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STUDY OF DENITRIFICATION AND REDUCTIVE DECHLORINATION PROCESSES APPLIED TO GROUNDWATER BIOREMEDIATION

Ph.D. thesis

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RESUM

L'aigua subterrània constitueix un dels principals recursos d'aigua dolça del nostre planeta. I, de fet, en moltes zones del món ha esdevingut un recurs primari per a la industrialització, la irrigació de zones agrícoles i el consum humà. Tot i així, al llarg de les últimes dècades, la industrialització massiva i l'agricultura intensiva han provocat la seva gradual contaminació i han comportant, en molts casos, la inutilització d'aquest recurs d'aigua a curt, mitjà i llarg termini.

Actualment, la bioremediació *in situ* és una de les tecnologies més prometedores per a l'eliminació de contaminants en aigües subterrànies. Es basa en l'ús de processos microbiològics de degradació dels contaminants. Molts d'aquests processos degradatius ja ocorren a les aigües subterrànies de manera natural, però generalment acostumen a estar limitats per alguna mancança en el medi. L'optimització i acceleració d'aquests processos naturals és el que es coneix amb el nom de bioremediació i pot donar lloc a la possibilitat d'eliminar o reduir la contaminació fins a uns nivells acceptables en un temps raonable. Tot i així, en molts països, l'aplicació de tecnologies de bioremediació *in situ* és encara inexistent o molt limitada. Aquest fet es deu a la complexitat de la tecnologia, que requereix integrar conceptes microbiològics, hidrològics i químics, entre d'altres.

La present tesi es basa en l'estudi dels processos de bioremediació com a tecnologies de descontaminació d'aqüífers. Concretament, es pretén estudiar la desnitrificació i la decloració reductiva com a tecnologies de bioremediació per eliminar, respectivament, els nitrats i els hidrocarburs alifàtics clorats (o CAHs, de *chlorinated aliphatic hydrocarbons*) de les aigües subterrànies contaminades. A més a més, es pretén aplicar tecnologies avançades que permetin millorar en el coneixement d'aquests processos.

L'aqüífer associat a la riera d'Argentona, situat a la mateixa localitat d'Argentona (Catalunya, Espanya), ha estat la zona d'estudi per tal d'investigar el procés de desnitrificació. En una primera part, s'han dut a terme experiments en *batch* amb aigua subterrània i sòl subsuperficial del mencionat aqüífer. A partir d'aquests primers estudis, s'ha observat la baixa capacitat de l'aqüífer per eliminar els nitrats de manera natural, però alhora s'ha vist la viabilitat d'aplicar un procés de bioremediació com és l'addició de matèria orgànica. Paral·lelament, s'ha estudiat la influència de diferents factors com ara la presència d'oxigen i la tipologia de donador d'electrons sobre el procés de desnitrificació.

Posteriorment, s'ha desenvolupat un model matemàtic per descriure el consum d'oxigen, de nitrats i de matèria orgànica per part de la població microbiana facultativa i heterotròfica present en el material d'aqüífer. Alguns paràmetres del model han estat calibrats i s'ha estudiat la qualitat d'aquests paràmetres. El model desenvolupat constitueix una primera aproximació per tal d'obtenir un model de desnitrificació *in situ*.

Per tal d'avançar en l'estudi del procés de desnitrificació en condicions naturals, s'han realitzat experiments en dinàmic simulant el flux d'aigua subterrània a través de l'aqüífer. L'eficiència d'injectar matèria orgànica en aquestes condicions s'ha demostrat. Paral·lelament, s'han estudiat els efectes hidrodinàmics de l'aplicació de la bioremediació i, els resultats han demostrat la importància de dissenyar acuradament les tecnologies de bioremediació a escala de camp. Per altra banda, s'ha descrit la desnitrificació en condicions dinàmiques integrant en un model matemàtic les reaccions bioquímiques i els processos de transport que tenen lloc a la columna experimental.

Finalment, s'han aplicat les noves tecnologies de biologia molecular per entendre els efectes de l'aplicació d'un procés de bioremediació a nivell microbià. Per una banda, l'aplicació de la tècnica de la reacció en cadena de la polimerasa a temps real (o real-time PCR, de *real-time polymerase chain reaction*) ha demostrat el creixement de la població microbiana i, concretament, de la població desnitrificant en el material d'aqüífer estimulat. Per altra banda, l'electroforesi en gel de gradient desnaturalitzant (o DGGE, de *denaturing gradient gel electrophoresis*) ha permès investigar els canvis en la població microbiana indígena del material d'aqüífer a causa de l'estimulació amb matèria orgànica.

Amb l'objectiu d'avançar en el coneixement dels processos de bioremediació en aigües subterrànies, s'ha estudiat també la decloració reductiva de CAHs. En aquest cas, s'han aplicat metodologies experimentals destinades a l'estudi de la possible aplicació d'una barrera reactiva permeable per eliminar una ploma que conté majoritàriament *cis*-1,2-dicloroetilè i clorur de vinil, i que flueix cap al riu Zenne, prop de la ciutat de Brussel·les (Bèlgica).

L'estudi ha inclòs experiments en *batch* per tal d'investigar el potencial degradatiu del material d'aqüífer i dels propis sediments del riu Zenne. Així mateix, també s'han dut a terme experiments en columna que simulaven el flux d'aigua subterrània a través dels sediments del riu o del material d'aqüífer. Els resultats han demostrat el gran potencial degradatiu dels sediments, que a la llarga es podrien potenciar com a biobarrera natural del sistema per tal de prevenir que les aigües contaminades arribin a l'aigua superficial del riu Zenne.

ABSTRACT

Groundwater constitutes one of the main resources of freshwater in our planet. Actually, in many world areas, it has become a primary resource for industrialization, irrigation of agricultural zones and human consumption. Nevertheless, throughout the last decades, massive industrialization and intensive agriculture have caused gradual groundwater contamination limiting the use of this important resource in many world areas.

Nowadays, *in situ* bioremediation is one of the most promising technologies to remove contaminants from groundwater. It is based on the use of microbial degradation processes that mostly occur naturally in groundwater, but they are usually limited by environmental conditions. Optimization and acceleration of these natural processes is known as bioremediation and it can lead to the possibility to eliminate or reduce contaminants up to acceptable levels in a reasonable time. Nevertheless, in many countries the application of *in situ* bioremediation technologies is still non-existent or very limited. This is due to the complexity of the technology, which requires the integration of microbiological, hydrological and chemical concepts, among others.

This thesis is based on the study of bioremediation processes as reliable technologies to remove contaminants from groundwater. Specifically, it is aimed to study denitrification and reductive dechlorination as bioremediation technologies to remove nitrates and chlorinated aliphatic hydrocarbons (CAHs), respectively, from polluted groundwater. In addition, it is aimed to apply advanced technologies which allow improving on the knowledge of these processes.

The aquifer associated to the Stream Argentona, located in Argentona, Catalunya (Spain), was selected as study site to investigate the denitrification process. In the first part, microcosm experiments containing groundwater and subsoil from the aquifer were performed. From these first studies it was observed the low capacity of the aquifer to eliminate nitrates under natural conditions, but, at the same time, it was noted the feasibility of applying a bioremediation process such as the addition of organic matter. In addition, the influence of different factors such as the presence of oxygen and the type of electron donor on the denitrification process was studied.

Afterwards, a mathematical model was developed in order to explain the microbiological processes that occur when stimulating the aquifer material with an organic carbon source. The model could successfully explain the consumption of oxygen, nitrates and organic matter by the indigenous facultative heterotrophic microbial population from aquifer. Some parameters of the model were calibrated from experimental data and the quality of these parameters was investigated. The developed model constitutes a first approach in order to have reliable models for *in situ* denitrification.

In order to advance in the study of the denitrification process in natural conditions, dynamic experiments were carried out simulating the groundwater flow through the aquifer. The efficiency of injecting organic matter under these conditions was demonstrated. At the same time, hydrodynamic effects of the process were observed, indicating the importance to design properly bioremediation technologies before its application in field-scale. Furthermore, an integrated model coupling the biochemical reactions and the transport processes inside the column was developed and applied to describe denitrification under dynamic conditions.

Finally, molecular microbiological techniques were applied to investigate microbial changes due to the application of enhanced denitrification. On the one hand, real-time polymerase chain reaction (real-time PCR) technique revealed the growth of microbial population, specially of denitrifying bacteria in aquifer material stimulated with an organic carbon source. On the other hand, denaturing gradient gel electrophoresis (DGGE) method allowed to investigate changes in the indigenous microbial community due to the amendment with organic matter.

In order to advance in the knowledge of bioremediation processes in groundwater, reductive dechlorination of CAHs in groundwater was studied. In this case, experiments at laboratory scale were applied, aimed to study the possible application of a permeable reactive barrier (PRB) to eliminate a CAH-contaminated plume, containing basically *cis*-1,2-dichloroethene and vinyl chloride, which flows to the River Zenne near Brussels, Belgium.

The study included batch experiments in order to investigate the degradation potential in aquifer and sediments of River Zenne, as well as column experiments which simulated the groundwater flow through the sediments of the river or the aquifer material. The results demonstrated the high degradation potential of the sediments, which in the long term could be enhanced to act as a natural biobarrier of the system in order to prevent groundwater contaminants from arriving at the surface water of the River Zenne.

MAIN ABBREVIATIONS

ASM1	Activated Sludge Model No. 1
CA	Chloroethane
CAHs	Chlorinated aliphatic hydrocarbons
cis-DCE	*cis*-1,2-dichloroethene
Ct	Threshold cycle
C/N	Carbon-to-nitrogen ratio
1,1-DCA	1,1-dichloroethane
DGGE	Denaturing gradient gel electrophoresis
DIC	Dissolved inorganic carbon
d.m.	Dry matter
DNA	Deoxyribonucleic acid
DNAPL	Dense non-aqueous-phase liquid
DNRA	Dissimilatory nitrate reduction to ammonia
DO	Dissolved oxygen
DOC	Dissolved organic carbon
FIM	Fisher information matrix
GC	Gas cromatography
HPLC	High performance liquid cromatrography
ICP	Inductively Coupled Plasma
ISB	*In situ* bioremediation
MPN	Most probable number
narG	nitrate reductase gene
nosZ	nitrous oxide reductase gene
NTC	No-template-control
OC	Organic carbon
OF	Objective function
OM	Organic matter content
ORP	Oxidation-reduction potential
PCE	Tetrachloroethene
PCR	Polymerase chain reaction
PRB	Permeable reactive barrier
qPCR	Quantitative PCR
RSD	Relative standard deviation
1,1,1-TCA	1,1,1-trichloroethane
TCE	Trichloroethene
VC	Vinyl chloride

NOMENCLATURE

A	Cross section area
$\Delta h/\Delta t$	Hydraulic gradient
b_H	Heterotrophic decay rate constant
D	Dispersion coefficient
ε	Effective porosity
foc	Fraction of organic carbon content
J_w	Flow rate
K	Hydraulic conductivity
K_b	Biomass inhibition coefficient
Kd	Distribution coefficient
K_{NO3}	Saturation coefficient for nitrate
$K_{NO3,I}$	Inhibition coefficient for nitrate
K_{NO2}	Saturation coefficient for nitrite
K_{O2}	Saturation coefficient for oxygen
$K_{O2,I}$	Inhibition coefficient for oxygen
K_{OC}	Saturation coefficient for organic carbon
KOC	Organic-carbon/water partitioning coefficient
η	Reduction factor for denitrification
t_d	Dead time
μ	Specific growth rate of a microbial population
$\mu_{max,H}$	Heterotrophic maximum growth rate
V	Pore volume
V_d	Dead volume
v_{H2O}	Porus velocity
X_H	Heterotrophic biomass concentration
$X_{H,0}$	Initial heterotrophic biomass concentration
α	Dispersivity
Y_H	Heterotrophic growth yield

Chapter 1

GENERAL INTRODUCTION

1. GROUNDWATER QUALITY

The European Union Water Framework Directive (Directive 2000/60/CE) defines groundwater as all the water which is below the surface of the ground in the saturation zone and in direct contact with the ground or subsoil. In addition, aquifer is defined as a subsurface layer or layers of rock or other geological strata of sufficient porosity and permeability to allow either a significant flow of groundwater or the extraction of significant quantities of groundwater.

Of the total water available worldwide, estimates are that only 2.5% is freshwater, which represents a global volume of 35.2 million km^3. From these, 30.1% is considered to be groundwater (10.6 million km^3). Therefore, groundwater constitutes one of the main resources of freshwater, providing useful functions and services to humans and the environment. On the one hand, it feeds springs and streams, supports wetlands and maintains land surface stability in areas of unstable ground. On the other hand, in many world areas, groundwater constitutes a major source of drinking water as well as an important resource to support agriculture and industry (UNESCO, 2006).

The Groundwater Directive (Directive 2006/118/CE) considers groundwater as the largest body of freshwater in the European Union, but also the most sensitive as well. The sources of groundwater contamination are many and diverse because, in addition to natural processes, practically every type of facility or structure installed by man, and each and every human physical activity, may eventually cause groundwater quality problems (Zaporozec, 2002). Throughout the world, most practices of urbanization, industrial development, agricultural activities and mining enterprises have caused groundwater contamination, limiting the use of this important resource.

2. GENERAL OVERVIEW OF BIOREMEDIATION

Bioremediation and natural attenuation (also referred as intrinsic bioremediation) are remediation technologies that rely on stimulated or natural degradation processes to clean up contaminants of concern. Basically, these technologies meld an understanding of microbiology, chemistry, hydrogeology and engineering into a cohesive strategy to control microbial degradation of specific organic or inorganic compounds (ITRC, 2002).

Groundwater bioremediation methods can be divided into *ex situ*, *on site* (i.e. pump and treat) and *in situ* remediation technologies. *Ex situ* and *on site* bioremediation methods are generally costly and difficult due to extraction of contaminated water from subsurface, transport to

treatment facilities (*ex situ* technology), treatment and recharging the underground. These disadvantages have led to an increasing interest in applying *in situ* bioremediation (ISB) technologies as more viable, environmentally friendly and economic technologies for the restoration of the polluted subsurface environment. When the rate of natural attenuation is too slow, ISB implies the creation of proper subsurface environmental conditions to allow microorganisms the degradation of contaminants. Therefore, the basic premise of bioremediation is to accelerate microbial activity by optimizing their environmental conditions.

Microorganisms have the natural capacity to degrade or destroy a wide range of contaminants by a diversity of enzymatic processes. They obtain energy by catalyzing oxidation-reduction reactions, where an organic or inorganic compound, termed electron donor, is oxidized (i.e. electrons are lost) and another chemical compound, termed electron acceptor, is reduced (i.e. electrons are gained). Engineered bioremediation strategies include: the addition of electron donors or acceptors to stimulate the growth of microorganisms involved in the bioremediation processes, the addition of nutrients that limit the growth of the microorganisms and/or the addition of microorganisms with the desired bioremediation capabilities, also known as bioaugmentation (Lovley, 2003).

One common type of bioremediation is the oxidation of toxic, organic contaminants to harmless products, often carbon dioxide (CO_2). Therefore, these contaminants serve as electron donors for microorganisms. Furthermore, oxygen is the most commonly used electron acceptor for microbial respiration. This process is known as aerobic degradation and it has been studied in detailed for an extensive range of organic contaminants, from aromatic hydrocarbons, such as benzene, to xenobiotics, such as pesticides (e.g. Mirgain *et al.*, 1995; Pruden *et al.*, 2003). Nevertheless, many polluted aquifers are often anoxic (i.e. lacking oxygen), and anaerobically oxidation of many contaminants using alternative electron acceptors such as nitrate, sulphate and Fe(III) oxides has also been reported (e.g. Cunningham *et al.*, 2001; Reinhard *et al.*, 2005).

Some contaminants serve as electron acceptors rather than electron donors in bioremediation reactions. In the present thesis, two main examples of this type of bioremediation are considered: the denitrification process, in which microorganisms reduce nitrate to the innocuous nitrogen gas by using an organic or inorganic compound as electron donor, and the reductive dechlorination, in which microorganisms remove chlorines from chlorinated aliphatic hydrocarbons (CAHs) by using these compounds as electron acceptors in their respiration processes.

3. STUDY CASES

As described above, two main contaminants in groundwater are studied in this thesis: nitrate and CAHs. It is important to mention that both contaminants have become one of the main threats of groundwater resources in industrialized world. In this section, characteristics of both types of contaminants are provided. In addition, remediation options for these contaminants, specially bioremediation, are described.

3.1. NITRATE CONTAMINATION

Since the 1970s, nitrate contamination of groundwater has become a significant environmental problem, with many parts of the world now reporting nitrate pollution in the subsurface environment (e.g. Liu *et al.*, 2005; Saadi and Maslouhi, 2003). In particular, in Europe it is estimated that around one third of groundwater bodies exceeds the nitrate guideline value (Directive 91/676/EEC).

3.1.1. The nitrogen cycle

Nitrogen can form a variety of compounds due to the different oxidation states it can assume. In the environment, most changes from one oxidation state to another are mediated by microorganisms. The movement and transformation of the different nitrogen compounds through the biosphere is described by the nitrogen cycle, a general diagram of which is provided in Figure 1.1.

The atmosphere constitutes the main reservoir of nitrogen in the environment, where nitrogen exists as nitrogen gas (N_2) (the atmosphere is 79% N_2). Fixation is the process in which the inert nitrogen gas is incorporated into a chemical compound so that it can be used by plants and animals. Fixation of nitrogen gas to organic nitrogen is predominantly accomplished by a limited number of bacteria that tend to be symbiotic with plants such as legumes. Moreover, high-energy natural events such as lightning can cause the fixation of smaller but significant amounts of N_2.

The conversion of organic nitrogen to the ammonium form (NH_4^+) is termed ammonification or mineralization. In general, this process occurs during decomposition of animal and plant tissue and animal faecal matter. On the contrary, immobilization or assimilation is the biochemical mechanism that converts mobile nitrogen compounds (mainly ammonium or nitrate) into

organic nitrogen to form plant protein and other nitrogen-containing compounds. In addition, ammonium in soil can be lost to water by leaching, can be adsorbed by clay minerals and/or organic matter and can be biological oxidized in the nitrification process. Nitrification is done in two steps, first ammonium is oxidized to nitrite (NO_2^-), and then nitrite is oxidized to nitrate (NO_3^-). Two specific autotrophic bacterial groups are involved in these processes, the ammonium oxidizing bacteria and the nitrite oxidizing bacteria, using inorganic carbon as their source of cellular carbon.

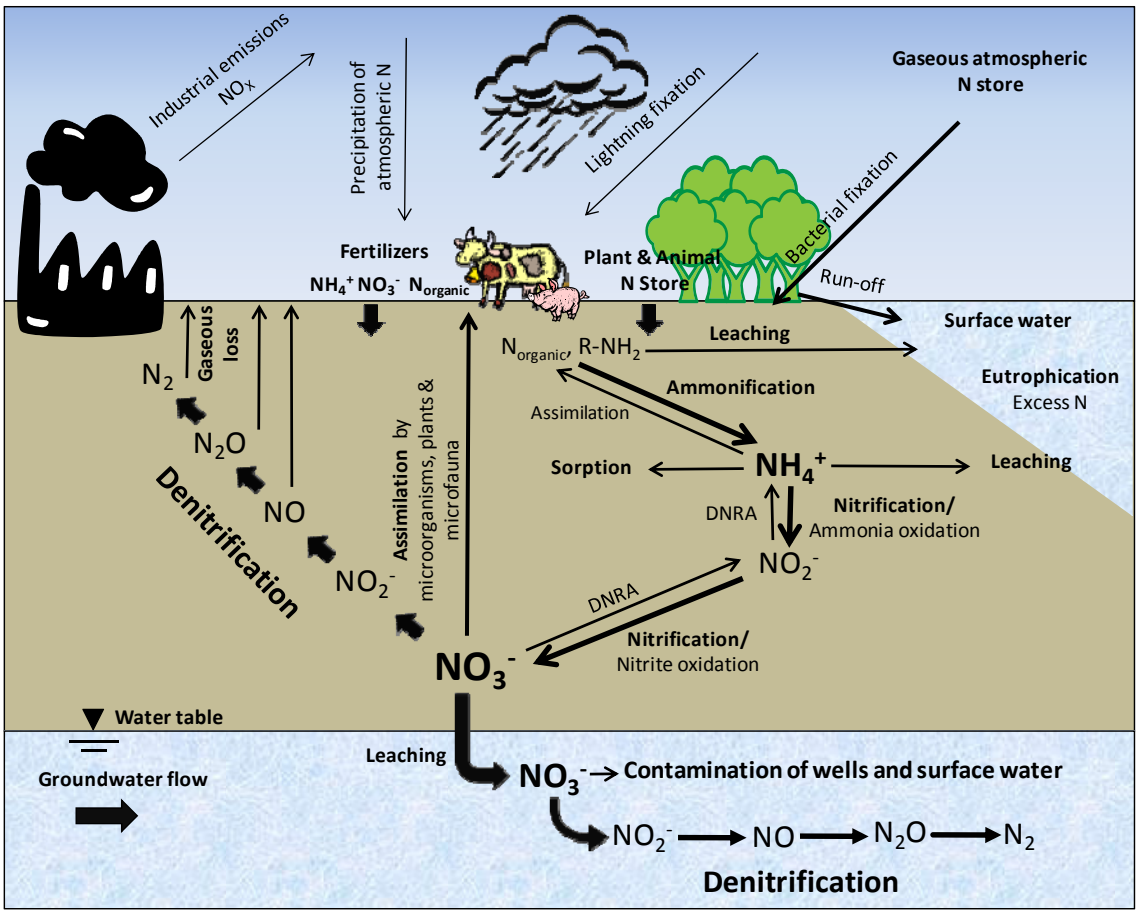

Figure 1.1. Schematic diagram of the nitrogen cycle and its influence upon the water environment. Adapted from Rivett *et al.* (2008).

Nitrate has a high solubility and potential for loss to groundwater by leaching. Once in the subsurface environment, nitrate most likely undergoes denitrification depending on the properties and prevailing conditions and migrates via advection and dispersion (Almasri, 2007; ITRC, 2002). Denitrification is the biological process that closes the nitrogen cycle, by reducing nitrate back to nitrogen gas under mostly anoxic conditions. It is not a direct conversion but a multistep process as described in Figure 1.1. Nitrate may also undergo dissimilatory nitrate reduction to ammonia (DNRA). DNRA is an anaerobic reduction reaction mediated by

fermentative bacteria. Generally, DNRA is favoured when nitrate is limiting and denitrification is favoured when electron donor is limiting (Kelso *et al.*, 1997; Korom, 1992).

The human activity has severely altered the nitrogen cycle. Some of the main alterations are due to the excessive use of fertilizers in crops, livestock, sewage waste and septic tanks, which increase the ammonia content and, in turn, the nitrate concentration in the soil and the hydrologic systems through leaching, groundwater flow and run-off. The nitrogen cycle is also modified by the fuel fossil combustion and forest burning increasing the global concentration of nitrous oxide (N_2O) and nitric oxide (NO) in the atmosphere. This favours the global warming, the ozone layer depletion and the atmospheric deposition of nitrogen.

In surface aquatic systems nitrogen pollution can lead to eutrophication, a nutrient-over enrichment which causes algal blooms, fish-kill events and species shifts. Moreover, nitrate and nitrite in drinking water have adverse effects on human health. Methaemoglobinemia, also known as blue baby syndrome, is a serious illness due to the conversion of nitrate to nitrite by the human body, which can interfere with the oxygen-carrying capacity of the blood. This illness is specially problematic in children. In addition, it seems that high nitrate levels in drinking water could be related to cancer (Wolfe and Patz, 2002).

3.1.2. Regulation and guidelines regarding nitrate and nitrite

The European Union and the World Health Organization (WHO) have both set the standard for nitrate in drinking water at 50 $mg \cdot L^{-1}$ (Directive 98/83/EC; WHO, 2004). The Nitrates Directive (Directive 91/676/EEC) requires protection of all natural freshwater and sets the same limit of 50 $mg \cdot L^{-1}$ nitrate, which applies to all groundwater regardless of its intended use, even though it is recognized that much lower nitrate concentrations, possible around 4.4-8.8 $mg \cdot L^{-1}$, may trigger eutrophication in surface waters (James *et al.*, 2005).

With regard to nitrite, a guideline value of 3 $mg \cdot L^{-1}$ has been established for short-term exposures, whereas a threshold of only 0.2 $mg \cdot L^{-1}$ is recommended for long-term exposures (WHO, 2004).

3.1.3. Nitrate remediation options

Different chemical, physical, physico-chemical and biological treatments have been developed to remove nitrate from water. Until now, the utility of conventional methods such as reverse osmosis, ion exchange and electrodialysis have been limited because they are expensive and

merely displace nitrate into a concentrated waste that may pose a disposal problem (e.g. Shrimali and Singh, 2001; Soares, 2000).

Biological denitrification is an important alternative, since it constitutes the only selective process for removing nitrate and transforming it into harmless nitrogen gas. As described in the nitrogen cycle, denitrification occurs naturally in the environment, when certain bacteria use nitrate as terminal electron acceptor in their respiratory process in the absence of oxygen. The denitrification reaction is carried out by a diversity of bacteria belonging taxonomically to the various subclasses of the *Proteobacteria*. Denitrifiers are common among the Gram-negative bacteria, such as *Pseudomonas*, *Alcaligenes*, *Paracoccus* and *Thiobacillus*, but also some Gram-positive bacteria, including *Bacillus*, can denitrify. Furthermore, some fungi and Archaea are also capable of denitrifying (Rittmann and McCarty, 2001; Zumft, 1997; Shoun, 1992). Due to this high taxonomic diversity, denitrifiers can be found in every sort of environmental niche (Gamble *et al.*, 1977), ensuring a potential for denitrification in most habitats. However, this process usually occurs at very low rates in the environment, generally due to the lack of suitable electron donors. Therefore, bioremediation of nitrate (usually referred as enhanced denitrification) consists in the provision of suitable electron donors which may be organic or inorganic compounds.

3.2. CHLORINATED ALIPHATIC HYDROCARBONS CONTAMINATION

CAHs such as tetrachloroethene (PCE, also known as perchloroethene), trichloroethene (TCE) and trichloroethane (TCA) are another type of contaminants which represent a threat for the quality of groundwater. In this section besides the statement of the main characteristics of CAHs compounds, an overview of degrading processes and remediation options is provided.

3.2.1. CAHs characteristics

CAHs, a family of compounds commonly used as solvents, constitute one of the most prevalent groundwater contaminants in industrialized areas (e.g. Stroo *et al.*, 2003). Since 1930s, chlorinated ethenes and ethanes such as PCE, TCE and 1,1,1-trichloroethane (1,1,1-TCA) have been widely used in industrial processes, mainly as cleaning solvents in dry-cleaning operations or as degreasing agents for metal surfaces (Rivett *et al.*, 2001; Vogel *et al.*, 1987). The improper storage, handling and disposal practices resulted in widespread groundwater contamination. These solvents are dense non-aqueous-phase liquids (DNAPL) able to penetrate deep below the water table where they slowly dissolve into the groundwater to

form long-living polluted plumes. These plumes may attain lengths of several kilometres due to the intrinsic characteristics of chlorinated solvents: low sorption, limited chemical reaction and often low biodegradation potential (Jackson, 1998; Mackay and Cherry, 1989). Table 1.1 summarizes the chemical and physical characteristics of some CAHs.

Table 1.1. Chemical and physical properties of the CAH compounds: PCE, TCE, *cis*-1,2-dichloroethene (*cis*-DCE), vinyl chloride (VC) and 1,1,1-TCA. Data from USEPA (1995).

Parameters	PCE	TCE	*cis*-DCE	VC	1,1,1-TCA
Formula	C_2Cl_4	C_2HCl_3	$C_2H_2Cl_2$	C_2H_3Cl	$C_2H_3Cl_3$
Molecular weight ($g \cdot mol^{-1}$)	165.8	131.4	96.9	62.5	133.4
Water solubility at 25°C ($g \cdot L^{-1}$)	0.2	1.0	3.5	2.7	4.4
Melting point (°C)	-19	-73	-80	-154	-30
Boiling point (°C)	121	87	60	-14	74
Density at 20°C ($g \cdot mL^{-1}$)	1.62	1.47	1.26	0.91	1.34
Henry's law constant ($atm \cdot m^3 \cdot mol^{-1}$)	$1.3 \cdot 10^{-2}$	$1.0 \cdot 10^{-2}$	$3.4 \cdot 10^{-3}$	$5.6 \cdot 10^{-2}$	$8.0 \cdot 10^{-3}$
log K_{oc} [a]	2.32-2.38	2	1.56-1.69	1.75	1.91-1.95
log K_{ow} [b]	3.40	2.29	1.86	0.60	2.49
MCL ($\mu g \cdot L^{-1}$) [c]	5	5	70	2	200

[a] Water-solid partitioning coefficient normalized to organic carbon content.
[b] Octanol-water partition coefficient.
[c] Maximum Contaminant Level (MCL): maximum concentration in drinking water systems.

Contamination of the groundwater with CAHs poses serious health problems due to the toxic and carcinogenic nature of these compounds (Vogel *et al.*, 1987). Therefore, stringent drinking water standards have been set in the low $\mu g \cdot L^{-1}$ range (Table 1.1).

3.2.2. Microbial degradation and abiotic transformation of CAHs

CAHs can be transformed in the subsurface through a range of biotic and abiotic processes. In terms of microbial metabolism, three major routes of CAHs transformation can be differentiated: (1) when these contaminants act as electron donor, (2) when they are transformed co-metabolically and (3) when they act as electron acceptor. An overview of these different processes together with abiotic transformations is provided below.

Electron donor reactions

In this type of reaction, microorganisms use the CAHs as energy and carbon source. It is reported that resistance of CAHs to act as electron donor and undergo oxidation increases with the number of chlorine substituents. Therefore, highly chlorinated compounds such as PCE and TCE tend to be recalcitrant under aerobic conditions, although degradation of both compounds under these conditions have been reported in some studies (Enzien *et al.*, 1994; Ryoo *et al.*, 2000; Sharma and McCarty, 1996). Concerning less chlorinated compounds, such as DCE and VC, several authors have demonstrated their oxidation under aerobic conditions; however, their oxidation under anaerobic conditions has also been reported. Aerobic oxidation of VC was demonstrated using a *Mycobacterium* isolated from a VC-contaminated soil (Hartmans and Debont, 1992) and a *Rhodococcus* isolated from a TCE-degrading mixed laboratory culture (Malachowsky *et al.*, 1994; Phelps *et al.*, 1991). In addition, Bradley and Chapelle (1998) reported significant mineralization of DCE and VC under aerobic, Fe(III)-reducing, sulphate-reducing and methanogenic conditions. However, their results corroborated that mineralization of both compounds was more significant under aerobic conditions.

