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Reija Kolehmainen

Natural Organic Matter Biodegradation and Microbial Community Dynamics in Artificial Groundwater Recharge

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Supervisor: Professor, Dr. Jaakko Puhakka
Laboratory of Environmental Engineering and Biotechnology
Tampere University of Technology
Tampere, Finland

Reviewers: Professor, Dr. Thor Axel Stenström
Swedish Institute for Infectious Disease Control
Water and Environmental Microbiology
Solna, Sweden

Docent, Dr. Ilkka Miettinen
National Public Health Institute
Environmental Health
Kuopio, Finland

Opponents: Professor, Dr. Martin Romantschuk
Department of Ecological and Environmental Sciences
University of Helsinki
Lahti, Finland

Docent, Dr. Ilkka Miettinen
National Public Health Institute
Environmental Health
Kuopio, Finland

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ABSTRACT

Artificial groundwater recharge (AGR) is a widely applied technique for producing drinking water by the infiltration of surface water into an appropriate geological formation, such as an esker. The continuous infiltration of surface water imposes the conduction of large quantities of natural organic matter (NOM) into an aquifer. Designing and implementing new areas for AGR requires a thorough understanding of the underlying biogeochemical and hydrological processes, including the mechanisms involved in the attenuation of NOM during infiltration. This study aimed to address the role of biodegradation in NOM removal and changes in microbial community structure during AGR for drinking water production. A bench-scale sand column and a fluidized-bed reactor (FBR) were used to quantify the proportion of mineralisation from dissolved organic carbon (DOC) removal and to reveal the influence of temperature on biodegradation. The spatial and temporal changes of microbial communities in the experimental systems were evaluated using culture independent molecular biology methods. The NOM removal and microbial community changes during infiltration were followed at three AGR sites in Finland; Hämeenlinna (site A), Jyväskylä (site B) and Tuusula (site C). In addition, extracellular enzyme activities and nutrient availability at the Tuusula site were determined.

The study showed efficient NOM removal along the flow path in both the experimental sand column and at the full-scale AGR sites. Reductions of total organic carbon (TOC) at sites A, B and C were 91%, 84% and 74%, respectively, in the winter, and 88%, 77% and 73%, respectively, in the summer. Similarly, total P decreased along the flow path at site C down to the detection limit. The proportion of organic N from total N decreased from 45% in the infiltration basin to 4 - 15% in the production well, most likely as a result of microbial metabolism. The average ratios of C:N:P indicated P limitation along the aquifer flow path and the natural groundwater. The nutritional conditions in the aquifer thus became highly oligotrophic. In the saturated sand column, on average 76% and 81% of TOC was removed within the first sampling port at 0.6 m and the entire length of 18.5 m, respectively. No accumulation of NOM was detected despite the long recharge period of 941 d. Increasing the hydraulic load from 0.3 $m^3(m^2d)^{-1}$ to 3.1 $m^3(m^2d)^{-1}$ did not affect TOC removal. This was likely due to the washout of NOM from the sand matrix due to the sudden increase in the flow rate. Large molecular size and aromatic NOM fractions were preferentially removed in the beginning of the flow path, which was likely due to sorption. However, no accumulation of smaller fractions was observed.

Biodegradation covered a substantial proportion of NOM removal in the sand bed. The $\delta^{13}C_{DIC}$ analysis revealed 32% to 52% DOC mineralisation in the sand column, depending on the temperature and hydraulic load. In both the continuous-flow FBR and the FBR batch tests, the highest average dissolved oxygen (DO) consumption rate was reached in summer (June-August), when lake water temperature was at its highest, followed by the fall, spring and winter. In the FBR batch tests, the DO consumption at low temperatures followed a typical kinetic curve for NOM biodegradation; i.e., a deep initial linear curve followed by a lower gradient. This illustrates the difference between rapidly and slowly biodegradable and non-biodegradable fractions. In the batch tests, a Q_{10} of 2.3 was found, which reveals the strong temperature dependency of NOM biodegradation. The analysis of $\delta^{13}C_{DIC}$ revealed 27% and 69% mineralisation of DOC in the FBR batch tests at 23 °C and 6 °C over 65 min and 630 min, respectively.

