70	48.06	86.13	39.91
17	-	-	90.40	39.59	93.39	49.09	84.55	39.28
18	-	-	89.60	39.20	93.30	47.86	85.10	39.13
19	-	-	90.80	39.07	94.37	48.95	85.58	40.19
20	84.79	49.29	90.94	39.55	97.03	49.07	86.85	39.66
21	84.30	49.11	91.66	39.71	98.75	48.60	86.15	39.80
22	84.21	49.11	88.16	39.64	100.77	49.80	85.25	38.72
25	84.25	49.11	86.29	39.74	99.61	50.50	88.42	39.08
26	84.42	49.11	87.50	39.61	-	51.10	87.11	38.58
27	84.71	49.11	87.39	39.81	91.10	48.44	86.25	38.86
28	83.33	49.11	89.00	39.74	-	48.73	87.06	38.72
29	83.82	49.11	90.80	40.42	-	48.15	85.73	38.88
30	84.14	49.11	91.00	41.19	99.91	49.80	86.48	38.72
31	84.00	49.11	91.00	40.81	97.27	48.06	83.98	40.19
32	85.68	49.11	92.00	40.96	96.83	48.39	86.23	38.50

APPENDIX 4: GLM ANOVA details for statistical comparison of Fe and Mn concentration between different microcosm sandstone types (reported in Chapter 6)

TRIAL III: METALS – Fe Concentrations

General Linear Model: Fe Conc versus Treatment, Day, MC

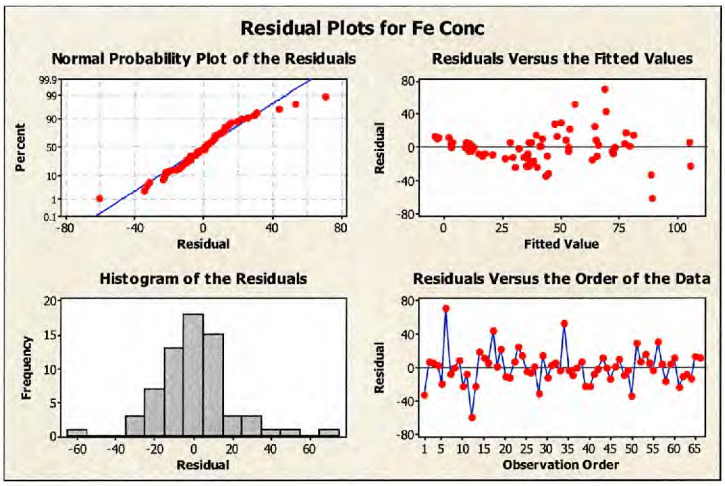
```
Factor          Type    Levels  Values
Treatment       fixed        3  3, 4, 5
Day             fixed       11  10, 23, 30, 37, 39, 41, 43, 45, 49, 52, 55
MC(Treatment)   random       6  3, 4, 5, 6, 7, 8
```

```
Analysis of Variance for Fe Conc, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS      F      P
Treatment        2  13737.3  13737.3  6868.6  14.41  0.029
Day             10  32935.3  32935.3  3293.5   6.19  0.000
MC(Treatment)    3   1429.6   1429.6   476.5   0.90  0.450
Error           50  26601.3  26601.3   532.0
Total           65  74703.5

S = 23.0657   R-Sq = 64.39%   R-Sq(adj) = 53.71%
```

Residual Plots




```
Tukey Simultaneous Tests

Response Variable Fe Conc
All Pairwise Comparisons among Levels of Treatment
Treatment = 3  subtracted from:

           Difference       SE of             Adjusted
Treatment    of Means  Difference  T-Value    P-Value
4             -28.68       6.955   -4.123     0.0004
5             -32.22       6.955   -4.634     0.0001

Treatment = 4  subtracted from:

           Difference       SE of             Adjusted
Treatment    of Means  Difference  T-Value    P-Value
5             -3.549       6.955  -0.5103     0.8667
```