Co-metabolism

When CAHs are degraded via co-metabolism, the degradation is catalyzed by an enzyme or cofactor that is fortuitously produced by the microorganism for other purposes. Therefore, for the co-metabolic process to occur, a growth-supporting substrate must be present, and the microorganism receives no known benefit from the degradation of the CAH. Co-metabolism of chlorinated compounds is best reported in aerobic environments (e.g. Semprini, 1997), although it can potentially occur under anaerobic conditions (Wiedemeier *et al.*, 1998).

Electron acceptor reactions (halorespiration)

Several anaerobic bacteria couple dechlorination of CAHs to the ATP synthesis with the halorespiration process (also referred as dehalorespiration or chloridogenesis). In this process, the CAH is used as an electron acceptor and a suitable electron donor such as hydrogen or an organic compound is required (El Fantroussi *et al.*, 1998; Holliger *et al.*, 1998; Smidt and de Vos, 2004).

Two main microbially mediated reductive dechlorination reactions are involved in halorespiration, hydrogenolysis and dichloroelimination. Hydrogenolysis, often simply known

as reductive dechlorination, involves the replacement of a chlorine substituent with an hydrogen atom. The reductive dechlorination of PCE to ethene proceeds through a series of hydrogenolysis reactions, where TCE, *cis*-DCE and VC are typical intermediates (Figure 1.2). In dichloroelimination two chlorines from adjacent carbons are removed and a double bond is formed. As indicated in Figure 1.2, dichloroelimination has been observed in some chlorinated ethanes (Figure 1.2) (Aulenta *et al.*, 2006; Ferguson and Pietari, 2000).

Several halorespiring bacteria of chlorinated ethenes have been isolated. These bacteria include members of different genus such as *Desulfitobacterium*, *Dehalobacter*, *Sulfurospirillum*, *Desulfuromonas*, *Dehalococcoides* and *Enterobacter* species (Holliger *et al.*, 1998; Smidt and de Vos, 2004). It is important to note that only members of the genus *Dehalococcoides* seem to be able to drive the dechlorination of VC to non-toxic ethene (Maymó-Gatell *et al.*, 1999). Reductive dechlorination of chlorinated ethanes has been reported for 1,1,1-TCA in *Dehalobacter* sp. strain TCA1 (Sun *et al.*, 2002) and for 1,2-dichloroethane (1,2-DCA) in *Desulfitobacterium dichloroeliminans* strain DCA1 (De Wildeman *et al.*, 2003).

Halorespiring microorganisms differ in their dechlorination abilities and electron donor requirements. Several strains are quite restrictive in terms of electron donor requirements, such as *Dehalobacter* and *Dehalococcoides,* which can only use hydrogen. Therefore, *in situ* bioremediation of CAHs is usually accomplished through the subsurface addition of selected carbon sources that can be fermented to hydrogen.

Abiotic transformations

Chloroethanes can undergo dehydrochlorination, an abiotic reaction involving the removal of a chlorine atom from one carbon and the simultaneous removal of a hydrogen atom from the adjacent carbon, resulting in the formation of a double bond. This reaction converts a chlorinated ethane into a less chlorinated ethene. An example of this process is the abiotic degradation of 1,1,1-TCA into 1,1-DCE (Figure 1.2) (Vogel *et al.*, 1987). Hydrolysis is another abiotic transformation that may undergo some chlorinated compounds. For example, chloroethane (CA) may undergo hydrolysis to ethanol (Figure 1.2) (Vogel *et al.*, 1987). However, it is known that most abiotic transformations are slow compared to biological reactions, provided that appropriate conditions are present such as sufficient substrate, nutrients and suitable microbial populations (Aulenta *et al.*, 2006; Vogel *et al.*, 1987).

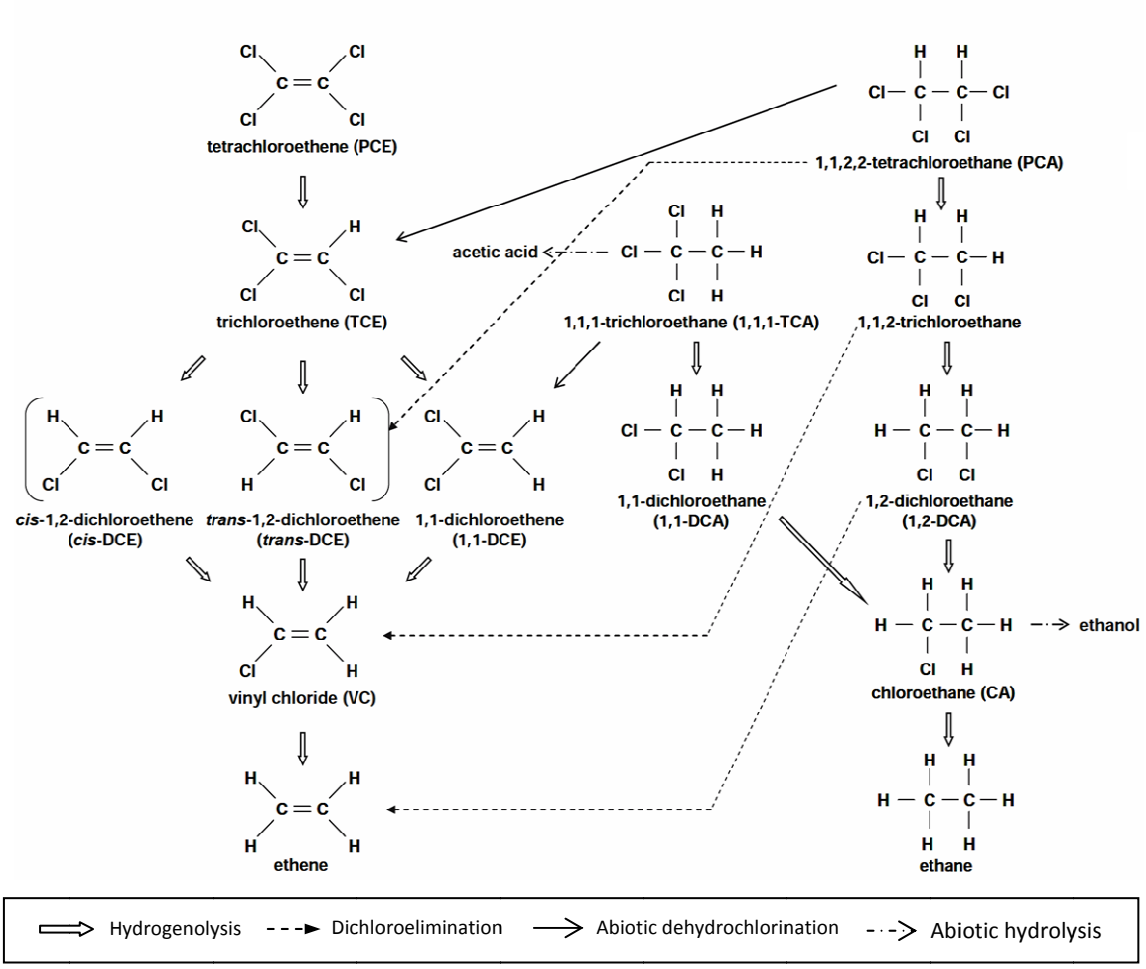

Figure 1.2. Reported anaerobic pathways for degradation of PCE, PCA and 1,1,1-TCA via biotic hydrogenolysis or dichloroelimination and abiotic dehydrochlorination or hydrolysis. From Hamonts (2009).

3.2.3. CAHs remediation options

CAHs are among the most difficult contaminants to clean up, particularly when their DNAPL source remains in the subsurface. Innovative *in situ* technologies, such as alcohol flushing, thermal technologies, oxidation and bioremediation have been developed recently (Stroo, *et al*., 2003). However, these technologies have not been thoroughly evaluated and, therefore, research is clearly needed to better understand them.

Bioremediation, mainly through reductive dechlorination, has received considerable attention as a reliable and cost-effective strategy for the removal of chlorinated solvents in groundwater. Over the last years, research has suggested that the transformation of CAHs into harmless non-chlorinated end-products can be practically achieved by enhancing bacterial dechlorination reactions in the field. Enhanced *in situ* reductive dechlorination has been

successfully applied for remediation of chlorinated solvent-contaminated sites (e.g. Major *et al.*, 2002).

As previously mentioned, reductive dechlorination is studied in the present thesis as a bioremediation mechanism to remove mainly *cis*-DCE and VC from polluted groundwater.

4. ADVANCED TOOLS AND TECHNIQUES FOR ASSESSING BIOREMEDIATION

Thus far in the literature, most bioremediation studies have been based on treatability tests, in which samples of contaminated environment were incubated in the laboratory and the rates of the contaminant degradation were documented. Such studies are necessary to provide an estimate of the potential metabolic activity of the microbial community but give little insights into the factors controlling the growth and metabolism of microorganisms involved in bioremediation processes.

Recent literature has pointed out the need to develop mathematical models to predict the activity of microorganisms during bioremediation processes as well as to design accurately bioremediation strategies. In addition, new technologies such as microbial molecular techniques have emerged complementing the existing methods to study microbial communities.

4.1. MODELLING BIOREMEDIATION PROCESSES

Modelling the transport and fate of pollutants in the subsurface environment is a great deal for the comprehensive implementation of ISB, since it permits to design properly bioremediation strategies and further predict the influence of input changes on the system. However, it requires describing not only the flow and direction of water movement but also the transformation of contaminants and other compounds which may influence on contaminant degradation and transport. Until now, feasible geochemical and hydrological models have been developed to simulate transport of contaminants in the subsurface. Obviously, microbial activity influences directly or indirectly the fate of contaminants and, as such, need to properly be included in these models. Therefore, firstly, the development of accurate models describing the microbial processes is required. This is why, in this thesis, the development of a microbial model describing the enhanced denitrification process in aquifer material was focused as a main point to study.

4.2. MOLECULAR TECHNIQUES

Historically, the assessment of microbial populations responsible for bioremediation reactions was based on traditional microbial analyses, which involved isolation and culture of specific microorganisms using their ability to grow on the pollutant of interest, followed by their identification based on morphological, physiological or metabolic traits. However, such culture dependent methods provided an unreliable representation of the microorganisms present in the environment, since it is known that most microorganisms are viable but not cultivable (e.g. Ward *et al.*, 1990).

Recently, technological advances in molecular biology have permitted a fast and a more reliable assessment of the type and abundance of microorganisms present in a polluted environment. Furthermore, such techniques may be used to obtain information about how these microorganisms respond to different environmental factors (Illman and Alvarez, 2009).

Deoxyribonucleic acid (DNA) is present in every independently living cell in order to translate the genetic information into working enzymes and other proteins. Molecular techniques rely on the principle that similar base sequences exist for genes that encode similar products in different microorganisms. Such techniques imply extracting DNA from environmental samples and assaying molecular markers (biomarkers). Mainly two different types of molecular markers are used in bioremediation studies. On the one hand, the most basic molecular target is the 16S rRNA, which enables to identify and quantify the presence of specific strains or groups of phylogenetically related microorganisms (e.g. identification and quantification of *Dehaloccoides* spp. involved in the reductive dechlorination of CAHs). On the other hand, functional genes encoding specific enzymes involved in bioremediation reactions may also be used as biomarkers (e.g. denitrifying genes involved in the denitrification pathway are used to study the denitrifying population).

A variety of molecular methods have been developed and applied to investigate microbial communities. In general, these approaches attempt to quantify the diversity or overall abundance of microbial populations, an overview of these methods is provided in Figure 1.3. As indicated, after DNA is extracted from environmental samples, it can be assayed directly by hybridization to gene probes. However, since the DNA of interest is often present at very low concentrations, most of the molecular techniques require DNA to be amplified first. This is accomplished by the Polymerase Chain Reaction (PCR), which implies amplifying the molecular markers further by using specifically designed oligonucleotide primers. The amplified material

(i.e. the PCR amplicon) is then available in sufficient quantity to investigate the diversity and community composition of the microorganisms possessing the target gene.

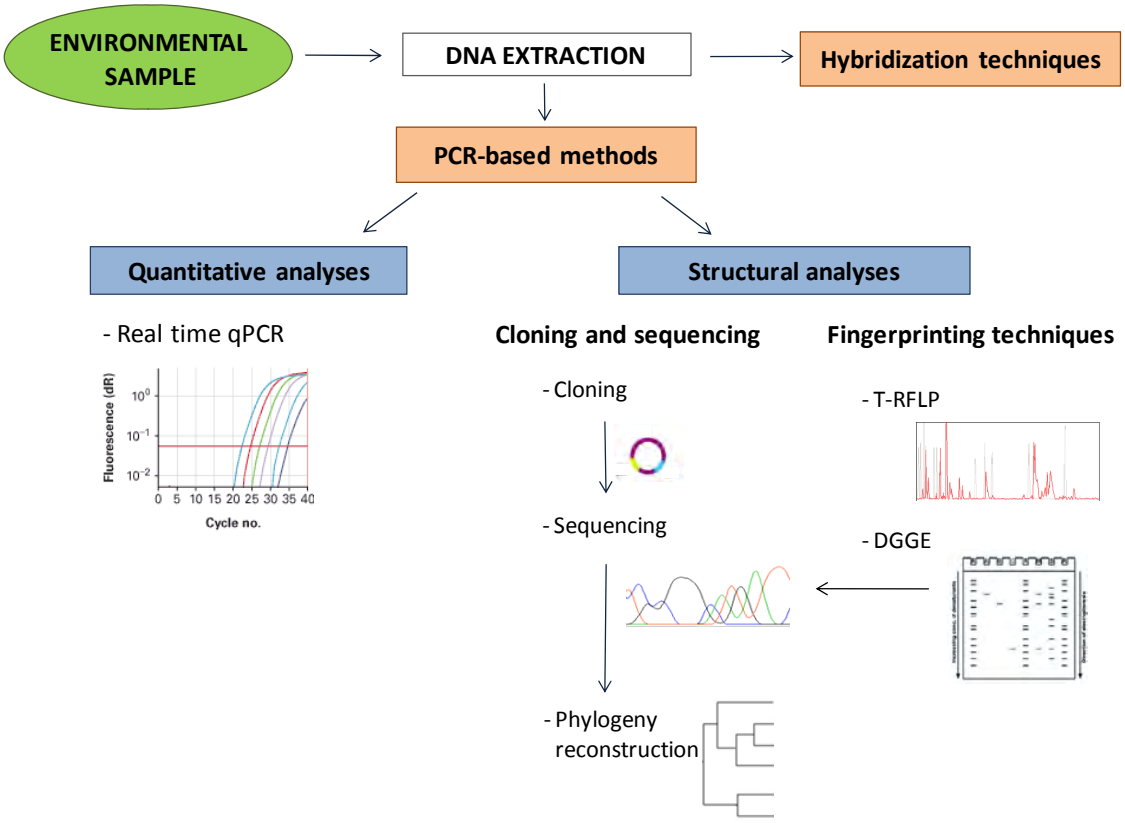

Figure 1.3. Schematic diagram of molecular techniques for examining microbial communities. Adapted from Wallenstein *et al.* (2006).

The abundance of a microbial population can be analyzed by quantitative PCR (qPCR, also known as real-time PCR), which is based on the use of a fluorescent dye that non-specifically binds double-strand DNA. The resulting fluorescent signal is directly proportional to the amount of PCR product present in the reaction, enabling quantification of the target DNA. Furthermore, following amplification of gene, the diversity and community composition of that gene in a mixed community can be assessed by molecular fingerprinting techniques or by cloning and sequencing techniques. Fingerprinting techniques, such as Terminal-Restriction Fragment Length Polymorphism (T-RFLP) and Denaturing Gradient Gel Electrophoresis (DGGE), are particularly useful to characterize microbial communities and evaluate the emergence or disappearance of specific strains or genes, even when the identity of the microorganism is unknown. On the one hand, T-RFLP involves cutting PCR products with restriction enzymes, separate them by gel electrophoresis and measure its length using a DNA sequencer. On the other hand, DGGE involves separating DNA fragments with the same length but with different sequences as they migrate under the influence of electrophoresis through a gel. Separation is

based on the fact that variations in the DNA sequence affect the melting behaviour of the secondary structure of DNA in polyacrilamide gels containing a linear gradient of a denaturing chemical. DGGE is less quantitative than T-RFLP but allows besides mapping shifts in the community structure, further sequencing and phylogenetic characterization of gel bands (Figure 1.3).

In this thesis, real-time PCR and DGGE techniques were selected as molecular methods to assess changes in the abundance and diversity of the microbial population in aquifer samples due to the application of enhanced denitrification.

5. RESEARCH MOTIVATIONS AND THESIS OUTLINE

Nitrate contamination in the subsurface and the consequent limitation of groundwater use has become one of the main environmental threats in Catalunya. This fact together with the water shortage in the country and the possibility to remove nitrate from groundwater by bioremediation motivated the research of this project. Denitrification, as a bioremediation technology of nitrate in groundwater, has generated an increasing interest within the research community and numerous studies have been published recently. However, there is still a lack of knowledge about the factors controlling the growth and activity of microorganisms responsible for the process, which often limits its implementation in the field. Most of published studies are based on the investigation of denitrification promotion, but little is known about the responses of microbial populations and its influence on nitrate removal kinetics. This fact motivated the investigation of the denitrification process from an interdisciplinary point of view by developing and coupling advanced tools to increase the comprehension of the process. On the one hand, modelling the microbial process could not be avoided to understand the kinetic process. Moreover, the application of new molecular techniques had also to be included to increase the comprehension of microbial responds to bioremediation treatments.

One of the main points of the phD was the experience of studying a second bioremediation process in a different laboratory. The opportunity to work in the Flemish Institute for Technological Research (VITO, Mol, Belgium), a consolidated research center in bioremediation topics, investigating the reductive dechlorination of CAHs, allowed improving on the knowledge of bioremediation technologies in groundwater. Furthermore, it is important to note that CAHs contamination in groundwater has become a main environmental threat

worldwide and the possibility to convert these contaminants to harmless end products by bioremediation processes has received an increasing interest over the last years.

Therefore, the present thesis is divided in two main parts:

The first part (*Denitrification*), the main part of the thesis, is divided in four chapters based on the study of the denitrification process as a bioremediation technology to remove nitrate from groundwater. First, it demonstrates the feasibility of applying enhanced denitrification to remove nitrate from contaminated groundwater and investigates the main factors determining the denitrification process (Chapter 3). Subsequently, it shows the development of a mathematical model to explain enhanced denitrification in aquifer material and the calibration of some of the model parameters (Chapter 4). This part also includes the study of the application of enhanced denitrification under experimental dynamic conditions (Chapter 5). Finally, the use of qPCR and DGGE techniques demonstrates changes in microbial community, in terms of abundance and diversity, due to the application of enhanced denitrification (Chapter 6).

The second part (*Reductive Dechlorination*) is based on the study of the bioremediation of CAHs and the factors determining it (Chapter 7). As previously mentioned, this part was carried out during a stay at VITO.

6. REFERENCES

Almasri, M.N. 2007. Nitrate contamination of groundwater: A conceptual management framework. *Environmental Impact Assessment Review*, 27, 220-242.

Aulenta, F., Majone, M. and Tandoi, V. 2006. Enhanced anaerobic bioremediation of chlorinated solvents: environmental factors influencing microbial activity and their relevance under field conditions. *Journal of Chemical Technology and Biotechnology*, 81, 1463-1474.

Bradley, P.M. and Chapelle, F.H. 1998. Microbial mineralization of VC and DCE under different terminal electron accepting conditions. *Anaerobe*, 4, 81-87.

Cunningham, J.A., Rahme, H., Hopkins, G.D., Lebron, C. and Reinhard, M. 2001. Enhanced *in situ* bioremediation of BTEX contaminated groundwater by combined injection of nitrate and sulfate. *Environmental Science & Technology*, 35, 1663-1670.

De Wildeman, S., Diekert, G., Van Langenhove, H. and Verstraete, W. 2003. Stereoselective microbial dehalorespiration with vicinal dichlorinated alkanes. *Applied and Environmental Microbiology*, 69, 5643-5647.

Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources, Council of the European Union, Official Journal L375, 31/12/1991. p. 1-12

Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption, Council of the European Union, Official Journal L330 05/12/1998. p.0032-0054

Directive 2000/60/CE of 23 October 2000 establishing a framework for community action in the field of water policy, European Parliament and the Council of the European Union, Official Journal L327, 22/12/2000. p. 1-72

El Fantroussi, S., Naveau, H. and Agathos, S.N. 1998. Anaerobic dechlorinating bacteria. *Biotechnology Progress*, 14, 167-188.

Enzien, M.V., Picardal, F., Hazen, T.C., Arnold, R.G. and Fliermans, C.B. 1994. Reductive dechlorination of trichloroethylene and tetrachloroethylene under aerobic conditions in a sediment column. *Applied and Environmental Microbiology*, 60, 2200-2204.

Ferguson, J.F. and Pietari, J.M.H. 2000. Anaerobic transformations and bioremediation of chlorinated solvents. *Environmental Pollution*, 107, 209-215.

Gamble, T.N., Betlach, M.R. and Tiedje, J.M. 1977. Numerically dominant denitrifying bacteria from world soils. *Applied and Environmental Microbiology*, 33, 926-939.

Hamonts, K. 2009. Structure and pollutant-degrading activity of the microbial community in eutrophic river sediments impacted by discharging chlorinated aliphatic hydrocarbon-polluted groundwater. Ph.D. thesis. Faculteit Bio-ingenieurswetenschappen. Katholieke Universiteit Leuven, Leuven (Belgium).

Hartmans, S. and Debont, J.A.M. 1992. Aerobic vinyl-chloride metabolism in *Mycobacterium Aurum* L1. *Applied and Environmental Microbiology*, 58, 1220-1226.

Holliger, C., Wohlfarth, G. and Diekert, G. 1998. Reductive dechlorination in the energy metabolism of anaerobic bacteria. *FEMS Microbiology Reviews*, 22, 383-398.

Illman, W.A. and Alvarez, P.J. 2009. Performance Assessment of Bioremediation and Natural Attenuation. *Critical Reviews in Environmental Science and Technology*, 39, 209-270.

Interstate Technology and Regulatory Cooperation (ITRC). August 2002. Technical/Regulatory Guidelines: A systemic approach to in situ bioremediation in groundwater. Including decision trees for in situ bioremediation of nitrates, carbon tetrachloride, and perchlorate.

Jackson, R.E. 1998. The migration, dissolution, and fate of chlorinated solvents in the urbanized alluvial valleys of the southwestern USA. *Hydrogeology Journal*, 6, 144-155.

James, C., Fisher, J., Russell, V., Collings, S. and Moss, B. 2005. Nitrate availability and hydrophyte species richness in shallow lakes. *Freshwater Biology*, 50, 1049-1063.

Kelso, B.H.L., Smith, R.V., Laughlin, R.J. and Lennox, S.D. 1997. Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. *Applied and Environmental Microbiology*, 63, 4679-4685.

Korom, S.F. 1992. Natural denitrification in the saturated zone - a review. *Water Resources Research*, 28, 1657-1668.

Liu, A.G., Ming, J.H. and Ankumah, R.O. 2005. Nitrate contamination in private wells in rural Alabama, United States. *Science of the Total Environment*, 346, 112-120.

Lovley, D.R. 2003. Cleaning up with genomics: Applying molecular biology to bioremediation. *Nature Reviews Microbiology*, 1, 35-44.

Mackay, D.M. and Cherry, J.A. 1989. Groundwater contamination: Pump-and-treat remediation 2. *Environmental Science & Technology*, 23, 630-636.

Major, D.W., McMaster, M.L., Cox, E.E., Edwards, E.A., Dworatzek, S.M., Hendrickson, E.R., Starr, M.G., Payne, J.A. and Buonamici, L.W. 2002. Field demonstration of successful bioaugmentation to achieve dechlorination of tetrachloroethene to ethene. *Environmental Science & Technology*, 36, 5106-5116.

Malachowsky, K.J., Phelps, T.J., Teboli, A.B., Minnikin, D.E. and White, D.C. 1994. Aerobic mineralization of trichloroethylene, vinyl-chloride, and aromatic-compounds by *Rhodococcus* species. *Applied and Environmental Microbiology*, 60, 542-548.

Maymó-Gatell, X., Anguish, T. and Zinder, S.H. 1999. Reductive dechlorination of chlorinated ethenes and 1,2-dichloroethane by "*Dehalococcoides ethenogenes*" 195. *Applied and Environmental Microbiology*, 65, 3108-3113.

Mirgain, I., Green, G. and Monteil, H. 1995. Biodegradation of the herbicide atrazine in groundwater under laboratory conditions. *Environmental Technology*, 16, 967-976.

Phelps, T.J., Malachowsky, K., Schram, R.M. and White, D.C. 1991. Aerobic mineralization of vinyl-chloride by a bacterium of the order actinomycetales. *Applied and Environmental Microbiology*, 57, 1252-1254.

Pruden, A., Sedran, M., Suidan, M. and Venosa, A. 2003. Biodegradation of MTBE and BTEX in an aerobic fluidized bed reactor. *Water Science and Technology*, 47, 123-128.

Reinhard, M., Hopkins, G.D., Steinle-Darling, E. and LeBron, C.A. 2005. *In situ* biotransformation of BTEX compounds under methanogenic conditions. *Ground Water Monitoring and Remediation*, 25, 50-59.

Rittmann, B.E. and McCarty, P.L. 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill, Singapore.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N. and Bemment, C.D. 2008. Nitrate attenuation in groundwater: A review of biogeochemical controlling processes. *Water Research*, 42, 4215-4232.

Rivett, M.O., Feenstra, S. and Cherry, J.A. 2001. A controlled field experiment on groundwater contamination by a multicomponent DNAPL: creation of the emplaced-source and overview of dissolved plume development. *Journal of Contaminant Hydrology*, 49, 111-149.

Ryoo, D., Shim, H., Canada, K., Barbieri, P. and Wood, T.K. 2000. Aerobic degradation of tetrachloroethylene by toluene-o-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Nature Biotechnology*, 18, 775-778.

Saadi, Z. and Maslouhi, A. 2003. Modeling nitrogen dynamics in unsaturated soils for evaluating nitrate contamination of the Mnasra groundwater. *Advances in Environmental Research*, 7, 803-823.

Semprini, L. 1997. Strategies for the aerobic co-metabolism of chlorinated solvents. *Current Opinion in Biotechnology*, 8, 296-308.

Sharma, P.K. and McCarty, P.L. 1996. Isolation and characterization of a facultatively aerobic bacterium that reductively dehalogenates tetrachloroethene to *cis*-1,2-dichloroethene. *Applied and Environmental Microbiology*, 62, 761-765.

Shoun, H., Kim, D.H., Uchiyama, H. and Sugiyama, J. 1992. Denitrification by fungi. *FEMS Microbiol Lett*, 73, 277-81.

Shrimali, M. and Singh, K.P. 2001. New methods of nitrate removal from water. *Environmental Pollution*, 112, 351-359.

Smidt, H. and de Vos, W.M. 2004. Anaerobic microbial dehalogenation. *Annual Review of Microbiology*, 58, 43-73.

Soares, M.I.M. 2000. Biological denitrification of groundwater. *Water Air and Soil Pollution*, 123, 183-193.

Stroo, H.F., Unger, M., Ward, C.H., Kavanaugh, M.C., Vogel, C., Leeson, A., Marqusee, J.A. and Smith, B.P. 2003. Remediating chlorinated solvent source zones. *Environmental Science & Technology*, 37, 224A-230A.

Sun, B.L., Griffin, B.M., Ayala-del-Rio, H.L., Hashsham, S.A. and Tiedje, J.M. 2002. Microbial dehalorespiration with 1,1,1-trichloroethane. *Science*, 298, 1023-1025.

U.S. Environmental Protection Agency. 1995. Contaminant specific fact sheets, Volatile organic chemicals. Technical version: EPA 811-F-95-004-T, EPA. Washington, DC.

UNESCO. 2006. Water, a shared responsibility. The United Nations World Water Development Report 2. Paris, France: UNESCO.

Vogel, T.M., Criddle, C.S. and McCarty, P.L. 1987. Transformations of halogenated aliphatic-compounds. *Environmental Science & Technology*, 21, 722-736.

Wallenstein, M.D., Myrold, D.D., Firestone, M. and Voytek, M. 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*, 16, 2143-2152.

Ward, D.M., Weller, R. and Bateson, M.M. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature*, 345, 63-65.

Wiedemeier, T.H., Swanson, M.A., Moutoux, D.E., Kinzie Gordon, E., Wilson, J.T., Wilson, B.H., Kampbell, D.H., Haas, P.E., Miller, R.N., Hansen, J.E. and Chapelle, F. 1998. Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water. U.S. Environmental Protection Agency, Report EPA/600/R-98/128, EPA, Washington, DC.

Wolfe, A.H. and Patz, J.A. 2002. Reactive nitrogen and human health: Acute and long-term implications. *Ambio*, 31, 120-125.

World Health Organization (WHO). 2004. Guidelines for Drinking Water Quality, third edition. WHO, Genova.

Zaporozec, A. 2002. Groundwater contamination inventory. A methodology guide. UNESCO.

Zumft, W.G. 1997. Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews*, 61, 533-616.

Chapter 2

OBJECTIVES

The main objective of this thesis is to investigate bioremediation processes as reliable technologies to remove contaminants from groundwater. Therefore, it is aimed to apply and integrate different methodologies, such as modelling and microbiological techniques, to improve on the knowledge of specific bioremediation processes. Two bioremediation processes are studied in this thesis: denitrification, which is the main process investigated, and reductive dechlorination.