In the infiltration basin sediments at site C, slightly higher biomass content, measured as volatile solids (VS), was present in the surface layer (from 6.4 mg VS g^{-1} to 13.8 mg VS g^{-1}) as compared to the bottom sediments (from 4.1 mg VS g^{-1} to 10.1 mg VS g^{-1}) (measured to a total depth of 10 cm). Similarly, the chlorophyll-a content was higher at the surface than in the bottom sediments, the quantity depending on the sampling date. In the sand column, the VS showed continuous adsorption and desorption of biomass at the 1.2 m sampling port with the average quantity of 6.4±0.8 mg VS g^{-1} dw sand. Most of the NOM was removed before the 1.2 m sampling port. In the FBR, on the other hand, the quantity of carrier biomass increased until a certain level, after which no more increase occurred during the course of time of the research experiment. The maximum biomass amount in the FBR was 13.1 mg VS g^{-1} dw carrier. A substantial decrease in cell counts occurred in aquifers at sites A, B and C. The average cell counts in raw waters varied from 7.4×10^5 cells ml^{-1} to 24.0×10^5 cells ml^{-1} and in extracted groundwaters from 0.5×10^5 cells ml^{-1} to 1.0×10^5 cells ml^{-1}. In the sand column, the average decrease in the cell counts by 0.6 m distance was from $20.2 \pm 5.7 \times 10^5$ cells ml^{-1} (n=23) in the raw water to $4.3 \pm 1.1 \times 10^5$ cells ml^{-1} (n=3) at 0.6 m distance. In the FBR, on average 35±5% fewer cells were present in the outlet water compared to the inlet water.

At sites A, B and C, the bacterial communities in raw waters and extracted groundwaters were diverse. Changes occurred during infiltration, which was shown by DNA extraction followed by the PCR of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE) fingerprinting. While the natural groundwater microbial community was diverse, it was different from that of the extracted groundwater in the AGR area. In the sand column, both the DGGE fingerprinting and the length heterogeneity analysis of amplified PCR products (LH-PCR) showed a change in the bacterial community already by 0.6 m distance as the community composition shifted from an Actinobacteria-dominated population to a diverse, mainly Proteobacterial community. Concurrently, a substantial decrease in DOC concentration and cell counts had occurred by that stage. The original lake water community changed overnight in the FBR feed tank amended with phosphate and nitrate. The feed tank community differed from the FBR outlet water community. While the water phase was dominated by Actinobacteria, Proteobacterial groups dominated in the biofilm. However, the dominance of the specific groups was not constant, which illustrates the dynamic nature of both communities.

Compared to raw water, substantial increases were detected in the specific extracellular enzyme activities (EEAs) of α-D-glucosidase, β-D-glucosidase, phosphomonoesterase, leucine aminopeptidase and acetate esterase when measured in the AGR aquifer at site C. This was paralleled with decreasing nutrient concentrations shown by strongly negative correlations between the measured EEAs and the nutrient pools. The trend of increasing EEA along the flow path thus indicates a decrease in the availability of nutrients for bacteria. The EEA in the basin sediment (down to 10 cm) and the pore water samples were of the same order of magnitude as the basin water, indicating similar nutritional conditions. The EEAs had strong positive correlations with each other, suggesting synergistic and cooperative functions.

In summary, the study demonstrated the substantial role of biodegradation in NOM removal during AGR. The biodegradation was shown to depend on seasonal changes in raw water characteristics. A change in environmental conditions was shown to be reflected by changes in the composition and the physiological functioning of the microbial community. The study contributed to the understanding of NOM removal mechanisms in AGR and provides information for different interest groups of water production.