TRIAL III: METALS – Mn Concentrations

General Linear Model: Mn Conc versus Treatment, Day, MC

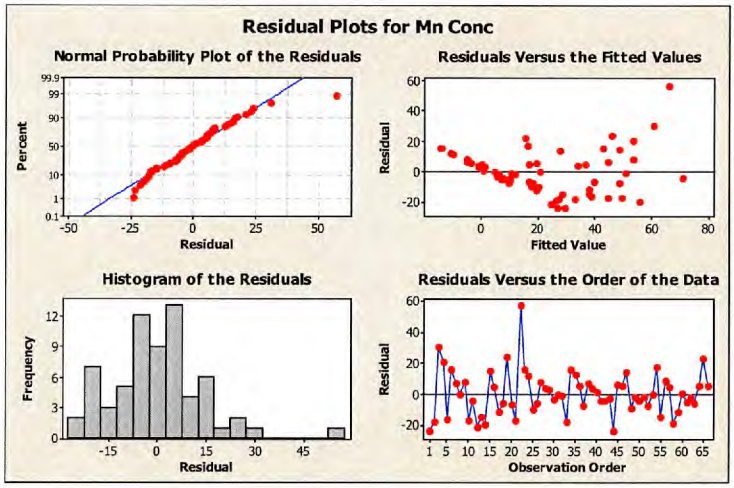
Factor	Type	Levels	Values
Treatment	fixed	3	3, 4, 5
Day	fixed	11	10, 23, 30, 37, 39, 41, 43, 45, 49, 52, 55
MC(Treatment)	random	6	3, 4, 5, 6, 7, 8

Analysis of Variance for Mn Conc, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	2	20232.1	20232.1	10116.0	220.41	0.001
Day	10	8564.4	8564.4	856.4	3.24	0.003
MC(Treatment)	3	137.7	137.7	45.9	0.17	0.914
Error	50	13213.1	13213.1	264.3		
Total	65	42147.3				

S = 16.2562 R-Sq = 68.65% R-Sq(adj) = 59.25%

Residual Plots



Tukey Simultaneous Tests

Response Variable Mn Conc
All Pairwise Comparisons among Levels of Treatment
Treatment = 3 subtracted from:

Treatment	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
4	-41.13	4.901	-8.392	0.0000
5	-31.08	4.901	-6.340	0.0000

Treatment = 4 subtracted from:

Treatment	Difference of Means	SE of Difference	T-Value	Adjusted P-Value
5	10.06	4.901	2.052	0.1104

APPENDIX 5: Statistical comparisons of DGGE banding data (reported in Chapter 7)

1. Compilation of data sets for ANOVA

Dice coeff. of similarity between pairs of biological replicates (BR):

64.3 (MC 7a/7b)

70.5 (MC 6a/6b)

17.7 (MC 4a/4b)

87.5 (MC 3a/3b)

92.4 (MC 5a/5b)

14.2 (MC 1a/1b)

This data set includes only replicate samples from single microcosms from Trial II.

Dice coeff. of similarity between pairs of samples taken from microcosm replicates for individual treatments (MR):

35.3 (Trial II: MC 8/MC 7a)

48.8 (Trial II: MC 8/MC 7b)

68.1 (Trial II: MC 6a/MC 5a)

66.9 (Trial II: MC 6a/MC 5b)

57.1 (Trial II: MC 6b/MC 5a)

54.0 (Trial II: MC 6b/MC 6a)

55.2 (Trial II: MC 4a/MC 3a)

51.4 (Trial II: MC 4a/MC 3b)

12.9 (Trial II: MC 4b/MC 3a)

17.6 (Trial II: MC 4b/MC 3b)

74.0 (Trial III: MC 8/MC 7)

62.2 (Trial III: MC 6/MC 5)

58.1 (Trial III: MC 4/MC 3)

This data set includes replicate comparisons from both MC Trial II and III. Control data were not included no duplicate MC reps of a singular control treatment type were analysed.