The specific objectives of this work are:

- To study the factors determining the denitrification potential in natural aquifer material impacted by nitrate contamination.
- To develop and calibrate a mathematical model describing the enhanced denitrification process in aquifer material.
- To study the denitrification process under dynamic conditions simulating the groundwater flow through an aquifer. To investigate the hydrodynamic effects of applying *in situ* bioremediation.
- To apply molecular techniques to study the response of microbial populations to enhanced denitrification processes.
- To study the reductive dechlorination potential of chlorinated aliphatic hydrocarbons (CAHs) in aquifer material and river sediments impacted by these contaminants. To investigate enhanced bioremediation of CAHs.

PART I - Chapter 3

STUDY OF THE FACTORS DETERMINING THE DENITRIFICATION PROCESS IN AQUIFER MATERIAL USING BATCH TESTS

1. INTRODUCTION

Nitrate is possibly the most widespread groundwater contaminant in the world and imposes a serious threat to drinking water supplies. In particular, in Catalunya (Spain), nitrate concentration in groundwater has increased over the last years, mainly due to the excessive application of chemical fertilizers and animal wastes to the crops. In fact, nowadays nitrate is considered the main cause of groundwater contamination in Catalunya. Directive 91/676/EEC concerning the protection of waters against pollution caused by nitrates from agricultural sources (and its transposition into Spanish law through Real Decreto 261/1996) obliged Member States to declare as vulnerable those surface areas where drainage could cause nitrate pollution, whether surface water or groundwater. To date, the Government of Catalunya has established a network of nitrate-vulnerable areas that involves almost half of the municipalities from the region (Figure 3.1).

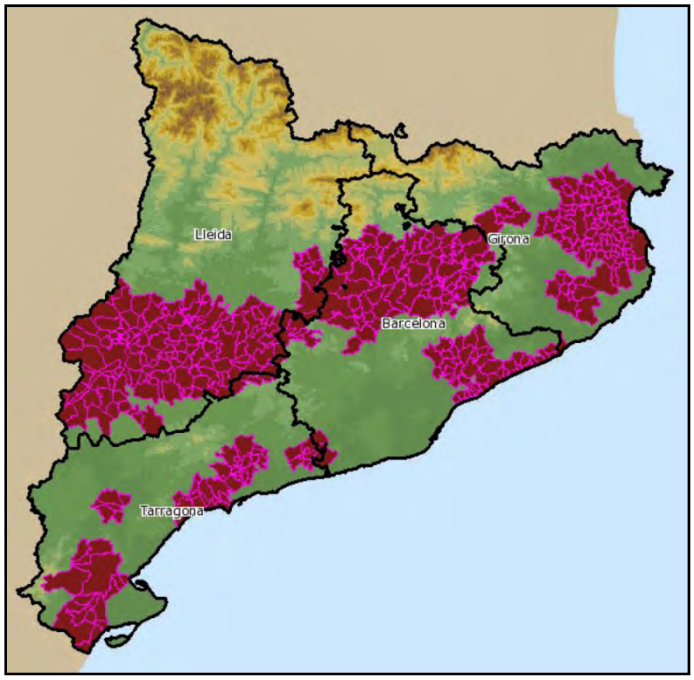

Figure 3.1. Nitrate vulnerable zones in Catalunya (Spain) according Decret 283/1998, Decret 476/2004 and Decret 136/2009. From Institut Cartogràfic de Catalunya (ICC).

The trends of rising nitrate over the world have led to focus the attention on the most effective method of its *in situ* removal, denitrification. As described in Chapter 1, denitrification is the biological process by which nitrate is transformed into nitrogen gas, through a sequence of enzymatic reactions as shown in equation 3.1.

$$\underset{\text{nitrate}}{NO_3^-} \Rightarrow \underset{\text{nitrite}}{NO_2^-} \Rightarrow \underset{\text{nitric oxide}}{NO_{(g)}} \Rightarrow \underset{\text{nitrous oxide}}{N_2O_{(g)}} \Rightarrow \underset{\text{nitrogen gas}}{N_{2(g)}} \quad (3.1)$$

Most of current knowledge about the denitrification process comes from the wastewater treatment field (e.g. Carrera *et al.*, 2003; Foglar *et al.*, 2005; Lee and Welander, 1996). However, denitrification studies in drinking water sources, such as groundwater, have recently been reported (e.g. Gómez *et al.*, 2000; Oa *et al.*, 2006; Schipper and Vojvodic-Vukovic, 2000). Nowadays, groundwater denitrification research is mainly focused on the influence of different environmental conditions on the process, which comprise the type of electron donor, nutrient availability or pH and temperature ranges, among others (Rivett *et al.*, 2008).

Electrons needed for denitrification can be originated from the microbial oxidation of organic or inorganic compounds and the denitrification processes are termed heterotrophic or autotrophic, respectively. Bacteria that use organic carbon as the energy source also tend to use it as a source of cellular carbon and are classified as heterotrophic bacteria, while those that use inorganic compounds as the energy source will normally use inorganic carbon (mainly from HCO_3^-) for cell construction and are termed as autotrophic bacteria. Most of denitrification based literature is related to the investigation of the heterotrophic process, although recent attention has been given to the autotrophic denitrification, with the main advantage of reducing microbial contamination in the treated water (Sierra-Alvarez *et al.*, 2007; Soares, 2000). Heterotrophic and autotrophic denitrification reactions are illustrated in equations 3.2 and 3.3, in which acetate and hydrogen act as electron donors, respectively, and biomass is represented as $C_5H_7O_2N$ (Rittmann and McCarty, 2001):

$$0.125CH_3COO^-_{(aq)} + 0.144NO^-_{3\,(aq)} + 0.144H^+_{(aq)} \rightarrow$$
$$0.066N_{2(g)} + 0.012C_5H_7O_2N_{(cell)} + 0.125HCO^-_{3(aq)} + 0.064\,CO_{2(g)} + 0.154H_2O \quad (3.2)$$

$$0.5H_{2(aq)} + 0.177NO^-_{3(aq)} + 0.177H^+_{(aq)} + 0.025CO_{2(g)} \rightarrow$$
$$0.086N_{2(g)} + 0.005C_5H_7O_2N_{(cell)} + 0.571\,H_2O \quad (3.3)$$

Conventionally, heterotrophic denitrification research has been based on the use of soluble carbon sources such as methanol, ethanol, acetate or sugar (Aslan, 2005, 2006; Chou *et al.*, 2003; Elefsiniotis and Li, 2006; Gómez *et al.*, 2000). However, recent attention has been given to the possibility of using alternative solid substrates such as shredded newspaper (Volokita et al., 1996), wheat straw (Aslan and Turkman, 2005; Soares and Abeliovich, 1998), crab-shell chitin (Robinson-Lora and Brennan, 2009) or sawdust (Schipper and Vojvodic-Vukovic, 2000), among others. The use of these carbonaceous solid materials seems to be a potential field in

the construction of Permeable Reactive Barriers (PRBs) for *in situ* groundwater nitrate bioremediation (Hunter, 2001; Su and Puls, 2007).

With regard to autotrophic denitrification, different inorganic compounds have also been tested. Several works have focused on the use of reduced-sulphur compounds (e.g. H_2S, FeS_2, S(0), $S_2O_3^{2-}$ or SO_3^{2-}) as viable alternative electron donors in organic carbon-limited systems (Campos *et al.*, 2008; Darbi *et al.*, 2002, 2003; Moon *et al.*, 2008; Sierra-Alvarez *et al.*, 2007; Soares, 2002; Zhang and Zeng, 2006). Nevertheless, the use of these compounds may be detrimental to water since sulphate concentration may increase to concentrations higher than recommended levels. Therefore, other autotrophic strategies such as the use of hydrogen gas as electron donor have recently been tested in laboratory-scale studies (Haugen, 2002; Schnobrich *et al.*, 2007; Smith *et al.*, 2005).

Most denitrifying bacteria are facultative anaerobes (Korom, 1992), that is microorganisms that can use oxygen or nitrate as terminal electron acceptor. However, the use of nitrate is thermodynamically less favourable than the reduction of dissolved oxygen for microorganisms. Numerous studies demonstrate how significant denitrification takes place only once the dissolved oxygen concentration falls below a certain low threshold. There is little consensus among studies but it seems reasonable to assume that denitrification will probably occur at dissolved oxygen concentrations below 1 $mg \cdot L^{-1}$ and perhaps below 2 $mg \cdot L^{-1}$ (Rivett *et al.*, 2008). Furthermore, presence of oxygen has been related to the incomplete denitrification and, therefore, to the accumulation of the intermediate products (equation 3.1) (Gómez *et al.*, 2002). This is important since nitrite is significantly more toxic than nitrate (WHO, 2004) and nitric oxide and nitrous oxide emissions contribute to acid rain and they are gases related to the ozone layer depletion and to the global warming (Rivett *et al.*, 2008). However, other factors such as the type of electron donor or nutrients availability have also been related to the stop of denitrification at intermediate stages (Hunter, 2003; Wang *et al.*, 2007).

In a different approach, several authors have investigated the use of pure culture of denitrifying bacteria to remediate nitrate contaminated groundwater. Moreno *et al.* (2005) studied the inoculation of denitrifying submerged filters for groundwater treatment with five different pure cultures of denitrifiers and demonstrated differences in terms of capacity to colonise the inert support, nitrite accumulation and denitrifying activity. Other authors have also studied the effects of abiotic factors on pure cultures of denitrifying bacteria such as oxygen, pH, nitrate and nitrite concentration or carbon source availability (Thomas *et al.*, 1994; Trouve *et al.*, 1998).

In addition to the denitrification process, nitrate can be reduced to ammonia by certain anaerobic bacteria. This process, termed dissimilatory nitrate reduction to ammonia (DNRA), occurs under similar conditions to those of denitrification, and therefore it is seen as a counterproductive process in denitrification studies. Nevertheless, it is less commonly observed in practice (Rivett *et al.*, 2008). The DNRA reaction proceeds as shown in equation 3.4, in which CH_2O represents organic matter (Environment Agency, 2005):

$$2CH_2O + NO_3^- + 2H^+ \rightarrow NH_4^+ + 2CO_2 + H_2O \quad (3.4)$$

The portioning of nitrate between DNRA and denitrification is believed to be controlled by the availability of electron donor: DNRA is the favoured process when the electron acceptor supplies are limiting, and denitrification is favoured when electron donor supplies are limiting (Kelso *et al.*, 1997).

Moreover, assimilation of nitrate into microbial biomass has also been reported as a nitrate removal process. Microorganisms capable of assimilatory nitrate reduction use nitrate, rather than ammonia, as a biosynthetic nitrogen source. In most microorganisms, this process occurs in the absence of more reduced inorganic nitrogen species. It is important to note that no net removal of nitrogen is accomplished by this process, since inorganic nitrogen is converted to organic nitrogen (van Rijn *et al.*, 2006).

2. OBJECTIVES

The main objective of this chapter is to study the denitrification process in batch tests containing groundwater and aquifer soil from a nitrate-contaminated aquifer. This main goal involved the following secondary purposes:

- To characterize aquifer soil and groundwater samples from a selected nitrate-contaminated area.
- To investigate the intrinsic denitrification potential of the aquifer system as well as the possibility to enhance denitrification.
- To study the influence of different factors on the denitrification process: type of electron donor, carbon-to-nitrogen (C/N) ratios and presence of oxygen.
- To compare experimental results with literature references.

3. MATERIALS AND METHODS

3.1. SITE DESCRIPTION

The studied site is located in Argentona (30 km NE from Barcelona city, Catalunya, Spain). It is an alluvial aquifer associated with the Stream Argentona. Specifically, aquifer material (i.e. subsoil and groundwater) was collected from a particular plot in this area, placed in the property of Ramon Lleonard (UTM: X450950, Y4599960). A site map of the sampling location is provided in Figure 3.2. This selection was based on both technical (i.e. accessibility, presence of sampling wells, previous information available, etc.) and environmental criteria (i.e. degree of nitrate pollution, thickness and nature of the aquifer, etc.).

Figure 3.2. Site map showing the selected sampling locations of soil and groundwater (▲) (Google Maps, 2009).

The studied aquifer mainly consists of unconsolidated sand and gravels overlying a granodiorite basement. According to piezometric maps, hydraulic gradient in the selected area is ~0.0125 $m \cdot m^{-1}$ and saturated thickness ranges between 10 and 20 meters. Main uncertainties are found in porosity (estimated to range from 15 to 25%), hydraulic conductivity (from 5 to 20 $m \cdot day^{-1}$) and hydraulic dispersivity (Grandia *et al.*, 2007).

Historic monitoring information about groundwater quality in the selected aquifer, and in particular in the sampling well from Ramon Lleonard's property, is available from Agència Catalana de l'Aigua (ACA) and indicates that nitrate concentration periodically exceeds the maximum threshold value of 50 $mg \cdot L^{-1}$ set by the Drinking Water Directive (Directive 98/83/EC) (Figure 3.3). This is believed to be due to the intense agricultural practices carried out in the area.

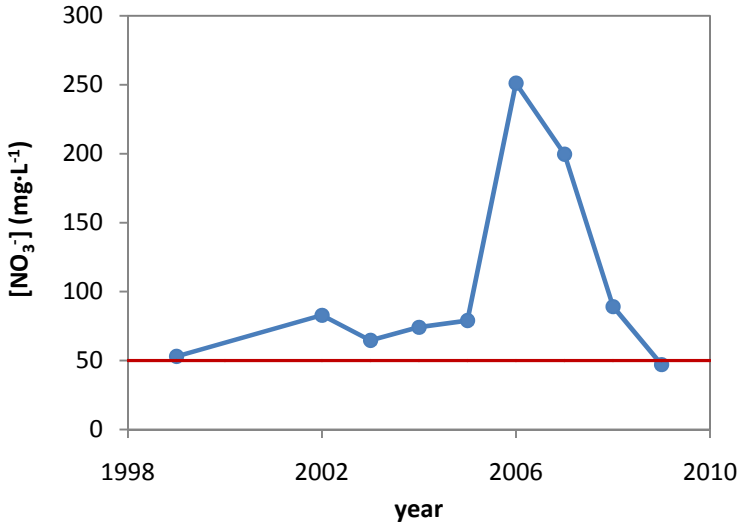

Figure 3.3. Measured nitrate concentrations in the sampling well located in the property of Ramon Lleonard over the last 10 years (ACA, 2009). The maximum concentration allowed in drinking water (Directive 98/83/EC) is indicated by the red line.

3.2. GROUNDWATER SAMPLING AND CHARACTERIZATION ANALYSES

Throughout the present work, three different sampling campaigns were carried out to collect aquifer material for laboratory experiments (May 2006, November 2007 and January 2009). In each sampling campaign, groundwater was sampled from the well located in the property of Ramon Lleonard, which is equipped with an electrical pump. In accordance with the recommendations from ACA (2005), the well was properly purged prior the collection of groundwater in order to ensure that samples were representative of groundwater quality.

Groundwater was placed in glass bottles and, immediately after collection, bottles were kept on ice in coolers for the transport to the laboratory, where they were stored in the refrigerator at 4°C until its use in experiments. An aliquot was separated the same day of collection in order to perform the characterization analyses detailed below.

3.2.1. Nitrogen species analyses

In early stages of this work, nitrate concentration in water samples was analysed following the cadmium reduction method (APHA, 1998) whereby nitrate was reduced to nitrite in the presence of cadmium. The nitrite produced like this, was determined by diazotizing with sulphanilamide and coupling with N-(1-naphtyl)-ethylendiamine to form a highly colored azo dye that was measured colorimetrically at 543 nm using a spectrophotometer (Shimadzu UV-1603). The detection limit of this method was 0.5 $mg \cdot L^{-1}$ NO_3^-. Afterwards, nitrate was analysed by High Performance Liquid Chromatography (HPLC, Agilent 2100 series) with a Waters 432 non-suppressed conductivity detector. A Hamilton PRP-X110 column packed with a polymeric anion exchanger was used. The mobile phase consisted of a 2 mM p-hydroxybenzoic acid solution where the pH was adjusted to 9.2 with NaOH. The analytical procedure followed was according to the method UNE-EN ISO 10304-1 (AENOR, 1995). The detection limit of this method was 1.5 $mg \cdot L^{-1}$ NO_3^-.

Nitrite was analysed by the sulphanilamide colorimetric method previously described without the cadmium reduction step (APHA, 1998), and using a Shimadzu UV-1603 specrophotometer. The detection limit was 0.005 $mg \cdot L^{-1}$ NO_2^-.

Ammonium was determined by means of a commercially available ammonium test kit (Merck, Ref 1.14400.0001) according to the manufacturer's instructions. The detection limit of this method was 0.05 $mg \cdot L^{-1}$ NH_4^+.

3.2.2. Carbon analyses

Organic carbon (OC) content in water was determined using a Shimadzu TOC 5050 analyzer. In fact, the purgeable fraction of dissolved organic carbon (DOC) was determined. According to the standard method (APHA, 1998), samples were filtered through a 0.45 µm nylon membrane filter and acidified with HCl to a pH lower than 2. Before, analyses samples were purged during a 20 minutes period to volatilise all the inorganic carbon present. The detection limit of the method was 1.0 $mg \cdot L^{-1}$ DOC.

Inorganic carbon (IC) content in water was also measured as dissolved inorganic carbon (DIC) by previously filtering the samples through a 0.45 µm nylon membrane filter and by using a Shimadzu TOC 5050 analyzer (APHA, 1998). The detection limit was 1.0 $mg \cdot L^{-1}$ DIC.

3.2.3. pH, ORP and conductivity

pH and oxidation-reduction potential (ORP) were measured directly using Crison electrodes (No. 52-02 and No. 52-62, respectively) coupled to a Crison GLP22 pH-meter. ORP values were normalised to the standard hydrogen electrode by the following expression (ITRC, 2002):

$$Eh = E_{obs} + Eh_{ref} \tag{3.5}$$

where Eh is the oxidation-reduction potential referred to hydrogen scale (V), E_{obs} is the observed reduction-oxidation potential of the reference electrode (V) and Eh_{ref} is the reduction-oxidation potential of the electrode related to the hydrogen electrode (V). Afterwards, Eh was expressed as pe using the following relationship:

$$pe = \frac{Eh \cdot F}{2.303 \cdot R \cdot T} \tag{3.6}$$

in which T is the temperature (K), F is the Faraday constant ($C \cdot mol^{-1}$) and R is the universal gas constant ($J \cdot mol^{-1} \cdot K^{-1}$).

The conductivity of water was determined by using a conductivity cell (Crison No. 52 92) coupled to a Crison conductivity meter GLP 32.

3.2.4. Anions and cations analyses

Sulphate and chloride concentrations were analysed by ion chromatography (Dionex ICS-2100) with ionic suppressor conductivity detection. The system was equipped with a Ionpac AS19 column (Dionex) and the mobile phase used was between 10 and 45 mM KOH. The detection limit of the method was 0.1 $mg \cdot L^{-1}$.

Cations (calcium, potassium, magnesium and sodium) were analysed by means of the same ion chromatography system (Dionex ICS-2100) equipped with a Ionpac CS16 column (Dionex). The mobile phase used when analysing cations contained methanosulphonic acid (30mM). The detection limit of the method was also 0.1 $mg \cdot L^{-1}$.

3.3. SOIL SAMPLING AND CHARACTERIZATION ANALYSES

In each sampling campaign, subsoil was collected from the same location, in the vicinity of the sampling well (Figure 3.2), at a depth of 1.5 m using an Edelman auger. Undisturbed soil samples were transferred into glass bottles and maintained refrigerated for the transport to the laboratory.

Once in the laboratory, all the samples were thoroughly manually homogenized and stored again in the glass bottles at 4°C. A small fraction was also separated to perform several chemical and microbiological analyses. Moreover, to complete the characterization of soil, a sieving and an X-ray diffraction (XRD) analyses were performed with soil collected in the first sampling campaign (2006). These two physical characteristics are supposed to be constant in the system over time.

3.3.1. Water content

Water content of soil samples was analysed immediately after collection. It was determined as the loss on mass after drying at 105°C overnight according to the method ISO 11465:1993 (ISO, 1993). The water content was expressed as a percentage using the following equation:

$$W_w = \frac{m_b - m_c}{m_b - m_a} \cdot 100 \tag{3.7}$$

in which W_w represents the water content of the sample (%), m_a is the mass of the empty dish used to weigh the sample (g), m_b is the mass of the dish containing the wet sample (g) and m_c is the mass of the dish containing the dry matter (after drying at 105°C) (g).

3.3.2. Nitrogen species analyses

Nitrate and ammonium from soil samples were extracted with a KCl solution (2M) according to Mulvaney (1994), and further analysed by following the cadmium reduction method and by means of the ammonium test kit, respectively, as detailed above (section 3.2.1).

Kjeldahl nitrogen, i.e. the sum of organic nitrogen, ammonia and ammonium, was determined according to the method described by Bremner (1996). Organic nitrogen was converted to ammonium with concentrated sulphuric acid. The ammonium produced like this, was further analysed from the amount of ammonia liberated by distillation of the digest with alkali.

3.3.3. Organic matter content

The organic matter content (OM) of the homogenized soil samples was calculated as the fraction of dry matter that was removed after 16 h at 400°C according to the method of Nelson and Sommers (1996).

3.3.4. pH and conductivity

Extractions with $CaCl_2$ (0.01M) and Milli-Q water were prepared to measure pH and conductivity of soil samples, respectively, according to the method UNE 77308:2001 (AENOR, 2001). Measurements were made using the electrodes previously detailed (section 3.2.3).

3.3.5. Phosphorous

Phosphorous was determined following the method described by Primo and Carrasco (1986). It was extracted from soil with sodium carbonate (0.5M) and further analysed as orthophosphate according to the method UNE-EN 1189 (AENOR, 1997).

3.3.6. Enumeration of denitrifiers

Enumeration of denitrifiers in aquifer soil samples was performed by the Most Probable Number (MPN) technique according to the method of Tiedje (1994). Briefly, 10 g of fresh soil was suspended in 90 mL of previously sterilized saline solution (0.85% NaCl) and homogenized by rotary shaking during 2 hours. At this point, soil suspension was diluted ten-fold in saline solution to 10^{-7}. Sterilised tubes containing 10 mL nutrient broth plus 5 mM KNO_3 and inverted Durham tubes were inoculated with 0.1 mL of each dilution in quintuple. Tubes were incubated at 27°C for two weeks. At that time, the denitrification process was measured by screening tubes for nitrate and nitrite disappearance by using the diphenylamine reagent. An additional confirmatory test was performed by checking bubble formation (N_2) in Durham tubes. Each time the analysis was performed, controls were done to ensure the quality of the results. Tubes that fulfilled both criteria (nitrate and nitrite removal and gas production) were considered positive for denitrifiers. MPN estimate of denitrifiers was derived by means of Microsoft Excel® (Microsoft) and the Solver tool as described in Briones and Reichardt (1999).

Measurements of all the chemical species and parameters described above were done in duplicate for all the samples to minimise undesirable biases (differences between duplicates were always lower than 5%).

3.4. MICROCOSM DENITRIFICATION TESTS

Four different sets of microcosm tests were performed to study the denitrification process in the aquifer material collected in Argentona. Experimental procedures of each set of tests are detailed in this section.

3.4.1. Set 1. Feasibility tests

A set of batch microcosm tests was devoted to test the general denitrification capacity of the aquifer system and to discern the ability of promoting this process with different electron donors. Twelve tests were prepared by suspending 15 g of homogenized soil in 140 mL of groundwater in sterilized 160 mL serum bottles. Aquifer material for this set of experiments had been collected in January 2009. In order to achieve an initial higher nitrate concentration (about 100 $mg \cdot L^{-1}$), groundwater was spiked with KNO_3 (Scharlau). A schematic diagram together with a picture of the microcosms is provided in Figure 3.4.

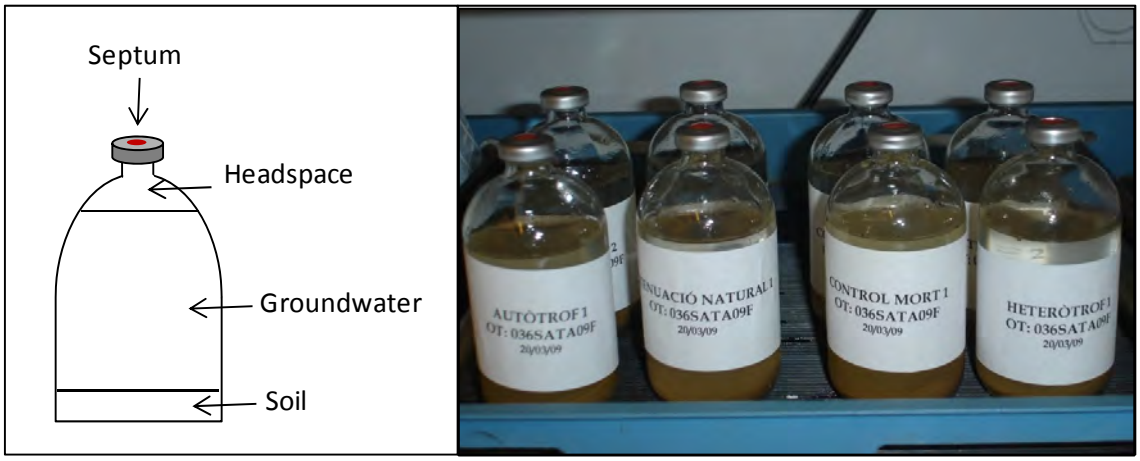

Figure 3.4. Schematic diagram and picture of microcosm tests performed in set 1.

Four different experimental conditions were tested: natural attenuation, abiotic control, heterotrophic denitrification and autotrophic denitrification. Details of each condition are given in Table 3.1. Natural attenuation and abiotic control conditions were prepared without addition of electron donor. Moreover, in the abiotic control, microbial population was inhibited by adding 1 mL of formaldehyde (37%, Scharlau). Stimulation of heterotrophic and autotrophic denitrification in the microcosms was performed by adding an organic carbon source (glucose, Scharlau) and an inorganic electron donor (sodium tiosulphate, Scharlau), respectively. It should be noted that no addition of inorganic carbon was carried out in the autotrophic test, since characterization analyses revealed a high DIC concentration in

groundwater (74.8 mg·L^{-1}). All the microcosm sets-up were prepared in duplicate to study the reproducibility of the process.

Table 3.1. Details of batch microcosm tests performed in set 1 (feasibility tests).

Microcosm condition	Soil (g)[a]	Ground-water (mL)	Tiosulphate (mg·L^{-1})	Glucose (mg·L^{-1})	Formal-dehyde (mL)
Natural attenuation	15 g	140	-	-	-
Abiotic control	15 g	140	-	-	1
Heterotrophic	15 g	140	-	180	-
Autotrophic	15 g	140	672	-	-

The addition of glucose and tiosulphate in the microcosms was done in stoichiometric excess. The stoichiometric reactions of heterotrophic and autotrophic denitrification considered were as follows:

$$S_2O_{3(aq)}^{2-} + 1.6NO_{3(aq)}^{-} + 0.2H_2O \rightarrow 2SO_{4(aq)}^{2-} + 0.8N_{2(g)} + 0.4H_{(aq)}^{+} \quad (3.8)$$

$$5C_6H_{12}O_{6(aq)} + 24NO_{3(aq)}^{-} \rightarrow 12N_{2(g)} + 24HCO_{3(aq)}^{-} + 18H_2O + 6CO_{2(g)} \quad (3.9)$$

Once all the tests were set up, serum bottles were sealed using aluminium crimp caps and rubber septa and immediately homogenized by shaking vigorously. The microcosms were subsequently stored in a dark thermostatic chamber at 17°C (Medilow, Selecta) to simulate the natural aquifer conditions. According to monitoring information, groundwater temperature in the sampling well ranges between 16.5 and 19.5°C (ACA, 2009).

Two millilitres samples to determine nitrate and nitrite were withdrawn twice a day using sterile needles and syringes. Afterwards, samples were filtered through a 0.22 µm nylon membrane filter and preserved at 4°C until the analyses were done. Nitrate and nitrite samples were stored respectively 4 or 2 days maximum before analyses, which were performed as previously described (section 3.2.1). Furthermore, once the tests were finished, 10-mL samples were collected and filtered through a 0.45 µm nylon membrane filter for ammonium measurement (section 3.2.1).

3.4.2. Set 2. Comparison between glucose and acetate

A set of microcosm tests was carried out in order to compare the effect of two different organic carbon sources on the denitrification process: glucose ($C_6H_{12}O_6$) and acetate ($C_2H_3O_2^-$). The selection of these substrates was made on the basis of their widespread availability, easy handling and low cost and because both are non-volatile and readily degradable organic

compounds suitable for bioremediation. A schematic diagram together with a picture of microcosms is provided in Figure 3.5.

The experimental protocol was as follows: an amount of 35 g of homogenized soil was transferred into a sterilized 350-mL glass flask, which was then filled with 300 mL of groundwater previously amended with the appropriate amount of external organic carbon source. Three different stimulation conditions were tested: 200 $mg \cdot L^{-1}$ acetate (added as sodium acetate, Scharlau), 200 $mg \cdot L^{-1}$ glucose (Scharlau) and a mixture (1:1) of acetate and glucose (100 $mg \cdot L^{-1}$ glucose + 100 $mg \cdot L^{-1}$ acetate). It is important to note that these three conditions contained the same amount of added OC, 80 $mg \cdot L^{-1}$ (Table 3.2).

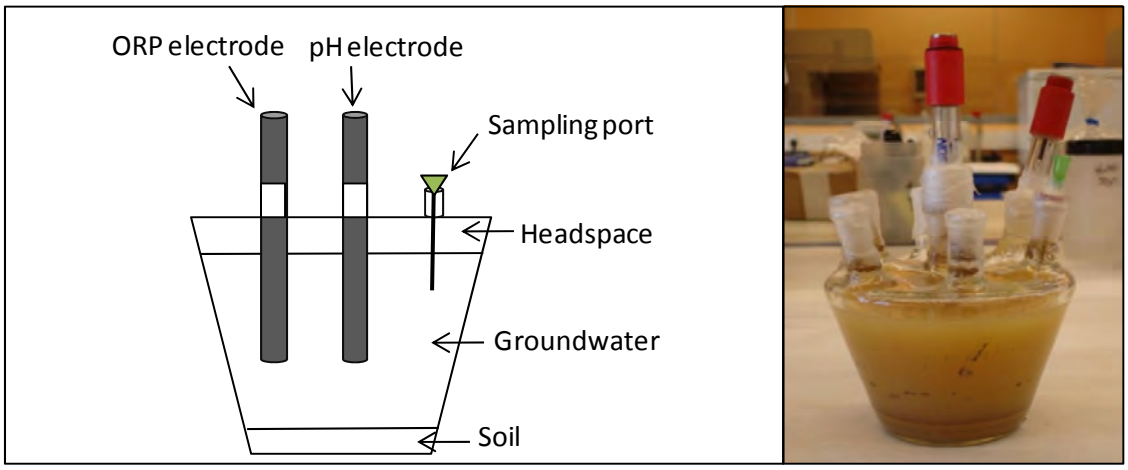

Figure 3.5. Schematic diagram and picture of microcosm tests performed in set 2.