TIIVISTELMÄ

Tekopohjaveden muodostuksen tarkoituksena on tuottaa talousvettä imeyttämällä pintavettä soveltuvaan geologiseen muodostumaan, kuten hiekkaharjuun. Jatkuvatoiminen pintaveden imeyttäminen altistaa akviferin suurille eloperäisen orgaanisen aineksen (NOM) määrille. Uusien tekopohjavesilaitosten toteuttaminen vaatii perusteellista ymmärrystä vallitsevista biogeokemiallisista ja hydrologisista prosesseista mukaan lukien orgaanisen aineksen pidättymiseen liittyvät mekanismit. Tämän tutkimuksen tavoitteena oli määrittää biohajoamisen rooli orgaanisen aineksen poistumasta sekä mikrobiyhteisön rakenteessa tekopohjaveden muodostuksessa tapahtuvat muutokset. Mineralisaation osuus liuenneen orgaanisen hiilen (DOC) poistumasta sekä lämpötilan vaikutus mineralisaatioon määritettiin pilot-mittakaavan hiekkakolonnissa ja laboratoriomittakaavan leijupetireaktorissa. Koelaitteistojen mikrobiyhteisöissä esiintyvät spatiaaliset ja temporaaliset muutokset määritettiin molekyylibiologisin menetelmin. Orgaanisen aineksen poistumista ja mikrobiyhteisödynamiikkaa tarkasteltiin Hämeenlinnan Ahveniston (laitos A), Jyväskylän Vuonteen (laitos B) ja Tuusulan Jäniksenlinnan (laitos C) tekopohjavesilaitoksilla. Tuusulan Jäniksenlinnan tekopohjavesilaitoksella määritettiin lisäksi solun ulkopuolisten entsyymien aktiivisuudet ja ravinteiden saatavuus.

Tutkimus osoitti eloperäisen orgaanisen aineen poistuman olevan tehokasta sekä tutkituilla tekopohjavesilaitoksilla että hiekkakolonnissa. Kokonaisorgaanisen hiilen (TOC) poistuma laitoksilla A, B ja C oli talvella 91 %, 84 % ja 74 % ja kesällä 88 %, 77 % ja 73 %, vastaavasti. Kokonais-fosforin pitoisuus väheni laitoksen C akviferissa lähelle määritysrajaa. Orgaanisen typen osuus kokonaistypestä väheni imeytysaltaan 45 %:n osuudesta vedenottokaivon 4 - 15 %:n osuuteen, todennäköisimmin mikrobimetabolian johdosta. Keskimääräisten C:N:P – suhteiden perusteella fosfori oli rajoittava ravinne tekopohjavesiakviferissa ja luonnontilaisessa pohjavedessä. Ravinneolosuhteet akviferissa olivat oligotrofiset. Saturoituneessa hiekkakolonnissa TOC-pitoisuus väheni ensimmäiseen näytteenottopisteeseen (0,6 m) mennessä keskimäärin 76 % ja koko kolonnin matkalla (18,5 m) 81 %. Merkkejä orgaanisen aineksen akkumulaatiosta ei esiintynyt pitkästä koejaksosta (941 vrk) huolimatta. Hydraulisen kuorman nosto arvosta 0,3 $m^3(m^2d)^{-1}$ arvoon 3,1 $m^3(m^2d)^{-1}$ ei vaikuttanut TOC-pitoisuuksiin. Tähän vaikutti todennäköisesti orgaanisen aineksen huuhtoutuminen hiekasta yhtäkkisen virtaaman noston seurauksena. Suuret orgaanisen aineksen kokofraktiot ja aromaattiset fraktiot poistuivat ensisijaisesti jo kolonnin alkupäässä, luultavimmin sorptiolla. Pienten kokofraktioiden akkumuloitumista kolonniin ei kuitenkaan havaittu.

Biohajoamisen osuus orgaanisen aineksen poistumasta hiekkakolonnissa oli merkittävä. Epäorgaanisen hiilen isotooppianalyysin ($\delta^{13}C_{DIC}$) perusteella liuenneen orgaanisen hiilen poistumasta 32 – 52 % perustui mineralisaatioon, lämpötilasta ja hydraulisesta kuormasta riippuen. Sekä jatkuvatoimisessa leijupetireaktorissa että leijupetireaktorilla tehdyissä panoskokeissa hapenkulutusnopeudet olivat korkeimmillaan kesäkuukausina (kesä-elokuu). Leijupetireaktorilla suoritetut panoskokeet osoittivat hapenkulutusnopeuksien noudattavan alhaisissa lämpötiloissa orgaanisen aineksen biohajoamiselle tyypillistä kineettistä käyrää, jossa käyrän lineaarinen alkupää laskee jyrkästi ja sitten loivenee. Tämä viittaa nopeasti ja hitaasti hajotettavan sekä hajoamattoman orgaanisen aineksen fraktioihin. Panoskokeissa havaittu lämpötilakerroin (Q_{10}) 2,3 osoittaa orgaanisen aineksen biohajoamisen olevan voimakkaasti lämpötilasta riippuvaista. Mineralisaation osuus $\delta^{13}C_{DIC}$ –analyysin perusteella oli 23 °C:ssa tehdyssä ja 65 min kestäneessä panoskokeessa 27 % ja 6 °C:ssa tehdyssä ja 630 min kestäneessä panoskokeessa 69 %.