Dice coeff. of similarity between pairs of samples taken from different microcosm treatment types (DT):

37.6 (Trial II: MC 8/MC 4a)

23.1 (Trial II: MC 8/MC 4b)

45.6 (Trial II: MC 8/MC 3a)

48.7 (Trial II: MC 8/MC 3b)

45.8 (Trial II: MC 7b/MC 4a)

11.1 (Trial II: MC 7b/MC 4b)

44.7 (Trial II: MC 7b/MC 3a)

42.4 (Trial II: MC 7b/MC 3b)

43.4 (Trial II: MC 6a/MC 4a)

14.2 (Trial II: MC 6a/MC 4b)

56.5 (Trial II: MC 6a/MC 3a)

57.1 (Trial II: MC 6a/MC 3b)

31.3 (Trial II: MC 6b/MC 4a)

7.1 (Trial II: MC 6b/MC 4b)

45.3 (Trial II: MC 6b/MC 3a)

44 (Trial II: MC 6b/MC 3b)

34.9 (Trial II: MC 4a/MC 5a)

35.9 (Trial II: MC 4a/MC 5b)

8.8 (Trial II: MC 4b/MC 5a)

8.9 (Trial II: MC 4b/MC 5b)

54.8 (Trial II: MC 3a/MC 5a)

35.4 (Trial II: MC 7a/MC 4a)

7.6 (Trial II: MC 7a/MC 4b)

26.1 (Trial II: MC 7a/MC 3a)

27.7 (Trial II: MC 7a/MC 3b)

52.4 (Trial II: MC 3a/MC 5b)

52.3 (Trial II: MC 3b/MC 5a)

52.3 (Trial II: MC 3b/MC 5b)

62.7 (Trial II: MC 8/MC 6a)

42.4 (Trial II: MC 8/MC 6a)

54.1 (Trial II: MC 8/MC 5a)

55.1 (Trial II: MC 8/MC 5b)

33.4 (Trial II: MC 7a/MC 6a)

19.2 (Trial II: MC 7a/MC 6b)

36.8 ((Trial II: MC 7a/MC 5a)

39.6 (Trial II: MC 7a/MC 5b)

41.2 (Trial II: MC 7b/MC 6a)

45.8 (Trial II: MC 7b/MC 6b)

50.0 (Trial II: MC 7b/MC 5a)

50.6 (Trial II: MC 7b/MC 5b)

Dice coeff. of similarity between pairs of samples taken from acetate Treatments and glucose treatments, respectively:

Acetate	Glucose
68.1 (Trial II: MC 6a/MC 5a)	17.7 (Trial II: MC 4a/MC 4b)
66.9 (Trial II: MC 6a/MC 5b)	55.2 (Trial II: MC 4a/MC 3a)
57.1 (Trial II: MC 6b/MC 5a)	51.4 (Trial II: MC 4a/MC 3b)
54.0 (Trial II: MC 6b/MC 6a)	
	12.9 (Trial II: MC 4b/MC 3a)
92.4 (Trial II: MC 5a/MC 5b)	17.6 (Trial II: MC 4b/MC 3b)
70.5 (Trial II: MC 6a/MC 6b)	87.5 (Trial II: MC 3a/MC 3b)

This group of comparisons is for similarities between acetate and glucose – ie, they have the same level of OC input.