Aquifer material for this set of experiments had been collected in the sampling campaign of 2006. A headspace of about 50 mL was maintained in all microcosm tests to provide aerobic conditions and better simulate the aquifer in Argentona, which according to field measurements presents a DO concentration about 5-6 $mg \cdot L^{-1}$ (ACA, 2009). The flasks were continuously mixed with a planar shaker rotating at 60 rpm in a dark thermostatic chamber at 17°C (Medilow, Selecta).

In this set of tests, flasks were equipped with pH and ORP electrodes (section 3.2.3) to measure these parameters throughout the experiments (Figure 3.5). Moreover, reactors contained a needle which, coupled with a syringe, permitted the collection of liquid samples to analyse nitrate, nitrite and DOC. Three millilitres samples were withdrawn twice a day until no more variations in the concentration of species of interest were observed (approximately 10 days). At the end of experiments, samples for ammonium measurement were also collected as previously described (section 3.4.1).

Table 3.2. Details of batch microcosm tests performed in set 2 (glucose and acetate comparison).

Microcosm condition	Soil (g)[a]	Ground-water (mL)	Glucose ($mg \cdot L^{-1}$)	Acetate ($mg \cdot L^{-1}$)	OC ($mg \cdot L^{-1}$)
Glucose	35	300	200	-	80
Acetate	35	300	-	200	80
Mixture glucose + acetate	35	300	100	100	80

Nitrate, nitrite and ammonium samples were preserved and analysed as detailed above (section 3.4.1). Samples for DOC were filtered through a 0.45 µm nylon membrane filter and preserved acidified at -20°C until analysis, which was always performed before one week after collection.

3.4.3. Set 3. Effect of glucose concentration

Set 3 was intended to study the effect of glucose concentration at promoting denitrification. Microcosms from this set contained aquifer material collected in 2006 and were performed following the same experimental protocol described in set 2 (Figure 3.5). Details of experimental conditions from each test are shown in Table 3.3. It should be noted that the test fed with glucose (200 $mg \cdot L^{-1}$) from set 3 correspond to the same test of glucose condition from set 2. Results from this test were included in both sets for comparison purposes.

Table 3.3. Details of batch microcosm tests performed in set 3 (effect of glucose concentration).

Microcosm condition	Soil (g)	Groundwater (mL)	Glucose ($mg \cdot L^{-1}$)	OC ($mg \cdot L^{-1}$)
Glucose (100 $mg \cdot L^{-1}$)	35	300	100	40
Glucose (200 $mg \cdot L^{-1}$)	35	300	200	80
Glucose (400 $mg \cdot L^{-1}$)	35	300	400	160

3.4.4. Set 4. Effect of dissolved oxygen concentration

In this set, a batch microcosm test was carried out to study the effect of oxygen concentration in the denitrification process. The test was performed in a 400 mL methacrylate flask provided with an adjustable lid that prevented the intrusion of air (Figure 3.6).

Aquifer material for this experiment had been collected in the sampling campaign of 2007. An amount of 35 g of homogenized soil was transferred into the flask, which was then completely filled with 325 mL of groundwater ensuring no headspace left in the flask. Again, groundwater had been previously amended with 200 $mg \cdot L^{-1}$ of glucose to promote the denitrification

process. The test was magnetically stirred in the liquid phase and maintained in a dark thermostatic chamber at 17°C (Medilow, Selecta).

ORP and pH were measured continuously by means of electrodes inserted in the liquid phase as described above (section 3.2.3). Moreover, the flask was equipped with a DO electrode (Crison No. 6050) coupled to a Crison OXI 49, which permitted to monitor oxygen on-line throughout the experiment. A sampling port to collect liquid samples was installed at the top of the flask by means of an Omnifit two-way valve (OM-1101) and an Omnifit cap adaptor (OM-2502). Three millilitres samples for nitrate, nitrite and DOC analyses were withdrawn twice a day by using sterile syringes. Each time the sampling was done, the lid of the flask was adjusted to the new microcosm volume preventing the formation of a headspace. Furthermore, at the end of the experiment, 10-mL samples for ammonium measurement were collected. All the analyses were performed according to the methods described in section 3.2.

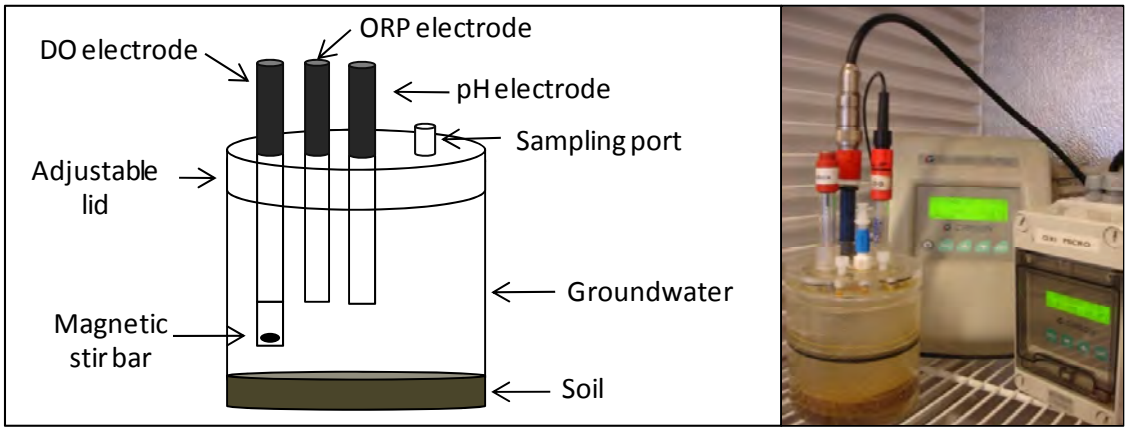

Figure 3.6. Schematic diagram and picture of microcosm tests performed in set 4.

4. RESULTS AND DISCUSSION

4.1. CHARACTERIZATION OF SOIL AND GROUNDWATER

Results of chemical and microbial analyses of aquifer material collected in Argentona in each sampling campaign are detailed in Table 3.4. It must be noticed the elevated nitrate concentration in groundwater, specially in the sampling campaigns from 2006 and 2007 (about 80 $mg \cdot L^{-1}$). Nevertheless, concentrations of other nitrogenous compounds such as ammonium and nitrite were below the detection limits. These results are typically found in aquifers from polluted areas, where the excess of nitrogenous compounds in soils, mainly organic nitrogen and ammonia, is transformed in soils via microbiologically mediated reactions. As a result,

nitrate is formed and, being a highly soluble ion, it passes through soils and reaches the groundwater (Soares, 2000).

Another important aspect from characterization results was the moderate content of DOC in groundwater (about 1-2 $mg \cdot L^{-1}$) and also the low content of organic matter in soil samples (0.2-0.3% dry matter). In fact, it is usual that subsurface environments present low organic carbon contents (i.e. they are oligotrophic) and, as such, intrinsic denitrification rates are usually slow leading to the accumulation of nitrate over decades (Kim *et al.*, 2002; Starr and Gillham, 1993).

Regarding the microbial analyses, it is important to point out the presence of denitrifiers in soil samples, indicating that denitrification activity might be possible in the selected location. It must be stressed that results of MPN of denitrifiers in all the soil samples collected were consistent with the range of published densities in soils using MPN-methods (between 10^4 and 10^6 $bacteria \cdot g^{-1}$ dry soil) (Chèneby *et al.*, 2000; Gamble, 1977; Weier and Macrae, 1992).

According to the US Department of Agriculture classification, the subsoil collected in 2006 consisted mainly of sands (<2000-50 μm) (Gee and Or, 2002). In particular, results of the sieving analysis were as follows: <50 μm (2.1%), 50-100 μm (0.4%), 100-150 μm (1.5%), 150-500 μm (10.5%), 500-1000 μm (33.1%) and >1000 μm (52.5%). From XRD analysis (0), the soil particles were found to be quartz and aluminium-silicates (mainly microcline intermediates and albite, together with small amounts of illite, montmorillonite and pargasite) in accordance with the reported high values of aluminium (between 30 and 100 $mg \cdot L^{-1}$) and silicon (20 $mg \cdot L^{-1}$) found in the aquifer (ACA, 2009).

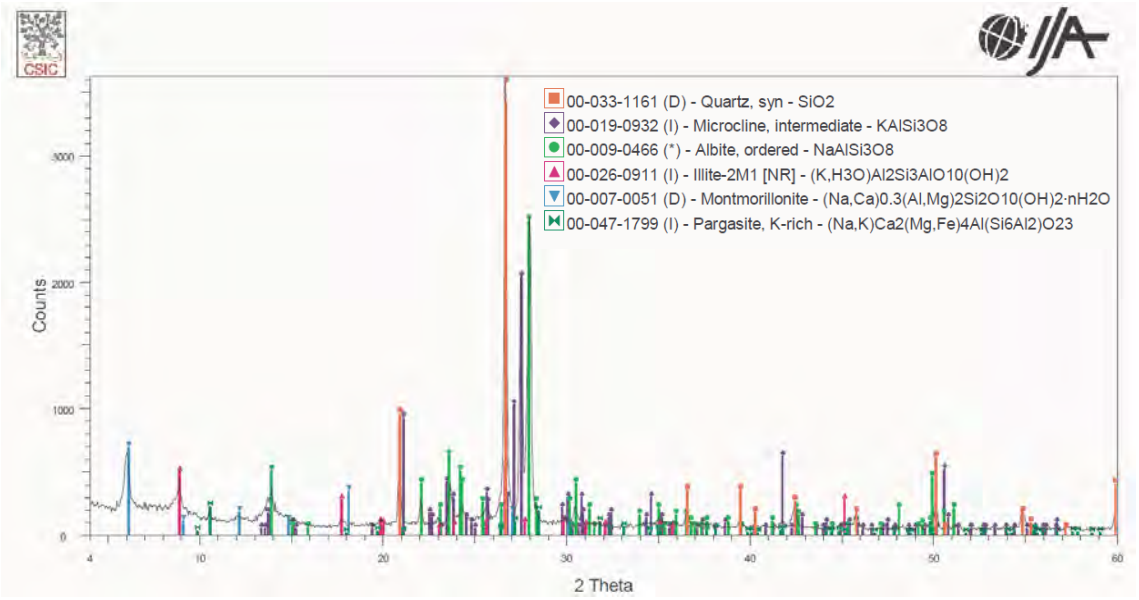

Figure 3.7. XRD analysis of the soil collected in Argentona in 2006.

Table 3.4. Microbial and chemical characteristics of groundwater and subsoil collected in the property of Ramon Lleonard. Results are the average of duplicates.

Sampling campaign →	May 2006		November 2007		January 2009	
Parameter ↓	Groundwater	Soil	Groundwater	Soil	Groundwater	Soil
pH	7.0	7.4	7.6	7.8	7.9	6.9
pe	7.6	-	7.0	-	6.7	-
Conductivity ($\mu S \cdot cm^{-1}$)	1014	146	813	229	970	125
Nitrate ($mg \cdot L^{-1}$ or kg^{-1} d.m.)[a]	79	250.4	76	159.8	45.8	143
Nitrite ($\mu g \cdot L^{-1}$)	<D.L.[b]	-	<D.L.	-	<D.L.	-
Ammonium ($mg \cdot L^{-1}$)	<D.L.	<D.L.	<D.L.	<D.L.	<D.L.	<DL
Kjeldahl nitrogen (g $N \cdot kg^{-1}$ d.m.)	-	1.0	-	0.6	<D.L.	0.3
Sulphate ($mg \cdot L^{-1}$)	87.6	-	89.3	-	80.4	-
Chloride ($mg \cdot L^{-1}$)	70.2	-	64.7	-	56.6	-
DOC ($mg \cdot L^{-1}$)	<D.L.	-	1.2	-	1.7	-
DIC ($mg \cdot L^{-1}$)	81.7	-	80.3	-	74.8	-
Water content (%)	-	10.5	-	11.9	-	13.4
OM (% d.m.)[c]	-	0.3	-	0.2	-	0.2
P total ($mg \cdot kg^{-1}$ d.m.)	-	153	-	41		12
Calcium ($mg \cdot L^{-1}$)	112.3	-	89.0	-	123.8	-
Potassium ($mg \cdot L^{-1}$)	1.9	-	2.5	-	2.5	-
Magnesium ($mg \cdot L^{-1}$)	23.0	-	23.6	-	23.6	-
Sodium ($mg \cdot L^{-1}$)	43.6	-	38.7	-	38.0	-
MPN denitrifier (cells·g^{-1} d.m.)	-	$1.3 \cdot 10^5$	-	$1.0 \cdot 10^5$	-	$5.8 \cdot 10^4$

[a] d.m.: dry matter.
[b] <D.L.: below detection limit.
[c] OM (% d.m.): organic matter content in % dry matter.

4.2. STUDY OF THE DENITRIFICATION PROCESS WITH AQUIFER MATERIAL IN MICROCOSM TESTS

As previously indicated, denitrification was evaluated in microcosm tests containing groundwater and subsoil from the study site. Four different sets of tests were performed in order to investigate the denitrification process in aquifer material and the influence of different parameters on the process. In this section results of each set are discussed.

4.2.1. Set 1. Feasibility tests

Feasibility tests were performed to study the intrinsic denitrification potential of the aquifer material and the capacity to promote heterotrophic and autotrophic denitrification.

Profiles of nitrate and nitrite concentrations over time in the microcosm tests are given in Figure 3.8. In the control tests (natural attenuation and abiotic control), the steady nitrate concentration coupled with the no production of nitrite was convincing evidence that denitrification cannot occur in aquifer material without the addition of an electron donor, at least under the assayed experimental conditions. This finding is in accordance with other denitrification studies in aquifers, which have suggested that the activity of denitrifying microorganisms is often limited by the availability of an electron donor (Smith and Duff, 1988; Starr and Gillham, 1993).

Microcosms amended with an external electron donor, i.e. glucose and tiosulphate, could successfully remove nitrate (nitrate removal efficiencies were above 95%). Nevertheless, the main difference between them was the required time to complete these removals. Addition of glucose resulted in complete reduction of nitrate and nitrite within 90.5 and 234 hours, respectively, whereas the addition of tiosulphate could only trigger complete nitrate and nitrite removal after 407 hours (in day 17th). In the tiosulphate-amended condition, a lag phase appeared to be present before the onset of denitrification (Figure 3.8A). This lag phase could be related to the low cell yield of autotrophic bacteria (Sierra-Alvarez *et al*., 2007) and it would reflect the time needed to increase the autotrophic denitrifying population to attain perceptible nitrate removals.

Differences were also manifested in the evolution of the intermediate nitrite (Figure 3.8B). In the abiotic control, nitrite concentration was always below the detection limit, demonstrating that microbial activity was poisoned, while in the natural attenuation test some nitrite production was observed, reaching a peak of 1.2 $mg \cdot L^{-1}$ in 66 h. However, accumulated nitrite

was reduced to below detection limit after 165 h. These results demonstrated that under natural conditions, microbial activity was present but limited. Main differences were found between heterotrophic and autotrophic batch tests. Heterotrophic tests showed a higher transient accumulation, with nitrite concentration reaching a peak of 41 $mg \cdot L^{-1}$ in 75.5 h and then gradually declining until a not detectable concentration after 234 h (in day 10th), whereas in the autotrophic tests, nitrite reached a peak of 6.2 $mg \cdot L^{-1}$ in 283 h and then it decreased to a concentration lower than 0.7 $mg \cdot L^{-1}$ in 407 h (in day 17th).

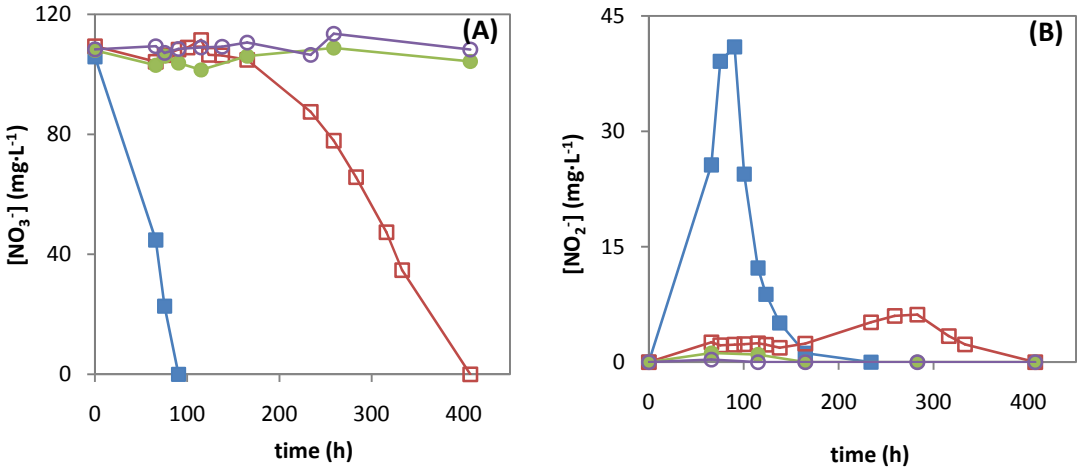

Figure 3.8. Profiles of nitrate (A) and nitrite (B) in microcosm tests (set 1) under natural conditions (●), in the abiotic control (○) and under the effect of stimulating factors: glucose (■) and tiosulphate (□). Data points are the average of duplicates.

It should be mentioned that sulphate was measured at the end of the autotrophic test and results indicated a concentration up to 284.2 $mg \cdot L^{-1}$ (initial concentration in groundwater was 80.4 $mg \cdot L^{-1}$). As previously stated, the increase of sulphate in sulphur-amended tests has been reported as one of the most important disadvantages of applying sulphur-based bioremediation strategies (Environment Agency, 2005). To date, no health-based guideline value has been established for sulphate in drinking water; however, taste thresholds have been set in the range from 250 to 1000 $mg \cdot L^{-1}$ depending on the nature of the associated cation to sulphate (WHO, 2004).

Overall, results from this set of microcosm tests clearly demonstrated that complete denitrification (i.e. nitrate and nitrite removal) could be achieved in aquifer material when an amendment with either glucose or tiosulphate was carried out. However, the type of electron donor had significant influences on the denitrification process in terms of overall rate of nitrate removal and transient accumulation of intermediates.

An additional result from this set of experiments was the reproducibility of the process. Results demonstrated that, under the same conditions, denitrification occurred in a similar way; in other words, profiles of nitrate removal and subsequent nitrite production and reduction were in accordance in duplicate tests (relative standard deviation (RSD) below 5% and 10% in most of the nitrate and nitrite duplicates, respectively).

4.2.2. Set 2. Heterotrophic denitrification: comparison between glucose and acetate

In this set of experiments the influence of the type of electron donor on the heterotrophic denitrification process was investigated by three different tests amended with glucose, acetate or a mixture (1:1) of glucose and acetate.

Results demonstrated that nitrate profiles were similar in the three microcosm tests (Figure 3.9), with nitrate concentrations gradually decreasing from 92 $mg \cdot L^{-1}$ to 66 $mg \cdot L^{-1}$ for glucose (nitrate removal 28%), 50 $mg \cdot L^{-1}$ for acetate (nitrate removal 46%) and 39 $mg \cdot L^{-1}$ for mixture acetate plus glucose (nitrate removal 58%) after 240 h of experiment (10 d). Therefore, slightly differences in the efficiency of removing nitrate by glucose and acetate were observed, although similar nitrate removals have been reported in literature. Chou *et al.* (2003) compared denitrification efficiency using glucose, acetate and methanol as carbon sources in activated sludge, and reported similar nitrate removal efficiencies when using acetate and glucose at the same C/N ratio. However, they observed an initial higher specific nitrate utilization rate when using glucose. Moreover, Sobieszuk and Szewczyk (2006) also reported similar denitrification removals for both acetate and glucose using *Ervinia* sp.

It should be noted that the initial nitrate concentration considered to calculate nitrate removals corresponded to the highest concentration observed in microcosm tests (92 $mg \cdot L^{-1}$). As indicated in Figure 3.9, an increase of nitrate concentration up to 92 $mg \cdot L^{-1}$ was observed in the first hours of the tests, which could be explained by the dissolution of nitrate present in soil (Table 3.4). However, it must be stressed that nitrate was stable over time in collected groundwater stored at 4°C, but it could not be preserved in collected soil samples. Differences between the theoretical nitrate content in the microcosms containing groundwater and soil from the sampling campaign of 2006 (about 108 $mg \cdot L^{-1}$ nitrate) and the maximum experimental concentration detected (92 mg $\cdot L^{-1}$) could be related to the loss of nitrate in soil samples due to storage. Wang *et al*. (2007) reported similar early increases of nitrate concentration in the liquid phase of anaerobic microcosm tests, which were related to the oxidation of organic nitrogen by residual oxygen present in the sediment. The same

phenomenon could also be related to the increase in nitrate content observed in this work, since Kjeldahl nitrogen concentration in soil indicated the presence of 1 g $N \cdot kg^{-1}$ dry soil. However, no evidence was obtained for this speculation since Kjeldahl nitrogen was not monitored during or after the microcosm tests.

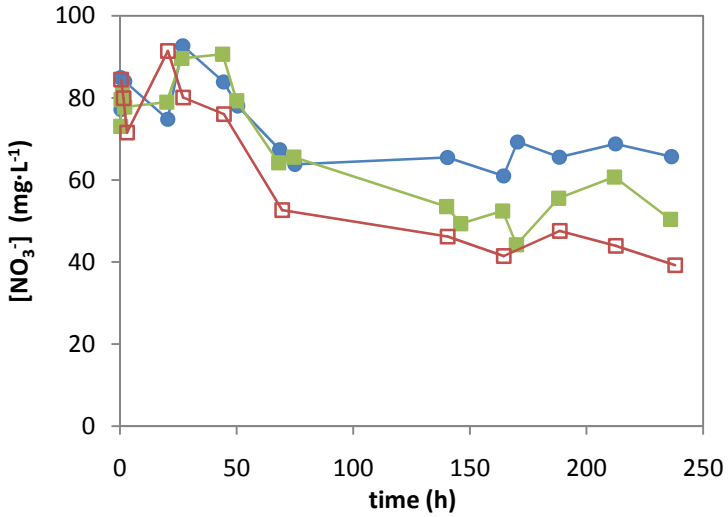

Figure 3.9. Nitrate profiles in microcosm tests amended with glucose (●), acetate (■) and a mixture (1:1) of glucose and acetate (□).

Differences in nitrite production and accumulation were also observed (Figure 3.10A). In the tests amended with either acetate or glucose as single carbon sources, a transient accumulation of nitrite was observed. The acetate-amended flask clearly showed a higher transient accumulation, with nitrite concentration reaching a peak of 8.0 $mg \cdot L^{-1}$ after 74.5 h and then gradually declining to 5.2 $mg \cdot L^{-1}$ in 240 h, whereas in the flask fed with glucose, nitrite reached a peak of 1.9 $mg \cdot L^{-1}$ within 44.4 h and then it decreased to 0.7 $mg \cdot L^{-1}$ until the end of experiment. Since nitrite is an important toxic in water, obtained results would point glucose as a preferred carbon source to enhance denitrification in groundwater systems. Nevertheless, this finding is in contrast with other studies that found higher nitrite accumulation in glucose-amended flasks than in acetate-amended flasks (Chou *et al.*, 2003; Wang *et al.*, 2007). Moreover, the microcosm amended with both glucose and acetate showed even higher and persistent nitrite accumulation than those amended with acetate or glucose as single carbon sources (Figure 3.10A). The reason why nitrite is not further reduced to nitrogen has been related to different factors in literature, such as the type of carbon source, the microorganism species, the oxygen content, the pH and the nitrate and phosphate concentration (Chou *et al.*, 2003; dos Santos *et al.*, 2004; Her and Huang, 1995; Hunter, 2003). For instance, Her and Huang (1995) suggested that nitrite formation depends on the C/N ratio.

Their study showed similar nitrite accumulations for glucose and acetate, which were quantified to be in the order of 21-23% at a molar C/N ratio of approximately 1.2, but less than 5% at molar C/N ratios between 2 and 10.

It should be stressed that nitrite concentration changed in few hours in the experiments and, therefore, peaks observed may not be the highest concentration achieved in the tests. In this way, it is important to mention that the investigation of nitrite production and accumulation was beyond the scope of this project and that this anion was only analysed in order to study the evolution of the denitrification process. Therefore, specially-designed tests would be needed to specifically investigate the behaviour of nitrite as an intermediate of the denitrification process and not definitive conclusions can be derived from the obtained results.

DOC consumption trends were similar regardless of the organic substrate (acetate or glucose) used. DOC contents clearly decreased from initial concentrations of 95 $mg \cdot L^{-1}$ to below 25 $mg \cdot L^{-1}$ at the end of the glucose- and acetate-amended tests (Figure 3.10B). The test amended with a mixture of acetate and glucose showed a higher initial DOC concentration (up to 110 $mg \cdot L^{-1}$), which decreased below 10 $mg \cdot L^{-1}$ after 164.5 h. This slightly higher initial amount of DOC compared to the tests amended with glucose and acetate as sole carbon sources could be related to the higher nitrate removal (Figure 3.9) and nitrite production (Figure 3.10A) observed in this test.

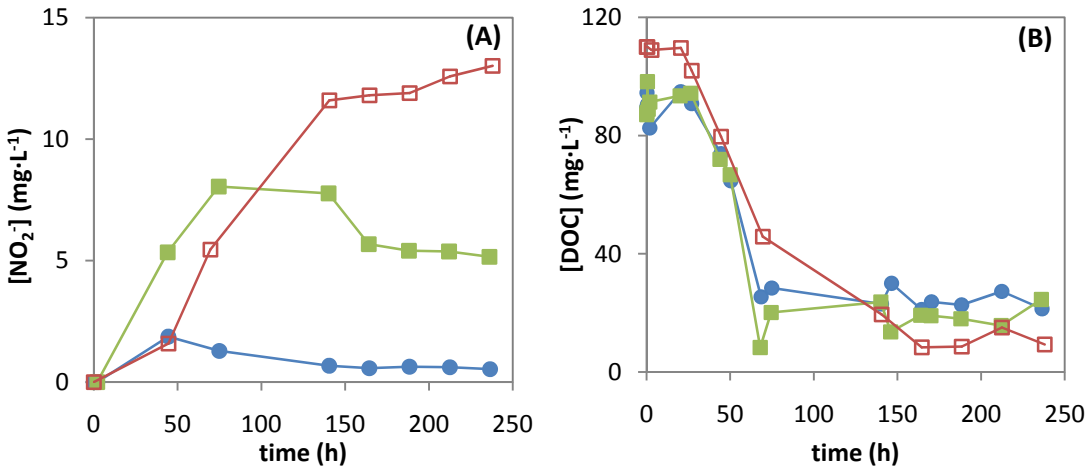

Figure 3.10. Nitrite (A) and DOC (B) profiles in microcosm tests (set 2) under the effect of stimulating factors: glucose (●), acetate (■) and a mixture (1:1) of glucose plus acetate (□).

As previously mentioned, in this set of microcosm tests pH and pe were monitored over time. Results of the three assayed conditions indicated that these parameters remained stable throughout the experiments (data not shown). On the one hand, it must be noted that pH remained mainly within the limits denitrification may occur (7.0<pH<8.5) (Brettar *et al.*, 2002;

Burchell *et al.*, 2007; Oa *et al.*, 2006) and, therefore, this parameter was not to be any limitation for denitrification to develop in the microcosm tests. On the other hand, pe measurements in the course of the tests were between 7.5 and 8.8. These values were higher than the expected ones.

It is noteworthy that, from a consideration of thermodynamic principles, the most stable species within a wide range of pH-pe is the gaseous nitrogen (N_2) (Figure 3.11). However, this equilibrium is not found in natural environments due to the catalyzing effect of bacteria in accelerating the denitrification process at lower redox potentials (Hiscock *et al.*, 1991). As previously mentioned, denitrification only takes place in limited oxygen concentrations, thus in reductive environments. Nevertheless, results from microcosm tests demonstrated that the water phase remained in oxidative conditions while denitrification was taken place. This could be explained taking into account the microenvironments concept suggested by Rivett *et al.* (2008), bacteria in aquifer soil do not necessarily experience the same oxygen concentrations as those measured in the water phase. In addition, it is suggested in this work that a stratification of the oxygen concentration may occur in the soil layer of the microcosms, i.e. the top layers of soil may remain aerobic while the deepest layers are anoxic. Therefore, the presence of anoxic conditions in the soil would have allowed the denitrification to occur. The stratification of the denitrification process has also been reported in the field-scale, for example by Hendry *et al.* (1983), who observed that shallow groundwater appeared to have oxidative conditions and significant nitrate concentrations, whereas reducing conditions existed at depth in the aquifer, were denitrification took place.

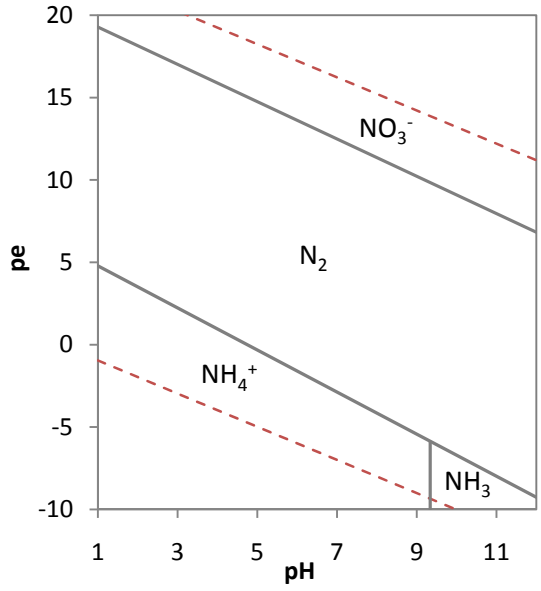

Figure 3.11. pe-pH diagram for the nitrogen system at 25°C. The diagram is drawn for a nitrate concentration of 0.01M by means of MEDUSA (Puigdomènech, 2004).