Tuusulan tekopohjavesilaitoksen imeytysaltaan pohjan pintaosassa biomassan määrä oli hieman suurempi (6,4 – 13,8 mg VS g^{-1}) kuin syvemmällä sedimentissä (4,1 – 10,1 mg VS g^{-1}) (kokonaissyvyys 10 cm). Myös klorofylli-a:n pitoisuus oli pintaosissa suurempi ja riippui näytteenoton ajankohdasta. Hiekkakolonnin hiekassa 1,2 m etäisyydellä esiintyi jatkuvaa biomassan kiinnittymistä ja irtoamista, keskiarvon ollessa 6,4±0,8 mg VS g^{-1} hiekkaa. Suurin osa orgaanisesta aineksesta oli poistunut tähän näytteenottopisteeseen mennessä. Leijupetireaktorissa puolestaan biomassan määrä lisääntyi koejakson alussa mutta myöhemmin sen määrä tasaantui. Biomassan enimmäismäärä koejakson aikana oli 13,1 mg VS g^{-1} kantaja-ainetta. Solumäärä väheni kaikilla tekopohjavesilaitoksilla huomattavasti raakavesien arvosta 7,4 – 24,0 × 10^5 solua ml^{-1} vedenottokaivojen arvoon 0,5 – 1,0 × 10^5 solua ml^{-1}. Hiekkakolonnissa solumäärä väheni jo ensimmäiseen näytteenottopisteeseen (0,6 m) mennessä raakaveden arvosta 20,2±5,7 × 10^5 solua ml^{-1} (n=23) arvoon 4,3±1.1 × 10^5 solua ml^{-1} (n=3). Leijupetireaktorista lähtevässä vedessä puolestaan oli keskimäärin 35±5 % vähemmän soluja kuin sinne johdetussa järvivedessä.

Ribosomaalisen 16S RNA-geenin monistus ja DGGE-profilointi (denaturaatiogeeli-elektroforeesi) osoittivat laitosten A, B ja C raakavesien ja tekopohjavesien bakteeriyhteisöjen olevan laajoja ja eroavan toisistaan. Laitoksen C luonnontilaisen pohjaveden bakteeriyhteisö oli samoin laaja ja erosi tekopohjaveden yhteisöstä. Hiekkakolonnin bakteeriyhteisöjen DGGE –ja LH-PCR-profilointi (PCR-monistettujen tuotteiden pituusvariaatioanalyysi) osoittivat merkittävän muutoksen yhteisön rakenteessa tapahtuvan jo ensimmäiseen näytteenottopisteeseen (0,6 m) mennessä Aktinobakteereiden osuuden vähetessä ja Proteobakteereiden osuuden kasvaessa merkittävästi. Bakteeriyhteisön lisäksi muutos tapahtui solumäärässä ja DOC-pitoisuudessa, jotka vähenivät merkittävästi. Järviveden bakteeriyhteisö muuttui vesisäiliössä, johon oli lisätty fosfaattia ja nitraattia ja josta vesi johdettiin leijupetireaktoriin. Leijupetireaktoriin johdetun veden bakteeriyhteisö muuttui edelleen leijupetireaktorissa. Vesifaasissa vallitsevana ryhmänä olivat Aktinobakteerit ja kantaja-aineen biofilmissä Proteobakteerit. Eri bakteeriryhmien vallitsevuus ei kuitenkaan ollut pysyvää mikä kuvastaa kummankin bakteeriyhteisön dynaamista luonnetta.