Dice coeff. of similarity between samples taken from acetate or glucose treatments paired with samples taken from the autotrophic control microcosm:

Acetate	Glucose
15.4 (Trial II: MC 6a/MC 2)	23.5 (Trial II: MC 4a/MC 2)
10.7 (Trial II: MC 6b/MC 2)	5.7 (Trial II: MC 4b/MC 2)
16.7 (Trial II: MC 5a/MC 2)	24.7 (Trial II: MC 3a/MC 2)
17.1 (Trial II: MC 5b/MC 2)	22.2 (Trial II: MC 3b/MC 2)

Dice coeff. of similarity between pairs of samples taken from different sandstone treatments:

Within sandstone	Between sandstone treatments
74 (Trial III: MC 8 TS/MC 7 TS)	70.9 (Trial III: MC 8 TS/MC 6 LG)
62.2 (Trial III: MC 6 LG/MC 5 LG)	58.4 (Trial III: MC 8 TS/MC 5 LG)
58.1 (Trial III: MC 4 DS/MC 3 DS)	48.4 (Trial III: MC 8 TS/MC 4 DS)
	54.9 (Trial III: MC 8 TS/MC 3 DS)
	80.1 (Trial III: MC 7 TS/MC 6 LG)
	58.3 (Trial III: MC 7 TS/MC 5 LG)
	47.9 (Trial III: MC 7 TS/MC 4 DS)
	61.0 (Trial III: MC 7 TS/MC 3 DS)
	48.7 (Trial III: MC 6 LG/MC 4 DS)
	55.2 (Trial III: MC 6 LG/MC 3 DS)
	66.7 (Trial III: MC 5 LG/MC 4 DS)
	57.9 (Trial III: MC 5 LG/MC 3 DS)

An initial normality tests and ANOVA comparing BR with MR revealed that Dice similarity values involving sample MC 4b were biased by the failure to recover any bands in this sample. Therefore, all data points involving MC 4b were excluded from the final analysis as outliers. Additionally, the replicates of MC 1, the sterile control were also removed owing to low band recovery. This meant that in effect, the ANOVA comparisons for data collected during Trial II only included samples extracted from biologically active treatments. The data that has been excluded is highlighted in yellow in the above list of data.

2. Test of normality of data sets and subsequent transformations

Data sets (excluding outliers) were checked for normality using the Anderson Darling test (data is normally distributed where $P > 0.05$); the results were:

BR: P= 0.24

MR: P= 0.062

DT: P = 0.017

The test showed that BR and MR were normally distributed, but DT was not ($P < 0.05$). A BOX-COX test was completed to determine the optimal transformation using a power relationship, which indicated that the original data should be raised to the power 1.5.

Transformed data sets:

BR (^1.5, normality test P = 0.46):

591.948
74.466
818.488
888.194

MR (^1.5, normality test P = 0.27):

209.731
340.902
561.980
547.191
431.474
396.817
410.118
368.506
46.332
73.836
636.572
490.553
442.858

DT (^1.5, normality test P = 0.10):

230.559
111.024
307.927
339.855
210.623
20.952
133.340
145.787
309.955
36.981
298.856
276.089
285.913
53.510
424.691
431.474
175.112
18.919
304.893
291.863
206.176
26.105
405.668
378.227
215.101
26.551
379.312
378.227

The other data sets, described below, did not require transformation:

Acetate treatments, normality test: P= 0.26

Glucose treatments, normality test: P= 0.13

Acetate & autotrophic similarity, normality test, P = 0.13

Glucose & autotrohpic similarity, normality test, P= 0.63

Within sandstones, normality test, P = 0.35

Between sandstones, normality test, P = 0.24

3. MINITAB outputs for ANOVA

BR compared to MR (transformed)

MTB> One-way ANOVA: BR^1.5, MR^1.5

```
Source  DF      SS      MS     F      P
Factor   1  139351  139351  2.81  0.113
Error   16  794300   49644
Total   17  933652

S = 222.8   R-Sq = 14.93%   R-Sq(adj) = 9.61%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N   Mean  StDev  ---+---------+---------+---------+------
BR^1.5   5  577.7  320.8          (--------------*-------------)
MR^1.5  13  381.3  178.6  (-------*--------)
                          ---+---------+---------+---------+------
                           300       450       600       750

Pooled StDev = 222.8
```

RESULT: There is no significant difference between BR and MR, P > 0.05.