Overall, results from this set of tests demonstrated slightly differences on the denitrification process, in terms of nitrate removal efficiency and nitrite production, due to the type of organic carbon substrate used. Furthermore, these differences were not in accordance with literature and could be related to other experimental factors.

A rather important result was that complete denitrification was not attained under the assayed experimental conditions. This could be related to the oxidative conditions measured in the microcosms, which is known that limit the denitrification activity.

4.2.3. Set 3. Effect of glucose concentration on denitrification removal

A new set of experiments was devoted to study the effect of electron donor concentration on the denitrification process. As previously mentioned, three different glucose concentrations were assayed in microcosm tests.

Results demonstrated that removal of nitrate was clearly affected by the glucose dose (Figure 3.12). Amendments of 100 $mg \cdot L^{-1}$ and 200 $mg \cdot L^{-1}$ glucose led to similar partial nitrate removals (33% and 29%, respectively), while amendments of 400 $mg \cdot L^{-1}$ glucose resulted in a significant higher nitrate removal (87%). This pattern corroborates that denitrification in these type of tests was organic carbon-limited. From these results it can be concluded that C/N molar ratios of 3.6 and 5.3 were not sufficient for complete denitrification, and that, under the experimental conditions assayed, a C/N of 11.6 was necessary for remarkable nitrate removals. This C/N ratio might seem in disagreement with Her and Huang (1995), who reported a minimum required C/N ratio of 2.2. However, the diversity of operational parameters among studies must be considered. The favourable conditions at which the denitrification experiments were conducted by Her and Huang (1995), such as using anaerobic conditions, an acclimatised denitrifying sludge, a nutrient-containing solution and a relatively high temperature (30°C), may account for the higher denitrification efficiencies (and lower C/N ratio required) than those obtained in this study.

The early increase in nitrate concentration was not observed in tests amended with 100 $mg \cdot L^{-1}$ and 400 $mg \cdot L^{-1}$ (Figure 3.12). Both experiments were performed several months later than experiments from set 2 and, as previously mentioned, a loss of nitrate in collected soil samples probably occurred.

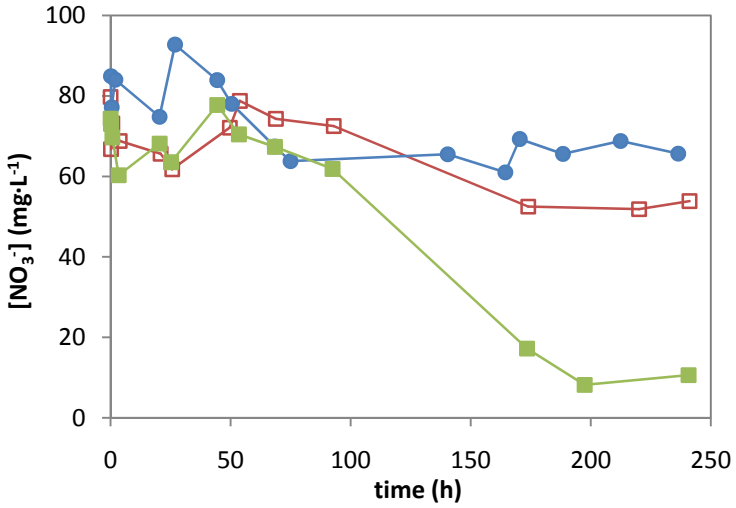

Figure 3.12. Nitrate profiles in microcosm tests under different glucose amendments: 100 mg·L^{-1} (□), 200 mg·L^{-1} (●) and 400 mg·L^{-1} (■).

Again, a transient weak accumulation of nitrite was observed (Figure 3.13). The accumulation was low in the flask amended with 100 mg·L^{-1} glucose, with a hardly perceptible peak less than 0.32 mg·L^{-1} after 48 h of experiment. More pronounced peaks of nitrite were observed in the other flasks, with maximum concentrations of 1.9 mg·L^{-1} (followed by a gradual drop to 0.7 mg·L^{-1} at the end of the experiment) and 1.1 mg·L^{-1} (falling to final concentrations under detection limits) in the flasks amended with 200 mg·L^{-1} and 400 mg·L^{-1} of glucose, respectively. As previously mentioned, it should be noted that the nitrite peak concentrations may not be the highest concentrations reached throughout the microcosm tests. A rather important result is that nitrite was accumulated when organic carbon source was not enough to achieve significant nitrate removals, this was specially true in the case of the amendment with 200 mg·L^{-1} of glucose. The same phenomenon has been reported by other authors (Gómez *et al.*, 2000; Her and Huang, 1995; Wang *et al.*, 2009).

Overall, results from this set of tests demonstrated that the increment of the dose of organic carbon source could overcome some negative effects observed (low nitrate removal and nitrite accumulation). As previously suggested in set 2, this could be explained because the high amount of oxygen present in the microcosm made the systems OC limited, in other words, the higher amount of electron acceptor needed a higher concentration of electron donor added. This finding is in accordance with results of Gómez *et al.* (2002), who studied the influence of dissolved oxygen on groundwater nitrate removal with a denitrifying submerged filter inoculated with an activated sludge and amended with different types and concentrations of organic carbon sources.

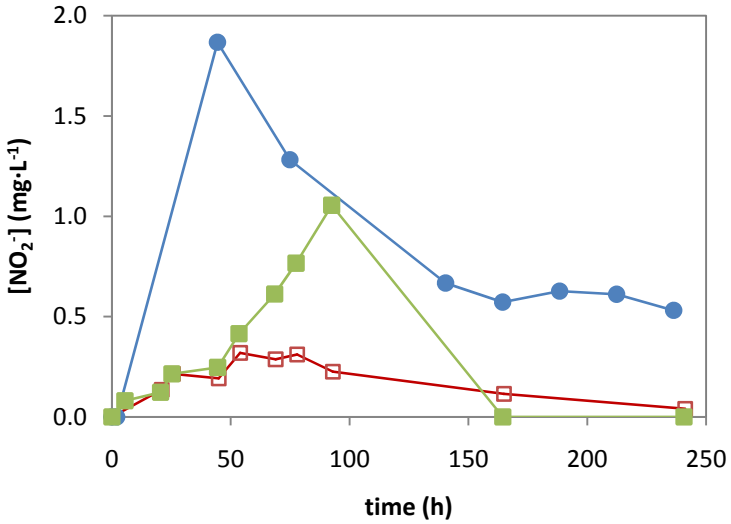

Figure 3.13. Profiles of nitrite in microcosm tests under different glucose amendments: 100 mg·L^{-1} (□), 200 mg·L^{-1} (●) and 400 mg·L^{-1} (■).

4.2.4. Set 4. Effect of oxygen on the denitrification process

Taking into account results form set 2 and set 3, a new test was performed to study the influence of the oxygen content on the denitrification process. In this experiment, no headspace was maintained in the reactor and, therefore, the oxygen content was limited.

Nitrate removal profile under the conditions from this test was significantly different from that observed in sets 2 and 3 (Figure 3.14A). Once DO concentration initially present in groundwater (approximately 9 mg·L^{-1}) was reduced to concentrations lower than 1 mg·L^{-1} nitrate was quickly consumed from an initial concentration of 76 mg·L^{-1} to below the detection limit within 46.5 h, resulting in an overall nitrate removal higher than 96%. This finding showed that, when an external organic carbon source is supplied, the activity of indigenous heterotrophic microorganisms can quickly exhaust DO in groundwater, and once the oxygen is almost depleted (~1 mg·L^{-1}) microorganisms can rapidly consume nitrate by denitrification. DOC was gradually consumed during the first 46.5 h, just as nitrate was reduced during the same elapsed time. This result indicates that DOC was consumed in the aerobic oxidation of glucose and in the denitrification process.

Nitrite sharply increased up to a concentration of 12 mg·L^{-1} (21.3% of initial nitrate concentration) (Figure 3.14B), which is high compared to those concentrations accumulated in sets 2 and 3. Nevertheless, nitrite concentration was reduced to below the detection limit after 48 h, indicating that this intermediate was not to be a potential contaminant in the

treated water. A similar profile of nitrite was obtained in the heterotrophic test from set 1 (Figure 3.8B), where nitrite reached a concentration of 41 $mg \cdot L^{-1}$ (50.7% of initial nitrate concentration) and then rapidly declined below the detection limit concentration. Thus, it can be suggested that when amending with glucose in a stoichiometric excess a hardly build up of nitrite occurs, although it easily decreases further to low levels. These results agree with Gómez *et al.* (2000), who reported an initial increase of nitrite concentration when increasing the dosage of carbon source in denitrifying submerged filters. Furthermore, her results also indicated that at high carbon source amendments, accumulated nitrite concentration could finally be removed.

Although the observed nitrite peak of 12 $mg \cdot L^{-1}$ might not be the highest concentration accumulated in the microcosm, results seem to indicate that the maximum nitrite accumulated was lower than the initial nitrate concentration, leading to the conclusion that nitrate and nitrite were simultaneously consumed in the test, but that the nitrate reduction to nitrite by denitrifiers was quicker than the subsequent conversion of nitrite to nitrogen gas.

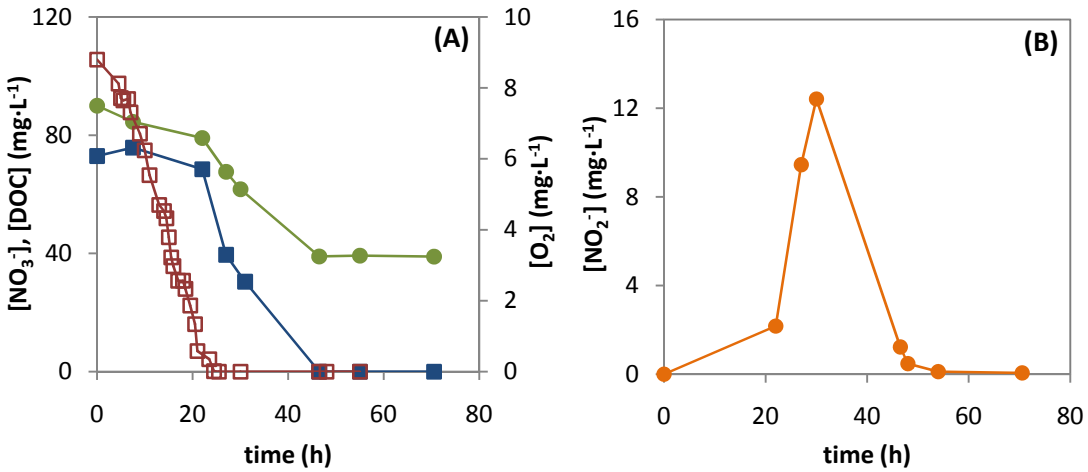

Figure 3.14. Profiles of (A) oxygen (□), nitrate (■) and DOC (●) and (B) nitrite (●) in microcosm test from set 4.

Contrary to experiments from set 2 and 3, in this test pe dropped from initial value of 6.0 to 4.7, corroborating that in this case anoxic conditions were attained in the liquid phase. Concerning the pH, it remained stable around neutrality as in the other sets.

4.3. MECHANISMS OF NITRATE AND CARBON CONSUMPTION

As stated above, two main reactions were involved in the batch tests: aerobic oxidation of organic matter and denitrification. Stoichiometry of these reactions was developed in order to study the theoretical carbon-to-oxygen (C/O) and carbon-to-nitrogen (C/N) ratios.

In heterotrophic processes, organic carbon plays a double role. First, it serves as electron donor in the redox reaction. Secondly, it serves as carbon source for cell synthesis through an assimilatory process into biomass. The coupling of these two reactions and the derivation of the stoichiometric overall reaction for aerobic oxidation of organic carbon and denitrification was reached by thermodynamic and bioenergetic principles (Rittmann and McCarty, 2001). This approach describes the mass balance in terms of the fraction of electrons transferred from donor to acceptor (fe) and to biomass (fs). The fractions of fe and fs sum up to 1.

The overall reactions were built combining the involved semi-reactions as follows (Rittmann and McCarty, 2001):

$$R = feRa + fsRc - Rd \tag{3.10}$$

where R refers to the overall stoichiometric reaction, Ra represents the semi-reaction of the electron acceptor, Rc represents the semi-reaction of the cell synthesis and Rd represents the semi-reaction of the electron donor. It is important to note that Rd has a negative sign because the donor is oxidized.

Furthermore, stoichiometric reactions were developed considering that nitrate took part in two reactions: as electron acceptor (denitrification reaction), and as the only nitrogen source in cell synthesis of bacteria (nitrate assimilation). Actually, it is known that ammonium is preferred as nitrogen source for bacteria, but that denitrifiers often use nitrate as nitrogen source for cell synthesis (Rittmann and McCarty, 2001). Moreover, it is considered in this work that heterotophs in aerobic conditions grow with nitrate, since analyses of groundwater from the studied site demonstrated that ammonium was not available (concentration lower than the detection limit, 0.05 mg·L^{-1}).

The semi-reactions involved in aerobic oxidation of organic matter and denitrification are as follows:

Oxygen as electron acceptor in aerobic respiration (Ra, aerobic):

$$\frac{1}{4}\,O_{2(g)} + H^{+}_{(aq)} + e^{-} \rightarrow \frac{1}{2}\,H_2O \tag{3.11}$$

Nitrate as electron acceptor in denitrification (Ra, anoxic):

$$\frac{1}{5}\,NO^{-}_{3(aq)} + \frac{6}{5}H^{+}_{(aq)} + e^{-} \rightarrow \frac{1}{10}\,N_{2(g)} + \frac{3}{5}\,H_2O \tag{3.12}$$

Nitrate as nitrogen source for bacteria cell synthesis (Rs):

$$\frac{1}{28}\,NO^-_{3(aq)} + \frac{5}{28}\,CO_{2(g)} + \frac{29}{28}H^+_{(aq)} + e^- \rightarrow \frac{1}{28}\,C_5H_7O_2N_{(cell)} + \frac{11}{28}\,H_2O \qquad (3.13)$$

Besides, electrons in the above reactions are supplied by glucose or acetate in this study (Rd):

$$\frac{1}{24}\,C_6H_{12}O_{6(aq)} + \frac{1}{4}\,H_2O \rightarrow \frac{1}{4}\,CO_{2(g)} + H^+_{(aq)} + e^- \qquad (3.14)$$

$$\frac{1}{8}\,CH_3COO^-_{(aq)} + \frac{3}{8}\,H_2O \rightarrow \frac{1}{8}\,CO_{2(g)} + \frac{1}{8}\,HCO^-_{3(aq)} + H^+_{(aq)} + e^- \qquad (3.15)$$

Denitrification reactions when using glucose and acetate were calculated assuming *fe* = 0.35 and *fs* = 0.65 (Rittmann and McCarty, 2001) and considering the general equation 3.10. Obtained reactions are detailed below.

Aerobic oxidation of glucose:

$$0.042C_6H_{12}O_{6(aq)} + 0.088\,O_{2(g)} + 0.023NO^-_{3(aq)} + 0.023H^+_{(aq)} \rightarrow$$
$$0.023C_5H_7O_2N_{(cell)} + 0.134CO_{2(g)} + 0.180\,H_2O \qquad (3.16)$$

Aerobic oxidation of acetate:

$$0.125\,CH_3COO^-_{(aq)} + 0.088\,O_{2(g)} + 0.023NO^-_{3(aq)} + 0.023H^+_{(aq)} \rightarrow$$
$$0.023C_5H_7O_2N_{(cell)} + 0.009CO_{2(g)} + 0.055\,H_2O + 0.125HCO^-_{3(aq)} \qquad (3.17)$$

Denitrification with glucose:

$$0.042C_6H_{12}O_{6(aq)} + 0.093NO^-_{3(aq)} + 0.093H^+_{(aq)} \rightarrow$$
$$0.035N_{2(g)} + 0.023C_5H_7O_2N_{(cell)} + 0.134\,CO_{2(g)} + 0.215\,H_2O \qquad (3.18)$$

Denitrification with acetate:

$$0.125CH_3COO^-_{(aq)} + 0.093NO^-_{3(aq)} + 0.093H^+_{(aq)} \rightarrow$$
$$0.035N_{2(g)} + 0.023C_5H_7O_2N_{(cell)} + 0.009\,CO_{2(g)} + 0.090\,H_2O + 0.125HCO^-_{3(aq)} \qquad (3.19)$$

It should be pointed out that the *fs* value is not a constant and it may be affected by a number of factors and environmental conditions, such as the available substrate, the organism decay rate or the biodegradable fraction of an active microorganism (Zhou, 2007). The use of a constant number is a common accepted approximation.

Equations 3.16 and 3.17 establish a theoretical C/O molar ratio of 2.9 and 2.8 for aerobic oxidation with glucose and acetate, respectively, whereas equations 3.18 and 3.19 establish a theoretical C/N molar ratio of 2.7 based on glucose and acetate denitrification.

In set 4, where the initial DO and nitrate concentrations were 8.8 $mg \cdot L^{-1}$ and 76 $mg \cdot L^{-1}$, respectively, a theoretical total amount of 49 $mg \cdot L^{-1}$ of OC would have been needed to consume oxygen and nitrate according to the stoichiometric ratios derived. This result correlates well with the experimental DOC consumption observed (51 $mg \cdot L^{-1}$), demonstrating that the processes considered (i.e. aerobic oxidation of organic matter and denitrification) were likely the ones taking place in the system.

In microcosm tests from set 2 and 3, where a headspace of about 50 mL was left, a total amount of 106 $mg \cdot L^{-1}$ of OC would have been needed to complete the oxygen and nitrate removal. This result clearly demonstrates that, except the test amended with 400 $mg \cdot L^{-1}$, all the experiments were limited by organic matter concentration. Nevertheless, as previously suggested, denitrification was likely taking place in anoxic layers or pockets of the soil.

As previously mentioned, under anaerobic conditions, nitrate can also be reduced to ammonium through the DNRA process. Analyses at the end of the experiments revealed the following ammonium concentrations: 0.2 $mg \cdot L^{-1}$ (autotrophic and heterotrophic tests from set 1 and test 200 $mg \cdot L^{-1}$ glucose from set 2 and 3), 0.3 $mg \cdot L^{-1}$ (test 200 $mg \cdot L^{-1}$ acetate from set 2), 0.05 $mg \cdot L^{-1}$ (test 100 $mg \cdot L^{-1}$ glucose from set 3 and test mixture glucose and acetate from set 2), 0.2 $mg \cdot L^{-1}$ (test 400 $mg \cdot L^{-1}$ glucose from set 3) and 0.9 $mg \cdot L^{-1}$ (set 4). These concentrations accounted for less than 4% of the total initial nitrate in all the tests. In view of these results, DNRA could be considered minor contributor to the observed nitrate removal. Volatilisation loss of ammonium in the form of NH_3 was disregarded since the pH in the tests was always below 8.5 (Figure 3.12). This finding is in contrast with results reported by Akunna *et al.* (1993), which indicated that in presence of glucose as carbon source nitrate reduction occurred mainly through the DNRA pathway. However, the anaerobic conditions at which experiments were performed by Akunna *et al.* (1993) could explain this discrepancy.

4.4. COMPARISON OF EXPERIMENTAL RESULTS WITH LITERATURE DATA

Results obtained in this study were compared with other published studies using acetate and glucose to promote denitrification in batch systems (Table 3.5). It is important to note that reported data are very sparse and that comparisons are extremely difficult. The wide range in values and formats is due to large differences in operational parameters, such as the initial nitrate concentration, the initial C/N ratio, the type of organic source, the type of inoculum and the initial concentration of denitrifying bacteria (and its composition), the nutrient medium for the bacteria population growth, the temperature, the operation way of batch reactors (e.g. with or without agitation, continuous addition of nitrate and/or organic source as long as they are consumed), previous acclimation of microbial population to nitrate and the duration of the experiment, among others (Akunna *et al.*, 1993; Chou *et al.*, 2003; Elefsiniotis and Li, 2006; Oa *et al.*, 2006; Tugtas and Pavlostathis, 2007; Wang *et al.*, 2007)

It can be observed that different types of inoculum are used among the studies (Table 3.5). Most works were conducted using activated sludge or enriched cultures (Akunna *et al.*, 1993; Chou *et al.*, 2003; Elefsiniotis and Li, 2006; Tugtas and Pavlostathis, 2007), whereas only a few used aquifer soil or sediment (Abdelouas *et al.*, 1999; Oa *et al.*, 2006; Wang *et al.*, 2007) in a similar way than in this project. Among all the cited studies, nitrate removal efficiencies appeared to be higher (>95%) when anaerobic conditions and high concentrations of organic carbon were applied, although nitrate removal was not necessarily through denitrification since high accumulations of nitrite and ammonium were observed in some studies (Abdelouas *et al.*, 1999; Akunna *et al.*, 1993; Wang *et al.*, 2007). On the contrary, at low C/N ratios like in this study (Chou *et al.*, 2003; Oa *et al.*, 2006) nitrate removals were mostly between 38-90% with increases observed at increased C/N ratios.

Furthermore, it is noteworthy that temperature used in the cited studies (Table 3.5) was commonly between 20-25°C, which may be appropriate for *ex-situ* treatment simulations but not for *in situ* treatments that occur in aquifers with lower temperatures. Mostly, the temperature has been related to an increase in the denitrification rate.

Table 3.5. Summary of published studies using acetate and glucose for denitrification in batch systems.

Initial NO_3^- ($mg \cdot L^{-1}$)	OC Source	OC ($mg \cdot L^{-1}$)	Molar C/N	Microorganisms source	T (°C)	time (d)	Final NO_3^- ($mg \cdot L^{-1}$)	% NO_3^- (removed)	Remarks	Reference
886	glucose	926	5.4	anaerobic sludge	n.r.	4	<D.L.[a]	99%	accumulation of NO_2^- and NH_4^+	(Akunna *et al.*, 1993)
886	acetate	823	4.8	anaerobic sludge	n.r.	4	<D.L.	99%	transient accumulation of NO_2^-	(Akunna *et al.*, 1993)
221	acetate	100	2.3	sludge from a	10	14	<D.L.	>95%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
221	acetate	200	4.7	sludge from a	10	14	<D.L.	>95%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
443	acetate	200	2.3	sludge from a	10	14	<D.L.	>95%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
443	acetate	400	4.7	sludge from a	10	14	<D.L.	>95%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
886	acetate	400	2.3	sludge from a	10	14	19[c]	98%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
886	acetate	800	4.7	sludge from a	10	14	19[c]	98%[c]	rates increasing with temperature	(Elefsiniotis and Li, 2006)
1329	acetate	1500[d]	n.r.	methanogenic	35	7	<D.L.	>99%	transient accumulation of NO_2^-	(Tugtas and Pavlostathis, 2007)
1329	glucose	1500[d]	n.r.	methanogenic	35	7	<D.L.	>99%	transient accumulation of NO_2^-	(Tugtas and Pavlostathis, 2007)
33	acetate	n.r[e]	2.0	activated sludge	n.r.	0.13	22	34.8%	transient accumulation of NO_2^-	(Chou *et al.*, 2003)
33	glucose	n.r.	2.0	activated sludge	n.r.	0.13	18	35.4%	accumulation of NO_2^-	(Chou *et al.*, 2003)
221	acetate	n.r.	0-29	anaerobic sludge	30	0.5	0-199	10-100%		(Her and Huang, 1995)
221	glucose	n.r.	0-29	anaerobic sludge	30	0.5	0-199	10-100%		(Her and Huang, 1995)
464	acetate	n.r.	≥1.25	local sediment	20	15	<D.L.	>99%	transient accumulation of NO_2^-	(Abdelouas *et al.*, 1999)
5.2	glucose	5333	5300	lake sediment	25	16	<D.L.	99%[c]	transient accumulation of NH_4^+	(Wang *et al.*, 2007)
10	acetate	2352	1215	lake sediment	25	16	0.1[c]	99%[c]	accumulation of NH_4^+	(Wang *et al.*, 2007)
233	acetate	68.2	1.5	subsurface soil	n.r.	25	133[c]	43%[c]	accumulation of NO_2^-	(Oa *et al.*, 2006)
92	glucose	95	5.3	aquifer material	17	10	66	28%	accumulation of NO_2^-	This study (set 2 and 3)
92	acetate	95	5.3	aquifer material	17	10	50	46%	accumulation of NO_2^-	This study (set 2)
92	mixture	110	6.2	aquifer material	17	10	39	57%	accumulation of NO_2^-	This study (set 2)
80	glucose	55	3.6	aquifer material	17	10	54	29%	low transient generation of NO_2^-	This study (set 3)
80	glucose	160	11.6	aquifer material	17	10	11	87%	low transient generation of NO_2^-	This study (set 3)
76	glucose	90	6.1	aquifer material	17	3	<D.L.	>96%	transient generation of NO_2^-	This study (set 4)

[a] <D.L.: below detection limit.

[b] WTP: Water Treatment Plant.

[c] Values estimated from figures reported in the referenced studies.

[d] Values expressed as chemical oxygen demand (COD)·L^{-1}.

[e] n.r.: not reported.

5. CONCLUSIONS

Feasibility microcosm tests with soil and groundwater from a nitrate-contaminated aquifer in Argentona, Catalunya (Spain) revealed that denitrification was limited by the availability of electron donors. Amendments with either glucose or tiosulphate successfully promote denitrification mediated by heterotrophic and autotrophic indigenous bacteria, respectively.

Main differences between heterotrophic and autotrophic denitrification were found to be related with the time needed to complete nitrate removal as well as with nitrite production. Heterotrophic process was faster but produced a higher transient accumulation of nitrite. In the autotrophic test, an important lag phase was observed before the onset of denitrification, moreover high accumulation of sulphate was detected.

Two different soluble organic carbon sources (acetate and glucose) were tested to promote heterotrophic denitrification. Similar nitrate removals were obtained with both substrates and main differences were related to nitrite production and accumulation, although a clear trend was not obtained.

Organic carbon limitation, resulting from aerobic respiration, was consistently associated with partial denitrification and significant nitrite accumulation in microcosm tests. Nevertheless, other limiting factors of denitrification such as the presence of oxygen, nutrient availability or the temperature, among others, could also be related with the accumulation of nitrite. More conclusive results about the behaviour of nitrite would require specific experiments that were beyond the scope of this project.

Stoichiometric reactions derived for aerobic oxidation of glucose and denitrification correlated well with the obtained experimental results, demonstrating that organic carbon was mainly consumed by aerobic oxidation and denitrification.

Ammonium generation was low (less than 4% of the initial nitrate present) and, therefore, nitrate removal through dissimilatory nitrate reduction to ammonia (DNRA) was considered a minor contributor to the overall nitrate removal.

6. REFERENCES

Abdelouas, A., Deng, L.J., Nuttall, E., Lutze, W., Fritz, B. and Crovisier, J.L. 1999. Réduction in situ des ions nitrate dans des eaux par les bactéries indigènes. *Comptes Rendus De L'Academie Des Sciences Serie Ii Fascicule a-Sciences De La Terre Et Des Planetes*, 328, 161-166.

UNE-EN ISO 10304-1. Determinación de iones fluoruro, cloruro, nitrito, ortofosfato, bromuro, nitrato y sulfato disueltos, por cromatografía en fase líquida. AENOR, 1995.

UNE 77308:2001. Calidad del suelo. Determinación de la conductividad eléctrica específica. AENOR, 2001.

UNE-EN 1189. Calidad del agua. Determinación de fósforo. Método espectrométrico con molibdato amónico. AENOR, 1997.

Agència Catalana de l'Aigua (ACA). 2005. Protocol: mostreig d'aigües subterrànies. Guia Pràctica. Departament de Medi Ambient i Habitatge. Generalitat de Catalunya.

Agència Catalana de l'Aigua (ACA). http://aca-web.gencat.cat/aca. 2009

Akunna, J.C., Bizeau, C. and Moletta, R. 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon-sources: glucose, glycerol, acetic-acid, lactic-acid and methanol. *Water Research*, 27, 1303-1312.

American Public Health Association (APHA). 1998. Standard methods for the examination of water and wastewater, 20th Edition. American Public Health Association , American Water Works Association and Water Environment Federation. Washington, DC, USA.

Aslan, S. 2005. Combined removal of pesticides and nitrates in drinking waters using biodenitrification and sand filter system. *Process Biochemistry*, 40, 417-424.

Aslan, S. and Turkman, A.E. 2005. Combined biological removal of nitrate and pesticides using wheat straw as substrates. *Process Biochemistry*, 40, 935-943.

Aslan, S. and Turkman, A. 2006. Nitrate and pesticides removal from contaminated water using biodenitrification reactor. *Process Biochemistry*, 41, 882-886.

Brettar, I. and Sanchez-Perez, J.M., Tremolieres, M. 2002. Nitrate elimination by denitrification in hardwood forest soils of the Upper Rhine floodplain - correlation with redox potential and organic matter. *Hydrobiologia*, 469, 11-21.

Briones, A.M. and Reichardt, W. 1999. Estimating microbial population counts by 'most probable number' using Microsoft Excel®. *Journal of Microbiological Methods*, 35, 157-161.

Bremner, J.M. 1996. Nitrogen-total. In: Sparks, D.L. (ed.). Methods of Soil Analysis. Part 3-Chemical Methods. Soil Science Society of America, Inc. Madison, Wisconsin, USA, pp. 1085-1121.

Burchell, M.R. and Skaggs, R.W., Lee, C.R., Broome, S., Chescheir, G.M. and Osborne, J. 2007. Substrate organic matter to improve nitrate removal in surface-flow constructed wetlands. *Journal of Environmental Quality*, 36, 194-207.

Campos, J.L., Carvalho, S., Portela, R., Mosquera-Corral, A. and Mendez, R. 2008. Kinetics of denitrification using sulphur compounds: Effects of S/N ratio, endogenous and exogenous compounds. *Bioresource Technology*, 99, 1293-1299.