Laitoksen C näytteistä mitatut α-D-glukosidaasin, β-D-glukosidaasin, fosfomonoesteraasin, leusiiniaminopeptidaasin ja asetaattiesteraasin spesifiset ekstrasellulaariset entsyymiaktiivisuudet (EEA) nousivat huomattavasti akviferissa raakaveden vastaavista arvoista. Nousu tapahtui samanaikaisesti ravinnepitoisuuksien laskiessa, mikä näkyi voimakkaana negatiivisena korrelaationa EEA-arvojen ja ravinnepitoisuuksien välillä. EEA-arvojen nousu indikoi bakteereiden ravinnesaatavuuden heikkenemistä virtausmatkalla. Imeytysaltaan pohjasedimentin ja sedimentin huokosvesien EEA-arvot olivat samaa suuruusluokkaa imeytysaltaan veden EEA-arvojen kanssa, mikä osoittaa pohjasedimentin ja altaan veden ravinneolosuhteiden olevan samankaltaisia. EEA-arvot korreloivat keskenään positiivisesti, mikä osoittaa ekstrasellulaaristen entsyymien toimivan synergisesti.

Tiivistäen voidaan sanoa, että tutkimus osoitti biohajoamisella olevan keskeinen rooli eloperäisen orgaanisen aineksen poistumassa tekopohjaveden muodostuksessa ja riippuvan veden laadussa tapahtuvista vuodenaikaisista muutoksista. Ympäristöolosuhteissa tapahtuvat muutokset heijastuivat muutoksina mikrobiyhteisöjen rakenteissa ja fysiologiassa. Tehty tutkimus lisää ymmärrystä orgaanisen aineksen poistumismekanismeista tekopohjaveden muodostuksessa ja antaa siten tietoa talousveden tuotannon eri sidosryhmille, kuten vesilaitoksille ja viranomaisille.

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TABLE OF CONTENTS

LIST OF ORIGINAL PAPERS

I. Kolehmainen, R.E., Kortelainen, N.M., Langwaldt, J.H., Puhakka, J.A., 2009. Biodegradation of natural organic matter in long-term continuous-flow experiments simulating artificial groundwater recharge for drinking water production. Journal of Environmental Quality, *in press*.

II. Kolehmainen, R.E., Crochet, L.M., Kortelainen, N.M., Langwaldt, J.H., Puhakka, J.A. Biodegradation of aqueous organic matter over seasonal changes: Bioreactor experiments with indigenous lake water bacteria. *Submitted*.

III. Kolehmainen, R.E., Tiirola, M., Puhakka, J.A., 2008. Spatial and temporal changes in Actinobacterial dominance in experimental artificial groundwater recharge. Water Research 42, 4525-4537.

IV. Kolehmainen, R.E., Langwaldt, J.H., Puhakka, J.A., 2007. Natural organic matter (NOM) removal and structural changes in the bacterial community during artificial groundwater recharge with humic lake water. Water Research 41, 2715-2725.

V. Kolehmainen, R.E., Korpela, J.P., Münster, U., Puhakka, J.A., Tuovinen, O.H., 2009. Extracellular enzyme activities and nutrient availability for bacteria during artificial groundwater recharge. Water Research, *in press*.

THE AUTHOR'S CONTRIBUTION

Paper I:
Reija Kolehmainen wrote the paper except for the part concerning the stable carbon isotope method, which was written by Nina Kortelainen. R. Kolehmainen is the corresponding author. She planned and performed the experimental work and interpreted the results except for the stable carbon isotope analysis, which was done by N. Kortelainen. Jörg Langwaldt advised on the experimental part.

Paper II:
Reija Kolehmainen wrote the paper except for the part concerning the stable carbon isotope method, which was written by Nina Kortelainen. R. Kolehmainen is the corresponding author. She planned and performed the analysis with the assistance of Ludivine Crochet except for the stable carbon isotope analysis, which was done by N. Kortelainen.

Paper III:
Reija Kolehmainen wrote the paper together with Marja Tiirola. R. Kolehmainen is the corresponding author. She planned the experimental work and performed the DGGE analysis. The LH-PCR and molecular cloning analysis as well as the interpretation of the results were performed together with M. Tiirola, who also performed the database examination.