BR^1.5 mean = 577.7, back-transformed to: 69.35

MR^1.5 mean = 381.3, back-transformed to: 52.58

(Residuals are normally distributed)

MR compared to DT (transformed)

MTB>One-way ANOVA: MR^1.5, DT^1.5

```
Source  DF      SS      MS     F      P
Factor   1  204795  204795  9.11  0.004
Error   39  876550   22476
Total   40 1081345

S = 149.9   R-Sq = 18.94%   R-Sq(adj) = 16.86%

                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level    N   Mean  StDev  --------+---------+---------+---------+-
MR^1.5  13  381.3  178.6                (----------*---------)
DT^1.5  28  229.4  135.3  (------*------)
                          --------+---------+---------+---------+-
                                240       320       400       480

Pooled StDev = 149.9
```

RESULT: There is a significant difference between MR and DT, P < 0.05.

BR^1.5 mean = 381.3, back-transformed to: 52.58

DT^1.5 mean = 229.4, back-transformed to: 37.47

(Residuals are normally distributed)

Similarity between acetate treatments compared to glucose treatments

MTB>One-way ANOVA: Ac, Glu-outliers

```
Source  DF    SS   MS     F      P
Factor   1    24   24  0.10  0.763
Error    7  1704  243
Total    8  1728

S = 15.60   R-Sq = 1.39%   R-Sq(adj) = 0.00%


                                 Individual 95% CIs For Mean Based on
                                 Pooled StDev
Level          N   Mean  StDev  ----+---------+---------+---------+-----
Ac             6  68.17  13.55          (------------*-----------)
Glu-outliers   3  64.70  19.84  (-----------------*-----------------)
                                 ----+---------+---------+---------+-----
                                    48        60        72        84

Pooled StDev = 15.60
```

RESULT: There is no significant difference between similarities within acetate treatments, compared to the similarities between glucose treatments

Acetate mean = 68.17

Glucose mean = 64.70

(Residuals are normally distributed)

Similarity between Acetate and autotrophic control compared to glucose with autotrophic control

One-way ANOVA: Ac/auto, glu/auto

```
Source  DF      SS      MS      F      P
Factor   1  123.61  123.61  21.26  0.006
Error    5   29.07    5.81
Total    6  152.69

S = 2.411   R-Sq = 80.96%   R-Sq(adj) = 77.15%


                           Individual 95% CIs For Mean Based on Pooled
                           StDev
Level      N    Mean  StDev    +---------+---------+---------+---------
Ac/auto    4  14.975  2.941    (------*-------)
glu/auto   3  23.467  1.250                       (--------*--------)
                           +---------+---------+---------+---------
                          12.0      16.0      20.0      24.0

Pooled StDev = 2.411
```

RESULT: There is a significant difference between similarities of acetate/auto treatments, compared to the similarities between glucose/auto treatments

Acetate/auto mean = 14.98

Glucose/auto mean = 23.47

(Residuals are normally distributed)

Similarity within sandstone types compared to similarity between sandstone types

MTB>One-way ANOVA: Within sandstone, Between sandstone

```
Source  DF      SS    MS     F      P
Factor   1    78.9  78.9  0.88  0.364
Error   13  1161.4  89.3
Total   14  1240.2

S = 9.452   R-Sq = 6.36%   R-Sq(adj) = 0.00%


                                    Individual 95% CIs For Mean Based on
                                    Pooled StDev
Level              N    Mean  StDev  --+---------+---------+---------+-------
Within sandstone   3  64.767  8.255    (-------------------*-------------------)
```

```
Between sandston  12  59.033  9.653     (---------*---------)
                                      --+---------+---------+---------+-------
                                      54.0      60.0      66.0      72.0

Pooled StDev = 9.452
```

RESULT: There is no significant difference between similarities within sandstones, compared to the similarities between sandstones

Within sandstone 64.77

Glucose/auto mean = 59.03

(Residuals are normally distributed)