Carrera, J., Vicent, T. and Lafuente, F.J. 2003. Influence of temperature on denitrification of an industrial high-strength nitrogen wastewater in a two-sludge system. *Water SA*, 29, 11-16.

Chèneby, D., Philippot, L., Hartmann, A., Henault, C. and Germon, J.C. 2000. 16S rDNA analysis for characterization of denitrifying bacteria isolated from three agricultural soils. *FEMS Microbiology Ecology*, 34, 121-128.

Chou, Y.J., Ouyang, C.F., Kuo, W.L. and Huang, H.L. 2003. Denitrifying characteristics of the multiple stages enhanced biological nutrient removal process with external carbon sources. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering*, 38, 339-352.

Darbi, A., Viraraghavan, T., Butler, R. and Corkal, D. 2002. Batch studies on nitrate removal from potable water. *Water SA*, 28, 319-322.

Darbi, A., Viraraghavan, T., Butler, R. and Corkal, D. 2003. Column studies on nitrate removal from potable water. *Water Air and Soil Pollution*, 150, 235-254.

Decret 283/1998, de 21 d'octubre de 1998, de designació de les zones vulnerables en relació amb la contaminacióde nitrats procedents de fonts agràries.

Decret 476/2004, de 28 de desembre de 2004, pel qual es designen noves zones vulnerables en relació amb la contaminació de nitrats procedents de fonts agràries.

Decret 136/2009, d'1 de setembre de 2009, d'aprovació del programa d'actuació aplicable a les zones vulnerables en relació amb la contaminació de nitrats que procedeixen de fonts agràries i de gestió de les dejeccions ramaderes.

Directive 91/676/EEC of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources, Council of the European Union, Official Journal L375, 31/12/1991. p. 1-12

Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption, Council of the European Union, Official Journal L 330 05/12/1998. p.0032-0054

dos Santos, S.G., Varesche, M.B.A., Zaiat, M. and Foresti, E. 2004. Comparison of methanol, ethanol, and methane as electron donors for denitrification. *Environmental Engineering Science*, 21, 313-320.

Elefsiniotis, P. and Li, D. 2006. The effect of temperature and carbon source on denitrification using volatile fatty acids. *Biochemical Engineering Journal*, 28, 148-155.

Environment Agency. 2005. Attenuation of nitrate in the sub-surface environment, Science Report SC030155/SR2.

Foglar, L., Briski, F., Sipos, L. and Vukovic, M. 2005. High nitrate removal from synthetic wastewater with the mixed bacterial culture. *Bioresource Technology*, 96, 879-888.

Gamble, T.N., Betlach, M. R. and Tiedje, J. M. 1977. Numerically Dominant Denitrifying Bacteria from World Soils. *Applied and Environmental Microbiology*, 33, 926-939.

Gee, G.W. and Or, D. 2002. Particle-Size Analysis. In: Dane, J.H. and Topp, G.C. (eds.). Methods of Soil Analysis. Part 4. Physical Methods. Soil Science Society of America. Madison, Inc. Wisconsin, USA. pp. 255-289.

Gómez, M.A., González-López, J. and Hontoria-García, E. 2000. Influence of carbon source on nitrate removal of contaminated groundwater in a denitrifying submerged filter. *Journal of Hazardous Materials*, 80, 69-80.

Gómez, M.A., Hontoria, E. and González-López, J. 2002. Effect of dissolved oxygen concentration on nitrate removal from groundwater using a denitrifying submerged filter. *Journal of Hazardous Materials*, 90, 267-278.

Grandia, F., Domènech, C., Jordana, S., Goscera, G., Arcos, D., Duro, L., Guimerà, J. and Bruno, J. 2007. Reactive transport modelling of denitrification plumes induced by organic matter injection. In: *Proceedings of the water pollution in natural porous media at different scales. Assessment of fate, impact and indicators. WAPO*^{2}. Barcelona (Spain).

Haugen, K.S., Semmens, M. J. and Novak, P. J. 2002. A novel in situ technology for the treatment of nitrate contaminated groundwater. *Water Research*, 36, 3497-3506.

Hendry, M.J., Gillham, R.W. and Cherry, J.A. 1983. An integrated approach to hydrogeologic investigations - a case-history. *Journal of Hydrology*, 63, 211-232.

Her, J.J. and Huang, J.S. 1995. Influences of carbon surface and C/N ratio on nitrate nitrite denitrification and carbon breakthrough. *Bioresource Technology*, 54, 45-51.

Hiscock, K.M., Lloyd, J.W. and Lerner, D.N. 1991. Review of natural and artificial denitrification of groundwater. *Water Research*, 25, 1099-1111.

Hunter, W.J. 2001. Use of vegetable oil in a pilot-scale denitrifying barrier. *Journal of Contaminant Hydrology*, 53, 119-131.

Hunter, W.J. 2003. Accumulation of nitrite in denitrifying barriers when phosphate is limiting. *Journal of Contaminant Hydrology*, 66, 79-91.

Institut Cartogràfic de Catalunya (ICC). http://www.atlesnacional.cat/icc/atles-nacional/medi-ambient/zones-vulnerables-per-nitrats. 2009.

ISO 11465:1993. Determination of dry matter and water content on a mass basis - Gravimetric method. International Organization for Standardization, 1993.

Interstate Technology and Regulatory Cooperation (ITRC). 2002. A systemic approach to in situ bioremediation in groundwater.

Kelso, B.H.L., Smith, R.V., Laughlin, R.J. and Lennox, S.D. 1997. Dissimilatory nitrate reduction in anaerobic sediments leading to river nitrite accumulation. *Applied and Environmental Microbiology*, 63, 4679-4685.

Kim, Y.S., Nayve, F.R.P., Nakano, K. and Matsumura, M. 2002. Screening and characterization of facultative psychrophilic denitrifiers for treatment of nitrate contaminated groundwater using starch-based biodegradable carriers. *Environmental Technology*, 23, 1017-1026.

Korom, S.F. 1992. Natural denitrification in the saturated zone - a review. *Water Resources Research*, 28, 1657-1668.

Lee, N.M. and Welander, T. 1996. The effect of different carbon sources on respiratory denitrification in biological wastewater treatment. *Journal of Fermentation and Bioengineering*, 82, 277-285.

Moon, H.S., Shin, D.Y., Nam, K. and Kim, J.Y. 2008. A long-term performance test on an autotrophic denitrification column for application as a permeable reactive barrier. *Chemosphere*, 73, 723-728.

Moreno, B., Gomez, M.A., Ramos, A., Gonzalez-Lopez, J. and Hontoria, E. 2005. Influence of inocula over start up of a denitrifying submerged filter applied to nitrate contaminated groundwater treatment. *Journal of Hazardous Materials*, 127, 180-186.

Mulvaney, R.L. 1994. Nitrogen - inorganic forms. In: Sparks, D.L. (ed.). Methods of Soil Analysis. Part 3-Chemical Methods. Soil Science Society of America, Inc. Madison, Wisconsin, USA, pp. 1123-1184.

Nelson, D.W. and Sommers, L.E. 1996. Total carbon, organic carbon and organic matter. In: Sparks, D.L. (ed.). Methods of Soil Analysis. Part 3-Chemical Methods. Soil Science Society of America, Inc. Madison, Wisconsin, USA. pp. 961-1010.

Oa, S.W., Kim, G. and Kim, Y. 2006. Determination of electron donors by comparing reaction rates for in situ bioremediation of nitrate-contaminated groundwater. *Journal of Environmental Science and Health Part A-Toxic/Hazardous Substances & Environmental Engineering*, 41, 2359-2372.

Primo, E. and Carrasco, J.M. (1987). Química Agrícola I: Suelos y fertilizantes. pp. 307-313. Editorial Alhabrama. Madrid.

Puigdomènech, I. 2004. MEDUSA: Make equilibrium diagrams using sophisticated algorithms.

Real Decreto 261/1996, de 16 de febrero, sobre protección de las aguas contra la contaminación producida por los nitratos procedentes de fuentes agrarias.

Rittmann, B.E. and McCarty, P.L. 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill. Singapore.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W.N. and Bemment, C.D. 2008. Nitrate attenuation in groundwater: A review of biogeochemical controlling processes. *Water Research*, 42, 4215-4232.

Robinson-Lora, M.A. and Brennan, R.A. 2009. The use of crab-shell chitin for biological denitrification: Batch and column tests. *Bioresource Technology*, 100, 534-541.

Schipper, L.A. and Vojvodic-Vukovic, M. 2000. Nitrate removal from groundwater and denitrification rates in a porous treatment wall amended with sawdust. *Ecological Engineering*, 14, 269-278.

Schnobrich, M.R., Chaplin, B.P., Semmens, M.J. and Novak, P.J. 2007. Stimulating hydrogenotrophic denitrification in simulated groundwater containing high dissolved oxygen and nitrate concentrations. *Water Research*, 41, 1869-1876.

Sierra-Alvarez, R., Beristain-Cardoso, R., Salazar, M., Gomez, J., Razo-Flores, E. and Field, J.A. 2007. Chemolithotrophic denitrification with elemental sulfur for groundwater treatment. *Water Research*, 41, 1253-1262.

Smith, R.L., Buckwalter, S.P., Repert, D.A. and Miller, D.N. 2005. Small-scale, hydrogen-oxidizing-denitrifying bioredctor for treatment of nitrate-pontaminated drinking water. *Water Research*, 39, 2014-2023.

Smith, R.L. and Duff, J.H. 1988. Denitrification in a sand and gravel aquifer. *Applied and Environmental Microbiology*, 54, 1071-1078.

Soares, M.I.M. 2000. Biological denitrification of groundwater. *Water Air and Soil Pollution*, 123, 183-193.

Soares, M.I.M. 2002. Denitrification of groundwater with elemental sulfur. *Water Research*, 36, 1392-1395.

Soares, M.I.M. and Abeliovich, A. 1998. Wheat straw as substrate for water denitrification. *Water Research*, 32, 3790-3794.

Sobieszuk, P. and Szewczyk, K.W. 2006. Estimation of (C/N) ratio for microbial denitrification. *Environmental Technology*, 27, 103-108.

Starr, R.C. and Gillham, R.W. 1993. Denitrification and organic carbon availability in two aquifers. *Ground Water*, 31, 934-947.

Su, C.M. and Puls, R.W. 2007. Removal of added nitrate in cotton burr compost, mulch compost, and peat: Mechanisms and potential use for groundwater nitrate remediation. *Chemosphere*, 66, 91-98.

Thomas, K.L., Lloyd, D. and Boddy, L. 1994. Effects of oxygen, pH and nitrate concentration on denitrification by Pseudomonas species. *FEMS Microbiology Letters*, 118, 181-186.

Tiedje, J.M. 1994. Denitrifiers. In: Weaver, R.W., Angle, J.S., Bottomley, P.S. (eds.) Methods of soil analysis. Part 2-Microbiological and biochemical properties. Soil Science Society of America, Inc. Madison, Wisconsin, USA, pp. 245-267.

Trouve, C., Chazal, P.M., Gueroux, B. and Sauvaitre, N. 1998. Denitrification by new strains of Thiobacillus denitrificans under non-standard physicochemical conditions. Effect of temperature, pH, and sulphur source. *Environmental Technology*, 19, 601-610.

Tugtas, A.E. and Pavlostathis, S.G. 2007. Electron donor effect on reduction pathway and kinetics in a mixed methanogenic culture. *Biotechnology and Bioengineering*, 98, 756-763.

van Rijn, J., Tal, Y. and Schreier, H.J. 2006. Denitrification in recirculating systems: Theory and applications. *Aquacultural Engineering*, 34, 364-376.

Volokita, M., Belkin, S., Abeliovich, A. and Soares, M.I.M. 1996. Biological denitrification of drinking water using newspaper. *Water Research*, 30, 965-971.

Wang, D.H., Huang, Q.H., Wang, C.X., Ma, M. and Wang, Z.J. 2007. The effects of different electron donors on anaerobic nitrogen transformations and denitrification processes in Lake Taihu sediments. *Hydrobiologia*, 581, 71-77.

Wang, Q.H., Feng, C.P., Zhao, Y.X. and Hao, C.B. 2009. Denitrification of nitrate contaminated groundwater with a fiber-based biofilm reactor. *Bioresource Technology*, 100, 2223-2227.

Weier, K.L. and Macrae, I.C. 1992. Denitrifying bacteria in the profile of a brigalow clay soil beneath a permanent pasture and a cultivated crop. *Soil Biology & Biochemistry*, 24, 919-923.

World Health Organization (WHO). 2004. Guidelines for drinking water quality, third edition. WHO, Genova.

Zhang, T.C. and Zeng, H. 2006. Development of a response surface for prediction of nitrate removal in sulfur — limestone autotrophic denitrification fixed-bed reactors. *Journal of Environmental Engineering*, 132, 1068-1072

Zhou, S. 2007. Stoichiometry of biological nitrogen transformations in wetlands and other ecosystems. *Biotechnology Journal*, 2, 497-507.

PART I - Chapter 4

MODELLING ENHANCED DENITRIFICATION IN MICROCOSM EXPERIMENTS

1. INTRODUCTION

An integral component of evaluating *in situ* bioremediation is the application of mathematical models to determine reaction rates. The most common approach for modelling biological processes and, in particular, denitrification has been the use of the Monod kinetics. Monod (1949) mathematically related the growth of a microbial population to the concentration of the substance limiting its growth with the following hyperbolic function:

$$\mu = \mu_{max} \cdot \left[\frac{S}{S + K_S}\right] \tag{4.1}$$

where μ and μ_{max} (T^{-1}) are the specific growth rate and the maximum growth rate of a biomass population, respectively, S ($M \cdot L^{-3}$) is the substrate concentration limiting the growth and K_S ($M \cdot L^{-3}$) is the saturation coefficient for substrate, which refers to the substrate concentration at which the growth rate is half the μ_{max} as indicated in Figure 4.1.

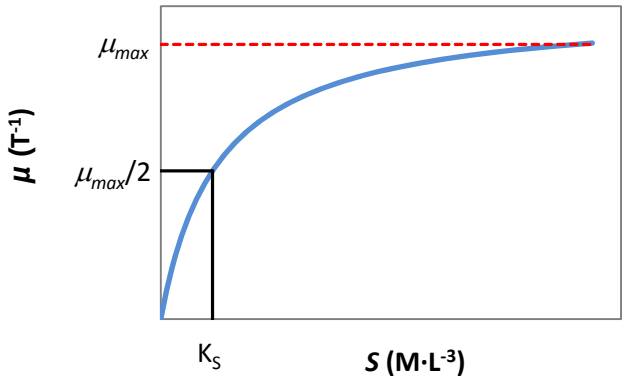

Figure 4.1. Monod relationship between the specific microbial growth rate (μ) and an essential substrate concentration (S).

Usually, a more sophisticated model is applied in biological processes, the so-called multiple-Monod kinetics, which consists in an extension of the Monod equation (equation 4.1) to handle the case in which the rate of microbial growth is limited by the concentration of one or more species other than a single growth substrate. Within this approach, Bae and Rittman (1996) supported the dual-limitation kinetics in which both the electron acceptor and the electron donor substrates limit the overall microbial growth rate. This kinetics has successfully been applied in several works to describe denitrification and other biological processes (Gu *et al.*, 2007; Kindred and Celia, 1989; Kinzelbach *et al.*, 1991; MacQuarrie and Sudicky, 2001; MacQuarrie *et al.*, 2001; Molz *et al.*, 1986; Schäfer *et al.*, 1998).

Furthermore, in literature, an evolving set of kinetic denitrification models of different complexity can be found, leading to simplified descriptions to represent nitrate removal such

as zero-order kinetics (Glass and Silverstein, 1998; Starr and Gillham, 1993) or fist-order kinetics, in which nitrate concentration depends on the nitrate concentration itself (Ocampo *et al.*, 2006) or on the substrate concentration (Sheibley *et al.*, 2003). In a different approach, some authors have also modelled the denitrification rate using a first-order Arrhenius kinetics (Andrews *et al.*, 1997a, b; Senzia *et al.*, 2002) to include the effect of the temperature.

The complexity of describing microbial processes in natural systems and the lack of proper kinetic and stoichiometric parameters have limited the development of denitrification models in the subsurface environment. Thus far in the literature, denitrification models are commonly applied in the wastewater treatment field (Henze *et al.*, 1987; Henze *et al.*, 2000; Pala and Bolukbas, 2005; v. Schulthess and Gujer, 1996). However, few studies simulating intrinsic denitrification (Lee *et al.*, 2006; Wriedt and Rode, 2006) or enhanced denitrification in groundwater systems (Killingstad *et al.*, 2002; Kinzelbach *et al.*, 1991) are found in literature.

Modelling of microbial processes is characterized by two important features. On the one hand, models are usually high-order non-linear systems including a large number of state variables and parameters (e.g. multiple-Monod models) and, on the other hand, there is a lack of reliable techniques for measurement of all the state variables of interest (Vanrolleghem *et al.*, 1995). In addition, a difficulty when modelling biological processes in environmental systems is the selection of the proper parameter values. To date, laboratory studies determining aquifer microbial characteristics are still limited and therefore, it is usual that research on denitrification in groundwater use parameters from the wastewater field (Lee *et al.*, 2006; Widdowson *et al.*, 1988) or estimate model parameters by experimental data fitting (Khalil *et al.*, 2005; Killingstad *et al.*, 2002; Vasiliadou *et al.*, 2006).

In order to overcome the mentioned problems, recent literature points out the need to study the possibility to identify unique values for model parameters, which is known as identifiability study. Two types of identifiability studies are described: the theoretical or structural identifiability which is related to the model structure and the available measured outputs, and the practical identifiability which is based on the experimental conditions and, therefore, on the available data (Petersen, 2001). Some works applying these types of analyses are reported in wastewater literature (Dochain and Vanrolleghem, 2001; Guisasola *et al.*, 2005; Jubany *et al.*, 2005; Petersen *et al.*, 2003) but, to date, no references have been found in the field of groundwater bioremediation modelling.

2. OBJECTIVES

The main objective of this chapter is to develop a mathematical model describing the evolution of nitrate, organic carbon (OC), dissolved oxygen (DO) and biomass when stimulating indigenous aquifer bacteria with an organic carbon source. This main scope involved the following specific objectives:

- To define the microbial kinetics involved in the main processes occurring when applying enhanced denitrification in aquifer systems.
- To develop the theoretical microbial stoichiometric reactions.
- To design and carry out specific experiments to evaluate the developed model and to calibrate specific parameters.
- To study the quality of the estimated parameters.

3. MATERIALS AND METHODS

3.1. EXPERIMENTAL DESIGN

Batch microcosm tests were carried out to calibrate and validate the denitrification model. The experimental design of the tests was the same as in set 4 from Chapter 3 (section 3.4.4). Therefore, 35 g of soil material and 325 mL of groundwater were transferred into a 400 mL-methacrylate flask provided with an adjustable lid that prevented the formation of a headspace in the reactor. In order to promote the denitrification process, the microcosms were amended with glucose (Scharlau).

Tests were maintained in a dark thermostatic chamber at 17°C (Medilow, Selecta) to simulate natural aquifer conditions. The flask was magnetically stirred and equipped with a DO electrode to monitor this parameter continuously. Samples for nitrate and dissolved organic carbon (DOC) were withdrawn twice a day with a sterile syringe. Each time the sampling was done, the lid of the reactor was adjusted to the new microcosm volume, preventing the intrusion of air.

This experimental design was chosen to simplify the modelling. As indicated in Chapter 3, the presence of oxygen in some tests probably caused that the denitrification process did not take place in the whole system but in anaerobic layers of the soil. With this new experimental design without headspace, the whole microcosm was considered to be in the same conditions and, therefore, was modelled as a continuous stirred reactor.

3.2. ANALYTICAL METHODS

Organic carbon (OC) content in microcosms was measured as DOC using a Shimadzu TOC 5050 analyzer. Nitrate was determined by High Performance Liquid Chromatography (HPLC, Agilent 2100 series). DO was continuously measured by means of a CRISON 6050 DO electrode inserted in the liquid phase of the microcosms and coupled with a CRISON OXI 49. Details of all the applied analytical methods are provided in Chapter 3 (section 3.2).

3.3. SIMULATION AND PARAMETER CALIBRATION

The software used for simulation was Simulink® (The MathWorks, Natick, MA), which is a software package for modelling, simulating and analyzing dynamic systems. It supports non-linear systems and provides an interactive graphical environment and a customizable set of block libraries that let an easy construction of models, keeping the structure and dealing with algebraic loops.

Model parameters were introduced using Matlab® (The MathWorks, Natick, MA). This software allows programming and generating series of simulations. In addition, Matlab includes some toolboxes useful for parameter estimation. In particular, in this project, the function 'fmincon.m' from Matlab was used for parameter calibration. This function was programmed in order to work as follows: call the simulink to generate the simulated values, calculate the objective function, propose new parameter values and decide when the convergence is fulfilled. For a better comprehension the calibration algorithm is detailed in Figure 4.2.

Parameter values were sought to minimize the least square objective function (OF), which compares the predicted model values with the experimentally measured data, as expressed in the following general equation:

$$OF = \left(\sum_{i=1}^{n} (exp_i - sim_i)^2 \right)^{1/2} \quad (4.2)$$

in which exp_i represents the experimentally measured concentrations of a particular solute, sim_i represents the predicted model concentrations of the same particular solute, and n is the number of experimental measures.

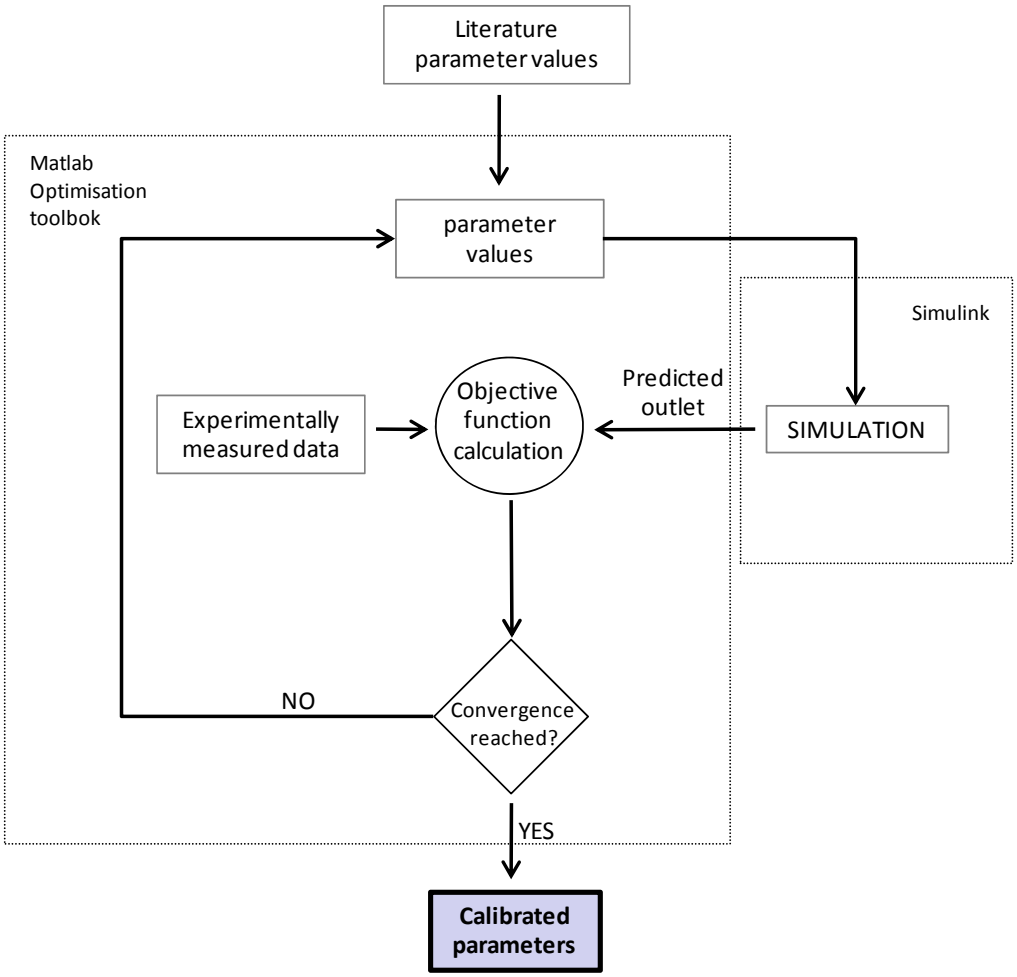

Figure 4.2. Schematic overview of the Matlab calibration algorithm based on parameter optimization.

3.4. EVALUATION OF ESTIMATED PARAMETER QUALITY

Practical identifiability of the optimal estimated parameters was studied to guarantee the reliability of the calibration from available experimental data. Practical identifiability is related to the quality of data and indicates if these data are informative enough to identify the model parameters and, more specifically, to give accurate values (Dochain and Vanrolleghem, 2001). The practical identifiability study was performed using contour plots of the objective function calculated after the modification of pairs of parameters around the optimum predicted values.

A rather important result of the practical identifiability study is the possibility to determine the parameter estimation error. Confidence intervals of estimated parameters were calculated through a numerical method based on the Fisher Information Matrix (FIM). The FIM matrix summarizes the quantity and quality of information obtained from experiments because it considers the sensitivity of calibrated parameters and the measurement errors of experimental data (Dochain and Vanrolleghem, 2001). The FIM matrix can be obtained as follows:

$$FIM = \sum_{i=1}^{n} \left(\frac{\partial y}{\partial p}(t_i, \bar{p})\right)^T Q_i \left(\frac{\partial y}{\partial p}(t_i, \bar{p})\right) \quad (4.3)$$

where the terms $\partial y/\partial p$ are the output sensitivity functions, which quantify the dependence of the model predictions on the parameters values, and Q_i is defined as the inverse of the measurement error covariance matrix, in which *s* is the measurement error.:

$$Q_i = (s^2)^{-1} \quad (4.4)$$

Assuming white measurement noise and no model mismatch, the inverse of the FIM provides the lower bound of the parameter estimation covariance matrix, which can be used for estimating the standard errors (σ) of the calibrated parameters ($\bar{p}$) (Dochain and Vanrolleghem, 2001):

$$\sigma(\bar{p}) = \sqrt{FIM^{-1}} \quad (4.5)$$

Finally, parameter confidence intervals can be obtained as follows:

$$\bar{p} \pm t_{\alpha;N-p}\sigma(\bar{p}) \quad (4.6)$$

for a confidence level specified as 100 (1-α)% with $N - p$ degrees of freedom and t-values obtained from the Student-*t* distribution (Dochain and Vanrolleghem, 2001).

The same approach has been previously used for estimating parameter confidence intervals in other models of biological processes. For instance, Dorado *et al.* (2008) used the FIM in a model simulating toluene abatement in gas biofilters and Guisasola *et al.* (2005) in a model describing nitrification in wastewater. However, previous reported studies have not been found in the field of groundwater bioremediation.

4. RESULTS AND DISCUSSION

4.1. THEORETICAL MODEL DEVELOPMENT

In this section, the development of a model to simulate denitrification and the attendant microbial population growth and decay is presented. The model also includes aerobic oxidation of organic carbon to better simulate a bioremediation process in aerobic aquifers. Therefore, the model intends to simulate the addition of an organic substrate, specifically glucose, in a nitrate-contaminated and initially aerobic aquifer system.

4.1.1. Microbial kinetics

As aforementioned, two redox reactions were considered in the development of the model, the aerobic oxidation of organic carbon and the denitrification, both of them mediated by the same microbial group, heterotrophs. The justification to only consider one microbial group is that most denitrifying bacteria are facultative anaerobes (Korom, 1992), that is microorganisms that ordinarily thrive as aerobes but are capable of switching to growth with nitrate as terminal electron acceptor. Consequently, the model includes only heterotrophic bacteria that can grow aerobically or in anoxic conditions depending on the dissolved oxygen concentration.

Heterotrophic microbial growth was modelled as the sum of two equations (Table 4.1). The first one representing the growth rate in aerobic conditions (oxygen was the terminal electron acceptor) and the second one representing the growth rate in anoxic conditions (nitrate was the terminal electron acceptor). Each growth equation was derived following the multiple-Monod kinetics and, therefore, included terms for substrate (OC) and the electron acceptor (i.e. oxygen or nitrate), in which K_{OC}, K_{O2} and K_{NO3} were the saturation coefficients for substrate, oxygen and nitrate, respectively. In this project, substrate was always expressed as OC which was assimilated to DOC in the experimental measures. An additional inhibition term was considered in the equation for anoxic conditions. This term suppressed anoxic growth as long as oxygen concentration exceeded a certain threshold value, which was expressed with the inhibition coefficient for oxygen ($K_{O2,I}$).

The maximum growth rate of heterotrophic bacteria ($\mu_{\max,\mathrm{H}}$) was considered to be a constant regardless of oxygen conditions. However, the parameter η was included in the growth equation of heterotrophs in anoxic conditions as a correction factor to adjust either the change in heterotrophic growth associated with anoxic conditions or the fact that only a portion of the heterotrophic biomass can denitrify (Batchelor, 1982).

Another process considered was the decay of heterotrophic bacteria (Table 4.1). Decay was modelled as a first order process with respect to heterotrophic biomass concentration (X_H). It was considered that this process was not dependent on aerobic/anoxic conditions and therefore a constant decay rate for heterotrophic bacteria was taken into account (b_H).

Table 4.1. Overview of the process rates considered in the model.