Paper IV:
Reija Kolehmainen wrote the paper and is the corresponding author. She planned and performed the experimental work and interpreted the results.

Paper V:
Reija Kolehmainen wrote the paper and is the corresponding author. She planned and performed the analysis with the assistance of Jaana Korpela, except for the analysis of amino acids, which was done by Uwe Münster. U. Münster assisted in the interpretation of the results.

ABBREVIATIONS

A	Arrhenius constant
AEST	Acetate esterase
AGR	Artificial groundwater recharge
α-Glu	α-D-glucosidase
AOC	Assimilable organic carbon
ATP	Adenosine triphosphate
BDOC	Biodegradable dissolved organic carbon
β-Glu	β-D-glucosidase
Chl-a	Chlorophyll-a
COD	Chemical oxygen demand
DAPI	4'-6-diamidino-2-phenylindole
$\delta^{13}C$	The isotopic ratio of carbon as a per mil (‰) difference relative to the international VPDB standard
DGGE	Denaturing gradient gel electrophoresis
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
E_a	Activation energy
EEA	Extracellular enzyme activity
FBR	Fluidized-bed reactor
f_{org}	The fraction of carbon in water DIC resulting from the oxidation of DOC
HMW	High molecular weight
HPLC	High performance liquid chromatography
HPSEC	High performanse size exclusion chromatography
HRT	Hydraulic retention time
HS	Humic substances
k	Oxygen uptake rate
LAP	Leucine aminopeptidase
LH-PCR	Length heterogeneity analysis of amplified PCR products
LMW	Low molecular weight
NOM	Natural organic matter
PCR	Polymerase chain reaction
PME	Phosphomonoesterase
POC	Particulate organic carbon
Q_{10}	Temperature coefficient
R	The universal gas constant
rRNA	Ribosomal RNA
SUVA	Specific ultraviolet absorbance
TOC	Total organic carbon
UVA	Ultraviolet absorbance
VPDB	Vienna Peedee Belemnite (fossil belemnite, *Belemnitella americana*, from the Late Cretaceous PeeDee Formation in South Carolina)
VS	Volatile solids

1 INTRODUCTION

1.1 Background

Artificial groundwater recharge (AGR) is used to increase the amount of groundwater available for abstraction through the percolation of surface water. This study focused on drinking water production by AGR through the continuous infiltration of lake or river water through an unconfined aquifer, where the physical, chemical and biological purification of water takes place during passage to production wells. The method has been practiced in many European countries and the USA with different kinds of applications for more than a century (Frycklund 1992). In Finland, the proportion of AGR is currently 13% and is likely to increase in the near future up to 30% due to the implementation of new large scale AGR sites (Isomäki *et al.* 2007). In other countries, the lack of potable water is also likely to increase drinking water production by AGR.

In Boreal regions, the main aim of AGR in drinking water production is the removal of natural organic matter (NOM) from raw water. The continuous infiltration of humic water imposes the conduction of large quantities of NOM into the aquifer causing a theoretical risk of severe environmental impacts, including aquifer clogging in the long term. Several factors affect NOM removal in an aquifer, such as water chemistry (especially NOM quality and quantity), aquifer material (Juhna *et al*. 2003), temperature, hydraulic load and retention time, infiltration distance (Helmisaari *et al*. 2003) and the microbial communities involved. Thus, an AGR aquifer is a highly complex environment where several biotic and abiotic processes vary in time and space.

The role of biodegradation in NOM removal is of great importance when determining the sustainability of AGR. Microorganisms utilize NOM as their energy and C source and convert it to CO_2, which is partly dissolved in water, partly evaporated and partly reutilised in microbial anabolism. NOM is not, however, easily biodegradable due to its complex polymeric structure that contains aromatic units and many types of covalent bonds and cross-linking within the organic macromolecular structure (Marschner and Kalbitz 2003, Thurman 1985, Alexander 1965). In an AGR aquifer, several factors simultaneously affect the biodegradation of NOM. These include the intrinsic NOM quality parameters, soil and solution parameters and external factors (Marschner and Kalbitz 2003). Microbial populations respond to a change in their environment in a way that maximizes their survival and rate of growth.