Process	Process rate ($M \cdot L^{-3} \cdot T^{-1}$)
1. Growth of heterotrophic bacteria (X_H)	
1.1 Growth of X_H in aerobic conditions	$\mu_{\max,H} \cdot \frac{OC}{OC + K_{OC}} \cdot \frac{O_2}{O_2 + K_{O_2}} \cdot X_H$
1.2 Growth of X_H in anoxic conditions	$\mu_{\max,H} \cdot \frac{OC}{OC + K_{OC}} \cdot \frac{NO_3^-}{NO_3^- + K_{NO_3^-}} \cdot \frac{K_{O2,I}}{O_2 + K_{O2,I}} \cdot \eta \cdot X_H$
2. Decay of heterotrophic bacteria (X_H)	$b_H \cdot X_H$

4.1.2. Kinetic parameters

Once the kinetic equations were established, a proper value for each kinetic parameter should be assigned. Initial guesses of the parameter values were done following a review of studies related to denitrification modelling (Table 4.2). It must be stressed that reported kinetic parameters are very scattered due to large differences in the way that authors selected them. Most of the reviewed studies obtained parameter values from other bibliographic sources (Lensing *et al.*, 1994; v. Schulthess and Gujer, 1996; Widdowson *et al.*, 1988). In other cases, some of the parameters were calibrated with specific experiments in order to fit the model prediction to experimental data (Doussan *et al.*, 1997; Killingstad *et al.*, 2002; Kinzelbach *et al.*, 1991; Schäfer *et al.*, 1998). In addition, studies that estimated all the parameters were also found. This is the case of Vasiliadou *et al.* (2006), who studied hydrogenotrophic denitrification in batch assays and estimated all the kinetic and stoichiometric parameters from their model (in total, 9 parameters) by experimental data fitting, using the least square method. Furthermore, differences between specific parameters such as maximum growth rates and decay rates among studies could result from the use of different experimental temperatures since it is known that both parameters are directly related to the temperature.

It is generally agreed that aerobic and denitrifying bacteria are the same microbial group, with the same kinetic constants. Differences between maximum growth rates of aerobic and denitrifying bacteria found in literature may be explained by the integration of the η parameter in the constant from denitrifiers. However, it is important to mention that some authors proposed significant differences between both maximum growth rates as, for example, Doussan *et al.* (1997). Moreover, other authors such as Schäfer *et al.* (2004) assumed also small difference between the decay rate of aerobic and denitrifying microorganisms (Table 4.2).

Table 4.2. Overview of published Monod kinetic reaction parameters in denitrification models.

Parameter	Symbol	Value	Units	Remarks	Reference
Maximum growth rate for aerobic bacteria	$\mu_{\max,O2}$	3	d^{-1}	Calibrated. Denitrification in aquifer material. Ambient temperature.	(Schäfer *et al.*, 1998)
		10		Calibrated. Denitrification in aquifer material. Ambient temperature.	(Doussan et al., 1997)
		1.5-2		Calibrated. *In situ* denitrification of groundwater.	(Kinzelbach *et al.*, 1991)
Maximum growth rate for denitrifying bacteria	$\mu_{\max,NO3}$	2.75	d^{-1}	Calibrated. Denitrification in aquifer material. Ambient temperature.	(Schäfer *et al.*, 1998)
		1.4		Calibrated. Denitrification in aquifer material. Ambient temperature.	(Doussan *et al.*, 1997)
		1.5-2		Calibrated. *In situ* denitrification of groundwater.	(Kinzelbach *et al.*, 1991)
		0.65		Calibrated. *In situ* denitrification of groundwater.	(Killingstad *et al.*, 2002)
		1.40		Calibrated. Denitrification in aquifer material at 15°C.	(Killingstad *et al.*, 2002)
Maximum growth rate for heterotrophic bacteria	$\mu_{\max,H}$	6.0	d^{-1}	Typical parameter values at 20°C and neutral pH. WWTP. [a]	(Henze *et al.*, 2000)
		1.13		Calibrated. WWTP.	(Pala and Bolukbas, 2005)
Decay rate constant for aerobic bacteria	b_{O2}	0.3	d^{-1}	Assumed value (10% of the maximum growth rate).	(Schäfer *et al.*, 1998)
		0.15		Calibrated. *In situ* denitrification of groundwater.	(Kinzelbach *et al.*, 1991)
Decay rate constant for denitrifying bacteria	b_{NO3}	0.275	d^{-1}	Assumed value (10% of the maximum growth rate).	(Schäfer *et al.*, 1998)
		0.15		Calibrated. *In situ* denitrification of groundwater.	(Kinzelbach *et al.*, 1991)
		0.05		Based on literature data.	(Zysset *et al.*, 1994)
		0.01		Calibrated. *In situ* denitrification of groundwater.	(Killingstad *et al.*, 2002)
Decay rate constant for heterotrophic bacteria	b_H	0.62	d^{-1}	Typical parameter values at 20°C and neutral pH. WWTP.	(Henze *et al.*, 2000)
		0.05		Calibrated. WWTP.	(Pala and Bolukbas, 2005)
Inhibition coefficient for oxygen	$K_{O2,I}$	6.4	mg $O_2 \cdot L^{-1}$	Calibrated. Denitrification in aquifer material. Ambient temperature.	(Schäfer *et al.*, 1998)
		0.001		Calibrated. Denitrification in aquifer material. Ambient temperature.	(Doussan *et al.*, 1997)
Saturation coefficient for oxygen	K_{O2}	0.2	mg $O_2 \cdot L^{-1}$	Typical parameter values at 20°C and neutral pH. WWTP.	(Henze *et al.*, 2000)
Saturation coefficient for nitrate	K_{NO3}	2.2	mg $NO_3^- \cdot L^{-1}$	Typical parameter values at 20°C and neutral pH. WWTP.	(Henze *et al.*, 2000)
		0.9		Calibrated. *In situ* denitrification of groundwater.	(Killingstad *et al.*, 2002)
		7		Calibrated. Denitrification in aquifer material. Ambient temperature.	(Schäfer *et al.*, 1998)
Saturation coefficient for substrate	K_S	20	mg COD $\cdot L^{-1}$ [b]	Typical parameter values at 20°C and neutral pH. WWTP.	(Henze *et al.*, 2000)
		343		Calibrated. WWTP.	(Pala and Bolukbas, 2005)

[a] WWTP: wastewater treatment plant.
[b] COD: chemical oxygen demand.

To be consistent in this work it was decided to use all the parameters from the same literature reference, the IWA Activated Sludge Model No. 1 (ASM1) at 20°C (Henze *et al.*, 2000). It should be noted that ASM1 parameters resulted from an extensive review from wastewater values and, therefore, constitute one of the most appropriate literature source for biological models.

It is well known that microbial mediated reactions are influenced by temperature but methods to adjust Monod parameters for temperature are scarce (MacQuarrie *et al*., 2001). For this reason, all the parameters from ASM1 were considered not to be dependent on the temperature, except for $\mu_{max,H}$ and b_H. In these cases, it was possible to recalculate them via a power law to incorporate the effect of working at a different temperature (i.e. 17°C) (Doussan *et al.*, 1997):

$$\mu_{max,H}(T) = \mu_{max,H}(20°C) \cdot 1.07^{(T-20)} \quad (4.7)$$

$$b_H(T) = b_H(20°C) \cdot 1.12^{(T-20)} \quad (4.8)$$

in which 20°C is the reference temperature and 1.07 and 1.12 are the constants describing the temperature influence on maximum specific growth rate and decay rate constant, respectively.

The ASM1 parameters are expressed in units of chemical oxygen demand (COD), typically used units in the wastewater field. However, when modelling biodegradation processes in environmental systems the use of COD units is not so well extended and it is preferred to work in mass units. Consequently, in this work two conversion factors, 0.38 g OC · g^{-1} COD and 0.49 g cells · g^{-1} COD, were used to convert COD units to equivalent mass units of organic carbon and cell biomass, respectively. These conversion factors were calculated by the following oxidation reactions of glucose and cells (cells considered as $C_5H_7O_2N$) (Rittmann and McCarty, 2001):

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (4.9)$$

$$4C_5H_7O_2N + 29O_2 \rightarrow 20CO_2 + 4NO_3^- + 14H_2O \quad (4.10)$$

A summary of the initial kinetic parameters used in simulations with the appropriate units is provided in Table 4.3.

Table 4.3. Initial kinetic parameters for the denitrification model at 17°C.

Parameter	Symbol	Value	Units
Maximum growth rate for heterotrophs	$\mu_{max,H}$	4.90	d^{-1}
Reduction factor for anoxic conditions	η	0.8	--
Saturation coefficient for nitrate	$K_{NO_3^-}$	2.21	mg NO_3^-·L^{-1}
Saturation coefficient for oxygen	K_{O_2}	0.20	mg O_2·L^{-1}
Saturation coefficient for organic carbon	K_{OC}	7.41	mg OC·L^{-1}
Inhibition coefficient for oxygen	$K_{O_{2,I}}$	0.20	mg O_2·L^{-1}
Decay rate constant for heterotrophs	b_H	0.44	d^{-1}

4.1.3. Stoichiometry

Development of stoichiometric reactions for aerobic oxidation of glucose and denitrification was performed following the theoretical derivation described in Chapter 3 (section 4.3). In this case, *fs* and *fe* values were considered to be 0.67 and 0.33, respectively, for heterotrophic bacteria growing in aerobic and anoxic conditions. These values were calculated from the yield coefficient for heterotrophs (Y_H) given by ASM1, 0.67 (Henze *et al.*, 2000). It should be stressed that *fs* is the dimensionless form of Y_H (g cell COD formed·g^{-1} COD oxidized) (Rittmann and McCarty, 2001). The developed stoichiometric reactions of microbial growth under aerobic and anoxic conditions are given in equations 4.11 and 4.12, respectively.

$$0.042C_6H_{12}O_{6(aq)} + 0.094\ O_{2(g)} + 0.022NO^-_{3(aq)} + 0.022H^+_{(aq)} \rightarrow$$
$$0.022C_5H_7O_2N_{(cell)} + 0.138CO_{2(g)} + 0.183\ H_2O \quad (4.11)$$

$$0.042C_6H_{12}O_{6(aq)} + 0.097NO^-_{3(aq)} + 0.097H^+_{(aq)} \rightarrow$$
$$0.037N_{2(g)} + 0.022C_5H_7O_2N_{(cell)} + 0.138CO_{2(g)} + 0.220\ H_2O \quad (4.12)$$

From these equations the theoretical stoichiometric ratios needed to complete the model were derived. Table 4.4 summarizes the stoichiometric parameters with the appropriate units used in simulations.

Table 4.4. Initial stoichiometric parameters for the denitrification model at 17°C.

Parameter	Symbol	Value	Units
Growth yield for heterotrophs	Y_H	0.91	mg cells·mg OC^{-1}
Ratio NO_3^-/OC in denitrification	Z	1.86	mg NO_3^-·mg OC^{-1}
Ratio O_2/OC in aerobic process	W	0.88	mg O_2·mg OC^{-1}
Ratio NO_3^-/OC in aerobic process	R	0.49	mg NO_3^-·mg OC^{-1}

4.1.4. Enhanced denitrification model

The complete mathematical formulation for rates of consumption or production of dissolved electron acceptors (i.e. nitrate and oxygen), electron donors (i.e. organic carbon) and biomass is presented in a matrix format in Table 4.5.

The matrix was constructed following the scheme of a typical Peterson matrix (Henze *et al.*, 1987), which is well extended in literature to describe biological models. All the components in the model are listed by symbol across the top of the table while their definitions are given across the bottom. The three processes considered are listed down the extreme left column while the rate expressions chosen to represent them are listed on the extreme right. The kinetic parameters chosen in the rate expressions are defined in the lower right corner. The body of the matrix contains the stoichiometric coefficients, which are defined in the lower left corner.

Table 4.5. Kinetic and stoichiometric model for enhanced denitrification in groundwater systems.

Component, i → / **Process, j ↓**	**OC**	**X_H**	**NO_3^-**	**O_2**	**Process Rate, ρ_j (M·L^{-3}·T^{-1})**
1. Growth of X_H					
1.1 Growth of X_H in aerobic conditions	$-\frac{1}{Y_H}$	1	$-\frac{R}{Y_H}$	$-\frac{W}{Y_H}$	$\mu_{\max,H} \cdot \frac{OC}{OC + K_{OC}} \cdot \frac{O_2}{O_2 + K_{O2}} \cdot X_H$
1.2 Growth of X_H in anoxic conditions	$-\frac{1}{Y_H}$	1	$-\frac{Z}{Y_H}$		$\mu_{\max,H} \cdot \frac{OC}{OC + K_{OC}} \cdot \frac{NO_3^-}{NO_3^- + K_{NO3}} \cdot \frac{K_{O2,I}}{O_2 + K_{O2,I}} \cdot \eta \cdot X_H$
2. Decay of X_H		-1			$b_H \cdot X_H$
Observed conversion rates [ML^{-3}T^{-1}]		$r_i = \sum_j \nu_{ij} \rho_j$			
Stoichiometric parameters Heterotrophic growth yield: Y_H Stoichiometric ratios: Z (NO_3^-/OC anoxic conditions) R (NO_3^-/OC aerobic conditions) W (O_2/OC)	Organic Carbon [M(OC)·L^{-3}]	Active heterotrophic biomass [M(cells)·L^{-3}]	Nitrate [M(NO_3^-)·L^{-3}]	Oxygen [M(O_2)·L^{-3}]	Kinetic parameters Heterotrophic growth and decay: $\mu_{max,H}$, K_{OC}, K_{O2},$K_{O2,I}$, K_{NO3}, b_H, Correction factor for anoxic growth of heterotrophs: η

4.2. MODEL SIMULATION AND CALIBRATION

The goal of this section is to test the developed microbial model with denitrification microcosm experiments and calibrate the most outstanding parameters. With this purpose, two different experiments were carried out.

It must be noticed that the obtained model (Table 4.5) is fourth order, since it includes four state variables (oxygen, nitrate, heterotrophic biomass and OC). It is an internal model where all the parameters keep their physical meaning. Furthermore, it is a non-linear continuous system and any of the available simulation algorithms in Matlab converged.

4.2.1. First experimental design: single pulse test

A first test was performed as detailed in section 3.1. It consisted in a three day experiment in which groundwater containing 76 $mg \cdot L^{-1}$ of nitrate (collected in the sampling campaign from 2007) was amended with 200 $mg \cdot L^{-1}$ of glucose and kept in contact with soil (also collected on 2007). Furthermore, initial dissolved oxygen concentration was 9 $mg \cdot L^{-1}$, which corresponds to the oxygen content of water in equilibrium with atmospheric oxygen at 17°C.

The applicability and accuracy of the developed model applying the parameters from Tables 4.3 and 4.4 was first evaluated by comparing model predicted profiles with experimental data (Figure 4.2). In this simulation, an initial biomass concentration ($X_{H,0}$) of $3.2 \cdot 10^{-3}$ $mg \cdot L^{-1}$ was used. This value was based on the Most Probable Number (MPN) of denitrifiers in the collected soil (10^5 $bacteria \cdot g^{-1}$ dry weight soil), the soil/water ratio used in the experiments and an assumed cell mass of $3 \cdot 10^{-13}$ $g \cdot bacteria^{-1}$ (Madigan *et al.*, 2000). It should be noted that biomass concentration was not measured throughout the experiment and, therefore, the consistency of the model for this state variable could not be evaluated.

In Figure 4.3 it can be observed that the model could reasonably explain the overall behaviour of the measured concentrations of oxygen, nitrate and DOC although, an obvious delay in time was present. Therefore, these results indicated that the developed model was appropriate to describe the processes involved when stimulating with glucose bacteria from the aquifer material in microcosm tests, but a better estimation of the input parameters was needed.

A simple screening sensitivity analysis was carried out by manually varying parameters. The sensitivity analysis was aimed to identify the model parameters influencing most significantly the model results. This analysis revealed that simulation results were sensitive to changes in

primarily three input parameters: $\mu_{max,H}$, b_H and $X_{H,0}$. Specially, the latter turned out to be crucial.

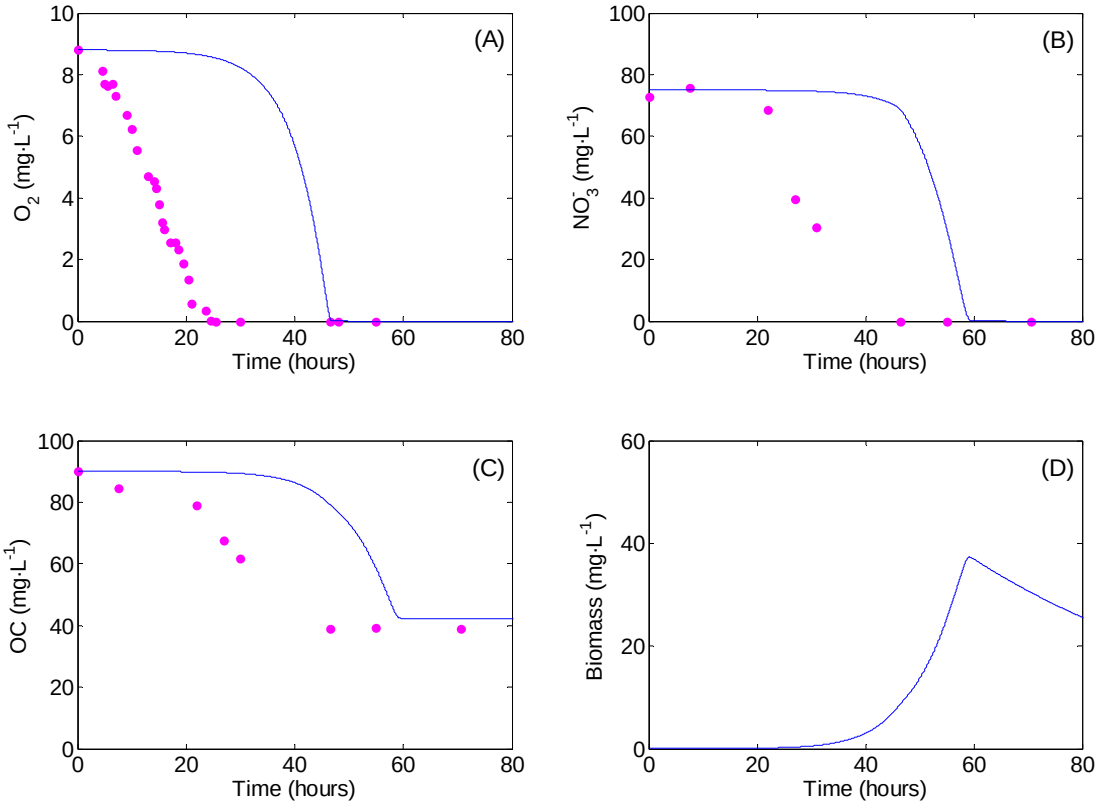

Figure 4.3. Single pulse test. Experimental results (●) and model predictions with initial parameters (Tables 4.3 and 4.4) (—): (A) dissolved oxygen, (B) nitrate, (C) organic carbon and (D) biomass (only model predictions).

Therefore, experimental data from the batch test was used to calibrate $\mu_{max,H}$, b_H and $X_{H,0}$. The objective function to be minimised was considered to be as follows:

$$OF = \left(\frac{1}{n}\sum_{i=1}^{n}(NO_{3,exp,i}^{-} - NO_3^{-}(\bar{p})_i)^2\right)^{1/2} + C \cdot \left(\frac{1}{m}\sum_{i=1}^{m}(O_{2,exp,i} - O_2(\bar{p})_i)^2\right)^{1/2} \quad (4.13)$$

in which $NO_{3,exp,i}^{-}$ and $NO_3^{-}(p)_i$ are vectors of *n* measured values and model predictions for nitrate at times t_i (*i* from 1 to *n*), $O_{2,exp,i}$ and $O_2(p)_i$ are vectors of *m* measured values and model predictions for oxygen at times t_i (*i* from 1 to *m*), and $\bar{p}$ is the vector of model parameters. The factor *C*, that multiplies the oxygen term, took a value of 10. This factor was included in the objective function to homogenize the order of magnitude of the two variables (oxygen and nitrate). Therefore, in the objective function, both nitrate and oxygen measures had the same weight, though *n* and *m* were different.

It should be emphasized that, in this work, it was considered that measures of oxygen and nitrate should be used to calibrate the model, whereas in reported studies, objective functions usually consider only one experimental measure to calibrate biological models (Dorado *et al.*, 2008; Jubany *et al.*, 2005; Khalil *et al.*, 2005). Obviously, nitrate was selected as the most outstanding variable to explain the denitrification process in the microcosm system. In addition, oxygen measures were considered to give reliable information about the initial conditions and, therefore, were included in the objective function to properly calibrate the $X_{H,0}$ parameter.

Organic carbon was not included in the objective function although it was also analysed throughout the experiment. The reason to reject this variable was to simplify the optimisation procedure. It should be noted that the consumption of OC in the model was directly related to the nitrate and oxygen removal by stoichiometric ratios, and therefore it was not necessary to include it for the parameter calibration.

The minimization of the objective function (equation 4.13) using the constrained optimization algorithm (Figure 4.2) gave the optimum parameter values. The upper and lower bounds for the parameters were previously established considering wide ranges that covered all the found literature parameters. Results of the estimated $\mu_{max,H}$, b_H and $X_{H,0}$ were 3.24 d^{-1}, $2.40 \cdot 10^{-3}$ d^{-1} and 0.92 $mg \cdot L^{-1}$, respectively (Table 4.6).

These results were significantly different from the initial considered values (Table 4.3). $X_{H,0}$ presented the largest deviation from the initial guess considered (almost three orders of magnitude). This could be explained by the uncertainty associated with the estimation of this parameter using the MPN and the following conversion to proper model units. Calibrated $\mu_{max,H}$ was observed to differ almost 34% from the initial considered value and, regarding the b_H, it should be noted that was optimised to a practically negligible value.

Model predictions by applying the calibrated parameters are shown in Figure 4.4. As it can be observed, when using these parameter values the output model concentrations of oxygen, nitrate and organic carbon fit well to the experimental data. Concerning the biomass, it can be seen that, unlike Figure 4.3D, mortality is almost unappreciated once the oxygen and nitrate concentrations are depleted, since the calibrated decay constant is almost zero.

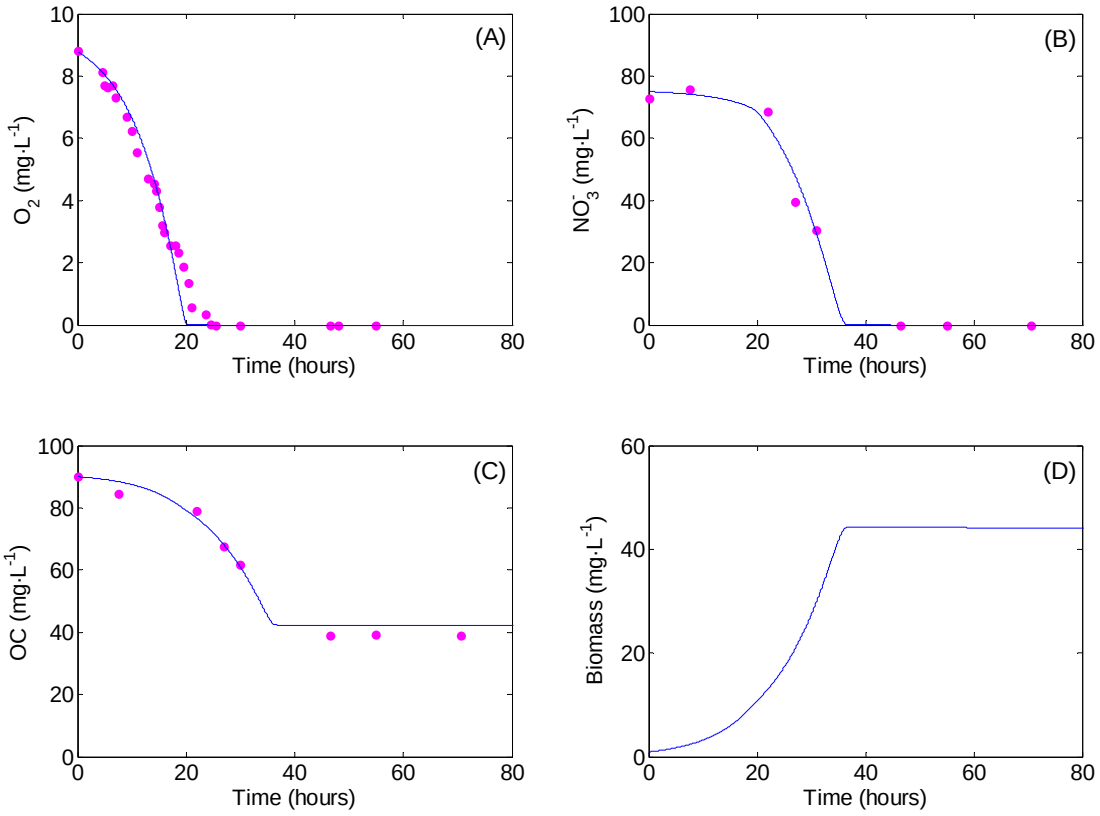

Figure 4.4. Single pulse test. Experimental results (●) and model predictions with the estimated parameters (Table 4.6) (—): (A) dissolved oxygen, (B) nitrate, (C) organic carbon and (D) biomass (only model predictions).

As previously mentioned, practical identifiability of the obtained parameters was analysed by means of contour plots of the objective function with respect to different pairs of parameters. Obtained results are depicted in Figure 4.5.

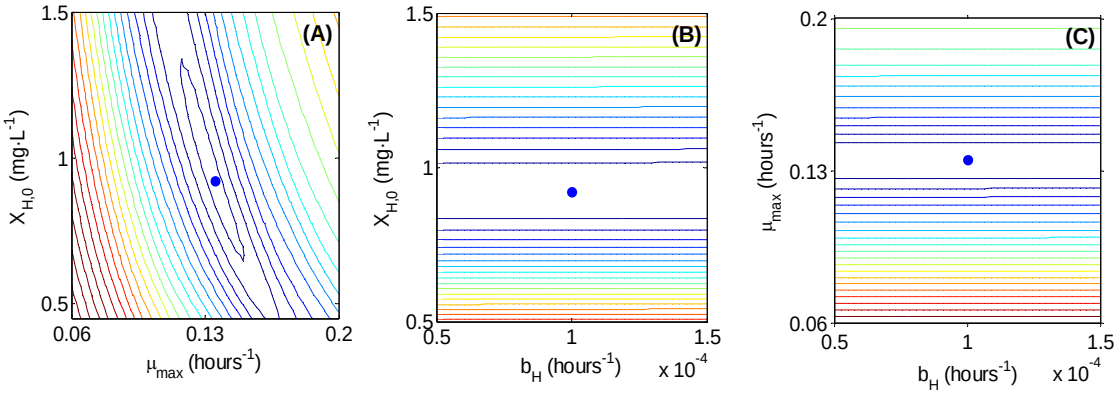

Figure 4.5. Contour plots of the objective function for pairs of parameters: (A) μ_{max}-$X_{H,0}$ (B) b_H-$X_{H,0}$ (C) b_H- μ_{max} and optimised parameters values (●).

A long valley is observed in Figure 4.5A, indicating that $\mu_{max,H}$ and $X_{H,0}$ are quite correlated parameters. This means that close to the minimum, a deviation of one of these parameters

could be compensated by a shift in the other parameter still producing a satisfying fit between experimental data and model predictions.

Figure 4.5 (B and C) shows that any value of b_H within a range near the optimised value produced the same fitting, which means that the objective function did not change. However, it should be noted that these figures may give an erroneous conclusion about this parameter. The sensitivity analysis proved that b_H was a sensitive parameter in this model. This can be observed by plotting the objective function with respect to $X_{H,0}$ and b_H (Figure 4.6) or with respect to $\mu_{max,H}$ and b_H (data not shown). Close to zero, the decay rate had no effect in the fitting of the model but, at higher values, the objective function became larger indicating a worst fitting of the model. Considering that the decay rate of heterotrophs cannot be negligible, this means that the experimental design was not appropriate to calibrate this parameter.

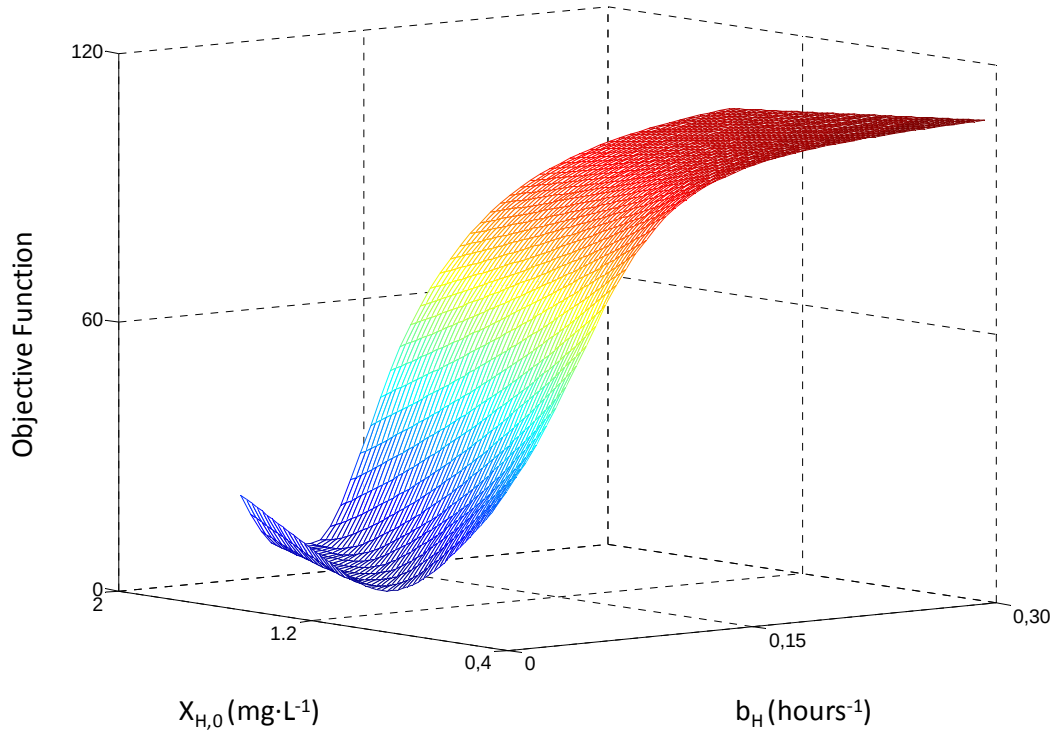

Figure 4.6. Plot of the objective function with respect to the initial biomass concentration and the decay rate constant in the single pulse experiment.

Although it has been demonstrated that this experiment was not useful for the parameters estimation, parameter confidence intervals were calculated. The general FIM expression (equation 4.3) was extended to take into account the two measured variables i.e. oxygen and nitrate:

$$FIM = \sum_{i=1}^{n} (\mathrm{Y}(\mathrm{t_i}, \overline{\mathrm{p}}))^T \; Q_i \; (\mathrm{Y}(\mathrm{t_i}, \overline{\mathrm{p}})) \tag{4.14}$$

where $\mathrm{Y}(\mathrm{t_i}, \overline{\mathrm{p}})$ refers to the output nitrate and oxygen sensitive functions with respect to the optimised parameters at times t_i (i =1 to n predicted values), and it was expressed as:

$$Y = \begin{pmatrix} \frac{\partial NO_3^-}{\partial \mu_{max,H}}(\mathrm{t_i}, \overline{\mathrm{p}}) & \frac{\partial NO_3^-}{\partial b_H}(\mathrm{t_i}, \overline{\mathrm{p}}) & \frac{\partial NO_3^-}{\partial X_{H,0}}(\mathrm{t_i}, \overline{\mathrm{p}}) \\ \frac{\partial O_2}{\partial \mu_{max,H}}(\mathrm{t_i}, \overline{\mathrm{p}}) & \frac{\partial O_2}{\partial b_H}(\mathrm{t_i}, \overline{\mathrm{p}}) & \frac{\partial O_2}{\partial X_{H,0}}(\mathrm{t_i}, \overline{\mathrm{p}}) \end{pmatrix} \tag{4.15}$$

And Q_i refers to the inverse of the measurement error covariance matrix. In this case, the Q_i was considered constant with time and was a 2x2 matrix:

$$Q = \begin{pmatrix} s_{NO3}^2 & 0 \\ 0 & s_{O2}^2 \end{pmatrix}^{-1} \tag{4.16}$$

where s_{NO3} and s_{O2} are the measurement errors for nitrate and oxygen calculated as the standard deviation of different measurements of sample replicates in different days. In this study s values of 1.5 mg·L^{-1} for nitrate and 0.04 mg·L^{-1} for oxygen were obtained.

Confidence intervals assessed through the FIM method considering a confidence level of 95% are indicated in Table 4.6. As it can be observed, calculated confidence intervals were very high compared to the obtained parameter values. And, even in the case of b_H, the confidence interval was higher than the estimated parameter value.

Table 4.6. Optimal estimated parameters and calculated confidence intervals with the single pulse test.

Parameter	Units	Value	Confidence interval
$\boldsymbol{\mu_{max,H}}$	d^{-1}	3.24	0.11
$\boldsymbol{b_H}$	d^{-1}	2.40·10^{-3}	0.09
$\boldsymbol{X_{H,0}}$	mg·L^{-1}	0.92	0.80

Overall, the analyses of the quality of the estimated parameters demonstrated that the experimental data was not appropriate to calibrate the model. The single pulse experiment made impossible to calibrate b_H, because mortality did not play an important role in such short experiment and calculated confidence intervals show the uncertainty associated with the model parameters. It should be pointed out that the experimental procedure made it difficult

to have many samples and this limitation could be directly related to the accuracy obtained in the estimated parameters.

In order to overcome this type of practical identifiability problems, different authors have proposed using experiments with fedbatch operation, that is, with injection of additional substrate at an optimal time in the course of the tests (Dochain and Vanrolleghem, 2001; Jubany *et al.*, 2005). Following this idea, a new batch experiment was performed.

4.2.2. Second experimental design: multiple pulse test

The new batch experiment was carried out following the experimental design detailed in section 3.1 and consisted of four consecutive nitrate and glucose pulses each time the nitrate concentration was depleted.

It is important to note that soil and groundwater for this test were collected in the sampling campaign from 2009, which means that subsurface water contained around 50 mg·L^{-1} of NO_3^- (Chapter 3, Table 3.4). In order to reproduce the nitrate conditions of other sampling campaigns, it was decided to adjust the initial nitrate concentration to about 100 mg·L^{-1} by means of KNO_3 (Scharlau). In addition, KNO_3 was used each time a new pulse was added. For an easier comprehension details of the experimental procedure are synthesized in Table 4.7.

Table 4.7. Experimental pulses of nitrate and glucose in the batch test.

Pulse	O_2 (mg·L^{-1})	NO_3^- (mg·L^{-1})	Glucose (mg·L^{-1})
1	9	100	200
2	<D.L.[a]	50	100
3	<D.L.	180	100
4	<D.L.	180	300

[a] <D.L.: below detection limit

As indicated in Table 4.7, DO was only present in the first pulse. Other pulses were done by injection in the microcosm few millilitres of nitrate- and glucose-highly concentrated groundwater thus, the addition of oxygen was practically negligible. Indeed, no oxygen was detected in the microcosm with the DO electrode after any of the three pulses.

Calibration of the goal parameters was performed following the same procedure that in the single pulse test and obtained values were 4.93 d^{-1}, 0.83 d^{-1} and 0.47 mg·L^{-1} for $\mu_{max,H}$, b_H and $X_{H,0}$, respectively (Table 4.8).

A rather important observation is that the obtained $\mu_{max,H}$ and b_H values were in the same order of magnitude that the literature values initially considered (Table 4.3). Optimised $\mu_{max,H}$ was practically equal to the ASM1 value (0.6% difference), whereas the obtained b_H differed about 47% from the initial considered value. Therefore, these results seem to indicate that the kinetic parameters from ASM1 are appropriate when simulating the stimulation of indigenous bacteria from aquifer. Moreover, the calibrated initial biomass concentration of 0.47 mg·L^{-1}, is consistent with, for example, the initial heterotrophic biomass concentration of 0.1 mg·L^{-1} assumed by MacQuarrie *et al.* (2001) in a model simulating the aerobic oxidation and denitrification of a wastewater plume in a shallow aquifer.

The objective function (equation 4.13) was calculated with different parameter values around the optimum as in the first experimental design (single pulse test). Figure 4.7 shows the contour plots of the objective function with respect to different pairs of parameters and the optimal parameter values. It can be observed that, for all pairs of parameters, closed contours were achieved with this test. In other words, this means that a small deviation in the parameters had a considerable effect on the objective function, and therefore the practical identifiability of the parameters was improved.

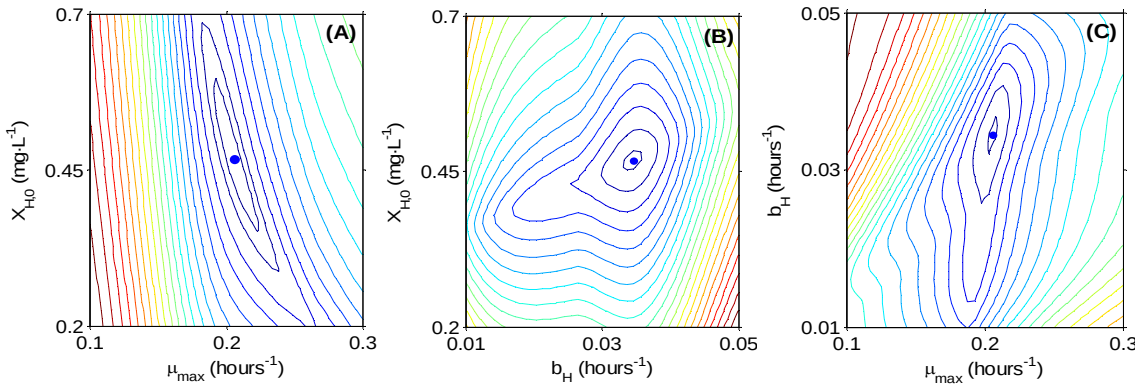

Figure 4.7. Contour plot of the objective function for pairs of parameters: (A) μ_{max}-$X_{H,0}$, (B) b_H-$X_{H,0}$ and (C) μ_{max}- b_H and optimised parameters values (●).

In Figure 4.8 the plot of the objective function with respect to $X_{H,0}$ and b_H is shown. A minimum of the objective function is clearly represented corroborating that parameters have a unique optimum value. Plots of the objective function with respect to the other parameters are not presented since they all have similar profiles.

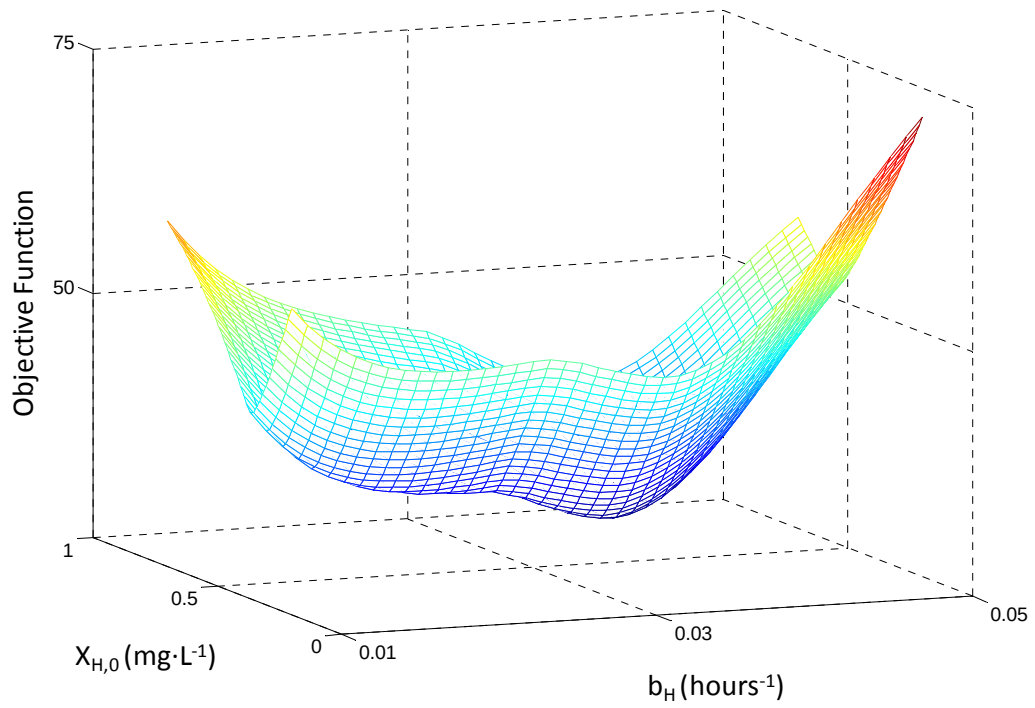

Figure 4.8. Plot of the objective function with respect to the initial biomass concentration and decay rate constant in the multiple pulse test.

Model predictions using the optimal estimated parameters together with the experimental results are depicted in Figure 4.9. It can be observed that the model described accurately oxygen and nitrate removal by heterotrophic bacteria, corroborating that it can properly be used to simulate enhanced denitrification in microcosms with aquifer material. To simulate OC, the initial experimentally measured DOC concentration in each pulse was used. However, the model could not properly fit DOC profile, specially in the second pulse (Figure 4.9C). Main differences were due to disagreement between the theoretical considered stoichiometric ratios (i.e. Z, W and R) and the experimentally ratios obtained. These differences could be explained mainly by two reasons. On the one hand, it should be remembered that the considered stoichiometric ratios were obtained following a theoretical development, which reasonably might not agree with environmental processes. In addition, f_s was considered as a constant value throughout the experiment, but it is known that it may change with numerous environmental factors, such as the available substrate or the biodegradable fraction of microorganisms, among others (Zhou, 2007). On the other hand, experimental characteristics such as the entrance of low quantities of oxyen in the microcosm or the presence of small amounts of other organic carbon substances could have influenced the experimental results. Concerning the biomass, it should be mentioned that the model predicted an important mortality each time oxygen and nitrate were depleted (Figure 4.9D).

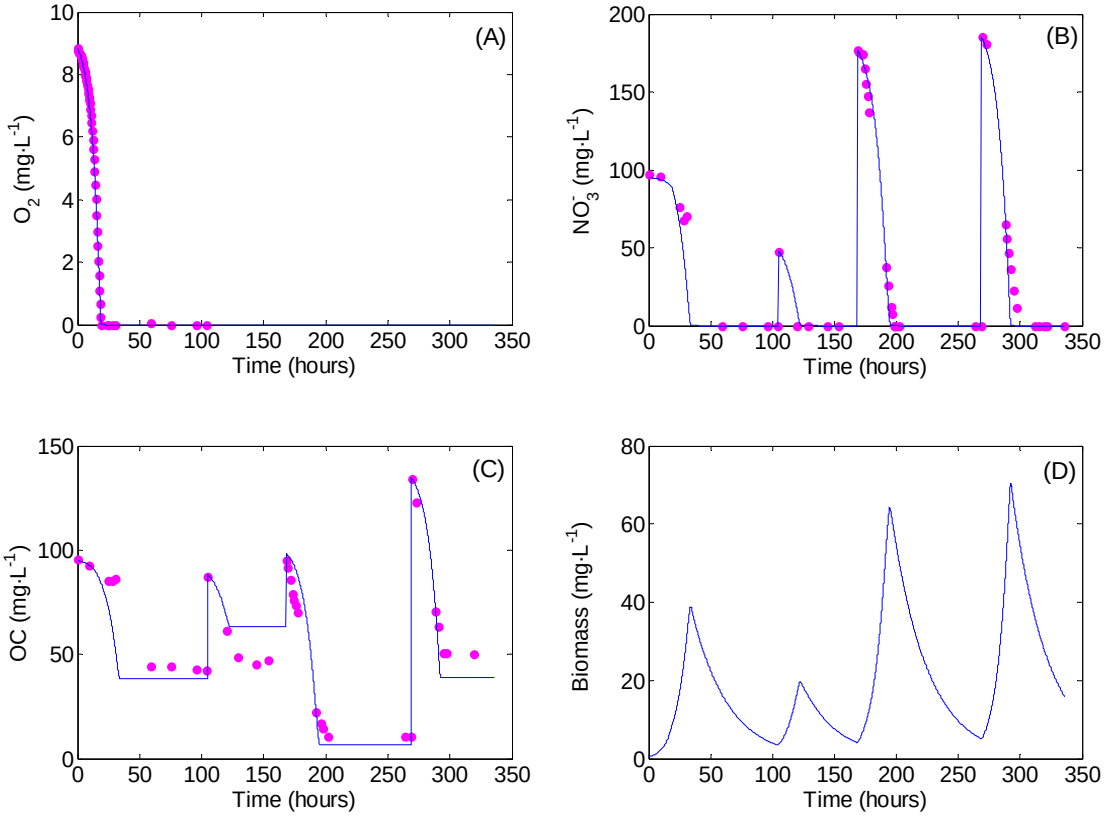

Figure 4.9. Multiple pulse test. Experimental results (●) and model predictions with the estimated parameters (Table 4.8) (—): (A) dissolved oxygen, (B) nitrate, (C) organic carbon and (D) biomass (only model predictions).

Calculated confidence intervals are indicated in Table 4.8. As previously reported by Dochain and Vanrolleghem (2001), it should be noted that these confidence intervals are very small since they do not consider modelling errors and only the measurement errors are included in the matrix Q_i. More reliable approaches to estimate confidence intervals can only be applied when working with a single state variable by evaluating the residual mean square (Dochain and Vanrolleghem, 2001). Although the method applied might underestimate the confidence intervals, it can be concluded that the use of the multiple pulse test resulted in a better calibration of the kinetic parameters with respect to the use of the one pulse test, as also indicated by the contour plots analyses (Figure 4.7).

Table 4.8. Obtained parameters and calculated confidence intervals with the multiple pulse test.

Parameter	Units	Value	Confidence interval
$\boldsymbol{\mu_{max,H}}$	d^{-1}	4.93	$7.0 \cdot 10^{-6}$
$\boldsymbol{b_H}$	d^{-1}	0.83	$9.1 \cdot 10^{-7}$
$\boldsymbol{X_{H,0}}$	$mg \cdot L^{-1}$	0.47	$2.7 \cdot 10^{-5}$

4.2.3. Model evaluation

The optimal estimated parameters obtained with the multiple pulse experiment were tested against experimental data from the single pulse test (section 4.2.1), and it was proved that there was reasonable agreement between model predictions and the measured data (Figure 4.10). Hence, it was clearly demonstrated that the new experiment design, consisting of four consecutive denitrification pulses, was useful to calibrate the goal parameters and that the developed model could explain the main processes involved in the microcosm tests.

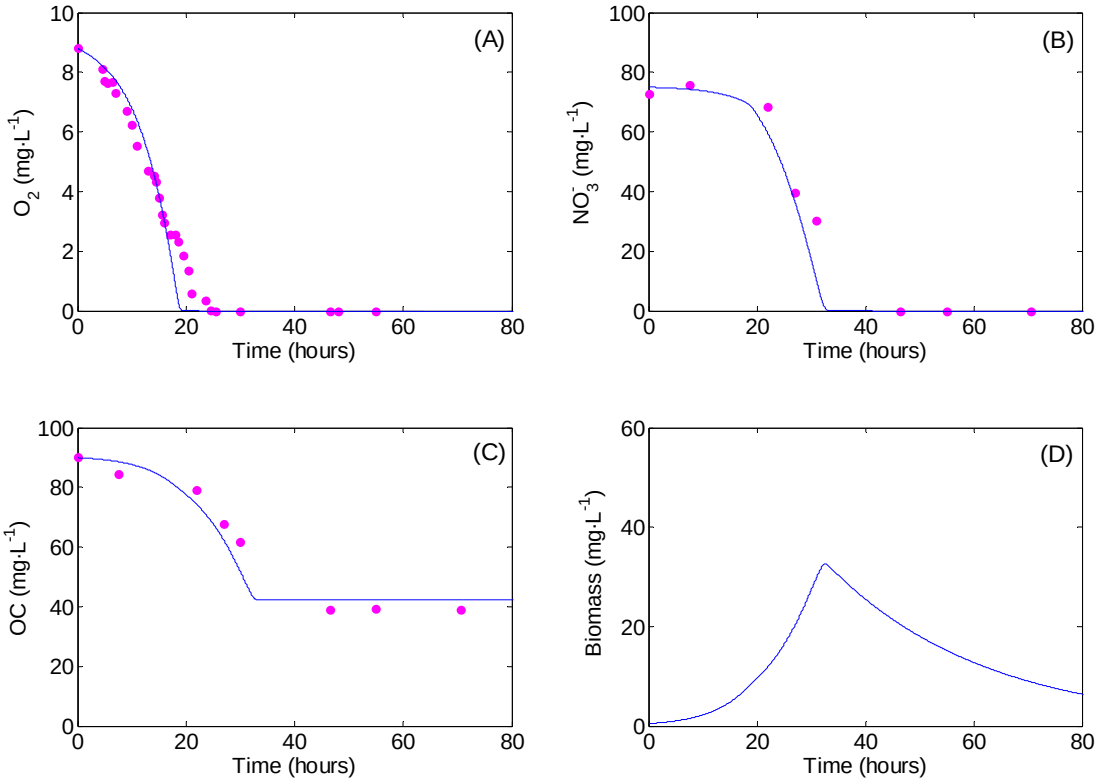

Figure 4.10. Single pulse test. Experimental results (●) and model predictions with the estimated parameters (Table 4.8) (—): (A) dissolved oxygen, (B) nitrate, (C) organic carbon and (D) biomass (only model predictions).

4.3. SUMMARY OF MODEL PARAMETERS

Table 4.9 summarizes all the model parameters required to describe the enhanced denitrification process in groundwater systems.

Table 4.9. Stoichiometric and kinetic parameters for the denitrification model at 17°C

Parameter	Symbol	Value	Units
KINETIC PARAMETERS			
Maximum growth rate for heterotrophs	$\mu_{max,H}$	4.93 [b]	d^{-1}
Reduction factor for denitrification	η	0.80 [a]	dimensionless
Saturation coefficient for nitrate	K_{NO3}	2.21 [a]	mg NO_3^- · L^{-1}
Saturation coefficient for oxygen	K_{O2}	0.20 [a]	mg O_2 · L^{-1}
Saturation coefficient for organic carbon	K_{OC}	7.41 [a]	mg OC · L^{-1}
Inhibition coefficient for oxygen	$K_{O2,I}$	0.20 [a]	mg O_2 · L^{-1}
Decay rate constant	b_H	0.83 [b]	d^{-1}
STOICHIOMETRIC PARAMETERS			
Growth yield for heterotrophs	Y_H	0.91 [a]	mg cells · mg OC^{-1}
Ratio NO_3^-/OC in denitrification	Z	1.86 [b]	mg NO_3^- · mg OC
Ratio O_2/OC in aerobic process	W	0.88 [b]	mg O_2 · mg OC
Ratio NO_3^-/OC in aerobic process	R	0.49 [b]	mg NO_3^- · mg OC
OTHER PARAMETERS			
Initial biomass concentration	$X_{H,0}$	0.47 [b]	mg · L^{-1}

[a] From Henze *et al.* (2000).
[b] Calibrated in this project with the multiple pulse test.

5. CONCLUSIONS

A mathematical model to describe enhanced denitrification in aquifer systems considering the aerobic oxidation of organic matter and denitrification itself was developed. Stoichiometric coefficients derived from the formulation of theoretical biological reactions of aerobic glucose oxidation and denitrification were included in the model.

Kinetic and stoichiometric parameters from an activated sludge model (ASM1) were initially used for model simulations. Results indicated that the model could explain the main processes involved in microcosm tests containing groundwater and soil from a nitrate-contaminated aquifer and amended with glucose to promote the denitrification process. The most sensitive parameters aimed to be calibrated were $\mu_{max,H}$, b_H and $X_{H,0}$.

A single denitrification test was not appropriate to calibrate the parameters. Practical identifiability analyses revealed that $\mu_{max,H}$ and $X_{H,0}$ were quite correlated parameters and b_H could not be calibrated. In addition, calculation of confidence intervals demonstrated the large uncertainty of the calibrated values.

A new experimental design consisting of four consecutive denitrification tests was applied. The estimated parameters using this experimental procedure were $\mu_{max,H}$ = 4.93 d^{-1}, b_H = 0.83 d^{-1} and $X_{H,0}$ = 0.47 $mg \cdot L^{-1}$. The optimised parameter values were consistent with published values, $\mu_{max,H}$ was practically equal to the initially considered ASM1 value (0.6% difference).

Predicted nitrate and oxygen profiles fit well with experimental results. Total predicted OC consumption due to nitrate and oxygen respiration slightly differ from the experimental results. This could be related to the uncertainty associated with microbial stoichiometric reactions and/or experiment characteristics.

The contour plots of the objective function and confidence intervals of the calibrated parameters showed that the four pulse experiment design improved parameter identifiability.

6. REFERENCES

Andrews, R.J., Lloyd, J.W. and Lerner, D.N. 1997a. Modelling of nitrate leaching from arable land into unsaturated soil and chalk - 1. Development of a management model for applications of sewage sludge and fertilizer. *Journal of Hydrology*, 200, 179-197.

Andrews, R.J., Lloyd, J.W. and Lerner, D.N. 1997b. Modelling of nitrate leaching from arable land into unsaturated soil and chalk - 2. Model confirmation and application to agricultural and sewage sludge management. *Journal of Hydrology*, 200, 198-221.

Bae, W. and Rittmann, B.E. 1996. A structured model of dual-limitation kinetics. *Biotechnology and Bioengineering*, 49, 683-689.

Batchelor, B. 1982. Kinetic-analysis of alternative configurations for single-sludge nitrification denitrification. *Journal Water Pollution Control Federation*, 54, 1493-1504.

Dochain, D. and Vanrolleghem, P. 2001. Dynamical modelling and estimation in wastewater treatment processes. London: IWA Publishing.

Dorado, A.D., Baquerizo, G., Maestre, J.P., Gamisans, X., Gabriel, D. and Lafuente, J. 2008. Modeling of a bacterial and fungal biofilter applied to toluene abatement: Kinetic parameters estimation and model validation. *Chemical Engineering Journal*, 140, 52-61.

Doussan, C., Poitevin, G., Ledoux, E. and Detay, M. 1997. River bank filtration: Modelling of the changes in water chemistry with emphasis on nitrogen species. *Journal of Contaminant Hydrology*, 25, 129-156.

Glass, C. and Silverstein, J. 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research*, 32, 831-839.

Gu, C.H., Hornberger, G.M., Mills, A.L., Herman, J.S. and Flewelling, S.A. 2007. Nitrate reduction in streambed sediments: Effects of flow and biogeochemical kinetics. *Water Resourc. Res.* 43.

Guisasola, A., Jubany, I., Baeza, J.A., Carrera, J. and Lafuente, J. 2005. Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation. *Journal of Chemical Technology and Biotechnology*, 80, 388-396.

Henze, M., Grady, C.P.L., Gujer, W., Marais, G.V.R. and Matsuo, T. 1987. A general-model for single-sludge waste-water treatment systems. *Water Research*, 21, 505-515.

Henze, M., Gujer, W., Mino, T. and van Loosdrecht, M. 2000. Activated sludge models ASM1, ASM2, ASM2D and ASM3. Scientific and technical report no.9. London: IWA Publishing.

Jubany, I., Baeza, J.A., Carrera, J. and Lafuente, J. 2005. Respirometric calibration and validation of a biological nitrite oxidation model including biomass growth and substrate inhibition. *Water Research*, 39, 4574-4584.

Khalil, K., Renault, P., Guerin, N. and Mary, B. 2005. Modelling denitrification including the dynamics of denitrifiers and their progressive ability to reduce nitrous oxide: comparison with batch experiments. *European Journal of Soil Science*, 56, 491-504.

Killingstad, M.W., Widdowson, M.A. and Smith, R.L. 2002. Modeling enhanced in situ denitrification in groundwater. *Journal of Environmental Engineering-Asce*, 128, 491-504.

Kindred, J.S. and Celia, M.A. 1989. Contaminant transport and biodegradation. 2. conceptual-model and test simulations. *Water Resources Research*, 25, 1149-1159.

Kinzelbach, W., Schäfer, W. and Herzer, J. 1991. Numerical modeling of natural and enhanced denitrification processes in aquifers. *Water Resources Research*, 27, 1123-1135.

Korom, S.F. 1992. Natural denitrification in the saturated zone - a review. *Water Resources Research*, 28, 1657-1668.

Lee, M.S., Lee, K.K., Hyun, Y.J., Clement, T.P. and Hamilton, D. 2006. Nitrogen transformation and transport modeling in groundwater aquifers. *Ecological Modelling*, 192, 143-159.

Lensing, H.J., Vogt, M. and Herrling, B. 1994. Modeling of biologically mediated redox processes in the subsurface. *Journal of Hydrology*, 159, 125-143.

MacQuarrie, K.T.B. and Sudicky, E.A. 2001. Multicomponent simulation of wastewater-derived nitrogen and carbon in shallow unconfined aquifers I. Model formulation and performance. *Journal of Contaminant Hydrology*, 47, 53-84.

MacQuarrie, K.T.B., Sudicky, E.A. and Robertson, W.D. 2001. Multicomponent simulation of wastewater-derived nitrogen and carbon in shallow unconfined aquifers II. Model application to a field site. *Journal of Contaminant Hydrology*, 47, 85-104.

Madigan, M.T., Martinko, J.M. and Parker, J. 2000. Brock biology of microorganisms. Upper Saddler River, NJ.

Molz, F.J., Widdowson, M.A. and Benefield, L.D. 1986. Simulation of microbial growth dynamics coupled to nutrient and oxygen transport in porous media. *Water Resources Research*, 22, 1207-1216.

Monod, J. 1949. The growth of bacterial cultures. *Annual Review of Microbiology*, 3, 371-394.

Ocampo, C.J., Oldham, C.E. and Sivapalan, M. 2006. Nitrate attenuation in agricultural catchments: Shifting balances between transport and reaction. *Water Resources Research*, 42.

Pala, A. and Bolukbas, O. 2005. Evaluation of kinetic parameters for biological CNP removal from a municipal wastewater through batch tests. *Process Biochemistry*, 40, 629-635.

Petersen, B., Gernaey, K., Devisscher, M., Dochain, D. and Vanrolleghem, P.A. 2003. A simplified method to assess structurally identifiable parameters in Monod-based activated sludge models. *Water Research*, 37, 2893-2904.

Petersen, B., Gernaey, K., and Vanrolleghem, P.A. 2001. Practical identifiability of model parameters by combined respirometric-titrimetric measurements. *Water Science and Technology*, 43, 347-355.

Rittmann, B.E. and McCarty, P.L. 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill. Singapore.

Schäfer, D., Schäfer, W. and Kinzelbach, W. 1998. Simulation of reactive processes related to biodegradation in aquifers - 2. Model application to a column study on organic carbon degradation. *Journal of Contaminant Hydrology*, 31, 187-209.

Senzia, M.A., Mayo, A.W., Mbwette, T.S.A., Katima, J.H.Y. and Jorgensen, S.E. 2002. Modelling nitrogen transformation and removal in primary facultative ponds. *Ecological Modelling*, 154, 207-215.

Sheibley, R.W., Jackman, A.P., Duff, J.H. and Triska, F.J. 2003. Numerical modeling of coupled nitrification-denitrification in sediment perfusion cores from the hyporheic zone of the Shingobee River, MN. *Advances in Water Resources*, 26, 977-987.

Starr, R.C. and Gillham, R.W. 1993. Denitrification and organic carbon availability in two aquifers. *Ground Water*, 31, 934-947.

v. Schulthess, R. and Gujer, W. 1996. Release of nitrous oxide (N2O) from denitrifying activated sludge: Verification and application of a mathematical model. *Water Research*, 30, 521-530.

Vanrolleghem, P.A., van Daele, M. and Dochain, D. 1995. Practical identifiability of a biokinetic model of activated-sludge respiration *Water Research*, 29, 2561-2570.

Vasiliadou, I.A., Pavlou, S. and Vayenas, D.V. 2006. A kinetic study of hydrogenotrophic denitrification. *Process Biochemistry*, 41, 1401-1408.

Widdowson, M.A., Molz, F.J. and Benefield, L.D. 1988. A numerical transport model for oxygen-based and nitrate-based respiration linked to substrate and nutrient availability in porous-media. *Water Resources Research*, 24, 1553-1565.

Wriedt, G. and Rode, M. 2006. Modelling nitrate transport and turnover in a lowland catchment system. *Journal of Hydrology*, 328, 157-176.

Zhou, S. 2007. Stoichiometry of biological nitrogen transformations in wetlands and other ecosystems. *Biotechnology Journal*, 2, 497-507.

Zysset, A., Stauffer, F. and Dracos, T. 1994. Modeling of reactive groundwater transport governed by biodegradation. *Water Resources Research*, 30, 2423-2434.