Studying the fate of NOM in an AGR aquifer is a challenge because of several co-occurring processes, the heterogeneity of NOM, analytical limitations and site specificity. Despite several studies performed on NOM biodegradation in lakes, no standard method exists for the quantification of NOM biodegradation. Also, aquifers used for AGR greatly differ from lakes in terms of lack of sunlight, the presence of a sand matrix and the constant flow of raw water. Therefore, studies performed with lake water assays cannot be directly compared with AGR.

Despite the long history of AGR, the scientific basis concerning NOM purification mechanisms and the microbial communities involved is insufficient. This information is a necessity when designing and implementing new large scale AGR sites for drinking water production, as well as for AGR management and the minimisation of adverse environmental impacts. Public concerns about possible negative impacts, land availability as well as legal aspects complicate the implementation of new areas for recharge. This study focused on the microbiological aspects of NOM removal by estimating the proportion of dissolved organic carbon (DOC) mineralisation in

the aquifer flow path (Paper I), evaluating the temperature dependency of biodegradation (Papers I and II), characterising the attached and free-living bacterial communities (Papers III and IV) and by estimating the extracellular enzyme activities (EEAs) and the nutrient availability in an AGR aquifer (Paper V).

1.2 Artificial groundwater recharge as a method for producing drinking water

Artificial groundwater recharge is based on the natural attenuation of water impurities when water travels through the aquifer sand bed. In Finland, several glaciofluvial deposits offer potential areas for recharging groundwater. AGR differs from natural groundwater formation in some significant respects (Frycklund 1992): in AGR, the raw water contains high amounts of NOM (including microorganisms), the quantity of infiltration is much higher, the infiltration is continuous and the residence time much shorter when compared to the formation of natural groundwater. The conditions in the subsurface will thus change due to infiltration.

Different methods can be applied for infiltration, such as basins, sprinkling networks (Fig. 1.1), channels, trenches or injection wells. Bank filtration occurs naturally through geological formations adjacent to water bodies and can be enhanced through continuous pumping. Bank filtration differs from other methods by having e.g. shorter retention times and is not considered in this study. In addition to drinking water production through continuous infiltration, groundwater recharge can be used in several other applications, such as the treatment of secondary wastewaters and storm waters, the storing of water for later use, the adjustment of groundwater levels and the prevention of salt water intrusion into an aquifer (Jensen 2001). The method is thus likely to become more popular in the future.

a) b)

Figure 1.1. Basin infiltration at Hämeenlinna AGR site (a). Sprinkling infiltration at Jyväskylä AGR site (b) (Photographs: R. Kolehmainen 2005).

The first AGR applications for drinking water production were initiated in Scotland and Germany in the 19th century (Kivimäki 1992). In Finland, infiltration was first used at the beginning of the 20th century. Currently, there are 25 AGR sites covering 13% of the country's total drinking water production with infiltration quantities varying from 100 to 22000 m^3d^{-1} (Isomäki *et al.* 2007). The sites under development will cover large land areas and use infiltration volumes up to 100 000 m^3d^{-1}. Natural groundwater and chemically treated lake or river water currently account for 48% and 40% of the drinking water production, respectively. The design parameters, such as

the size and constitution of the geologic formation, desirable infiltration distance, residence time and the quality demand for raw water and thus the need for pre-treatment vary greatly between the sites (Frycklund 1992).

The benefits that AGR provides include efficient NOM removal, uniform water quality, large capacity, the possibility to suspend infiltration, minimal need for chemical additions and the equalization of seasonal lake water temperatures (Isomäki *et al.* 2007). The chemical and microbiological characteristics of raw waters used for infiltration, however, differ from the characteristics of precipitation and, therefore, the environmental impacts of AGR cannot be completely avoided. Both basin and sprinkling infiltration require large land areas. Sprinkling infiltration imposes abnormally wet conditions on forest vegetation. This results in changes in vegetation and an increase in the pH of the organic forest floor (Helmisaari *et al.* 1999, Lindroos *et al.* 1998). The increase in pH may initiate nitrification and thus cause the potential flux of nitrate into groundwater (Paavolainen *et al.* 2000).

The accumulation of NOM, excessive biomass proliferation, the generation of gas and mineral precipitation carry with them the theoretical risk of the clogging of the basin sediments and the deeper aquifer in the long-term. As Helmisaari *et al.* (2003) summarise, clogging is influenced by the raw water quality (especially the colloids and pH), the grain size distribution and mineral composition of the aquifer material as well as the infiltration rate. The authors therefore suggest that the risk of clogging can be controlled by process optimization including the pre-treatment of raw water, the selection of suitable material for the basin sediments, optimizing the depth of the basin sediments and the water layer, adjusting the infiltration rate, regularly cleaning the basin sediments and pausing the infiltration process and/or changing sprinkling infiltration areas. The breaks are, however, likely to adversely affect the microbial populations responsible for NOM biodegradation. It has been illustrated that microbial populations in a sand column efficiently prevented clogging when compared to a column in which the microorganisms had been eliminated (Albrechtsen *et al.* 2001).

1.3 Characteristics of Boreal humic lake waters

In Boreal regions, the removal of NOM from surface water is the main objective of drinking water production. This is due to the reactions between NOM and disinfection chemicals resulting in disinfection by-product formation and the function of NOM as a nutrient source for microorganisms in the water distribution system. NOM refers to the sum of all natural C-containing substances and is a complex mixture of plant and animal residues, microorganisms as well as substances synthesised microbiologically or chemically from the decomposition products. In Finland, the recommended value for total organic carbon (TOC) in treated water is < 2 mg l^{-1} (Finnish Ministry of Social Affairs and Health 1994).

Boreal waters can be characterised as having a brown colour, low pH, low conductivity and alkalinity as well as a high content of humic substances (HS) (Keskitalo and Eloranta 1999). About 10% to 20% of DOC has known structures including fatty acids, carbohydrates, amino acids and hydrocarbons. The majority is thus comprised of HS, which are coloured, polyelectrolytic, organic acids with molecular weights from a few hundred to thousands (Hedges 1988, Thurman 1985). The average elemental composition of humic substances is 50% C, 4 to 5% H, 35 to 40% O, 1 to 2% N and <1% S and P and the major functional groups include carboxylic acids, phenolic hydroxyl, carbonyl and hydroxyl groups (Thurman 1985). Based on

two surveys, the median TOC concentration of Finnish lake waters varies annually from 7.6 mg l^{-1} to 12 mg l^{-1} (Kortelainen 1999). The high content of HS in Boreal lakes results from the large peatland catchments and the climatic conditions. In addition, the flat topography of countries such as Finland provides favourable conditions for NOM accumulation (Kortelainen 1993). Both the quantity and quality of NOM varies temporarily and spatially and is affected by, e.g., the seasonal runoff volumes and the growing periods. In addition to affecting the extent of mineralisation, the origin of DOC also affects the size and mineralisation constants of the labile and stable DOC pools (Kalbitz *et al*. 2003).

Humic substances are present in water as dissolved molecules, colloidal suspensions and particulate matter. Based on the solubility in aqueous acids and basis, HS are divided into humic acids, fulvic acids and humins. A typical characteristic of humic waters is their strong absorbance of UV radiation. Thus, oligotrophic lakes can also be humic despite their low content of DOC (Keskitalo and Eloranta 1999, Thurman 1985). A proposed model for a humic acid fraction of HS is shown in Fig. 1.2.

Aqueous NOM originates from autochthonous and allochthonous sources. The first is produced within the lake (primary production) and the latter in the catchment area (Münster and Chróst 1990). Allochthonous NOM has thus gone through several transformation processes and is considered stable and refractory. NOM contains both living and nonliving material in a ratio that varies seasonally and between different locations. Due to the heterogeneous nature of NOM, no single analytical method exists for its quantification. Organic carbon, which comprises about 50% of NOM, is generally used for the quantification.

Prevailing environmental conditions in Boreal areas result in low nutrient contents, different food web structures and species composition compared to lakes in southern Europe (Münster 1999a). Furthermore, HS efficiently bind nutrients, thus substantially affecting their availability for microorganisms. The growth and survival of a particular species thus depends on its ability to utilise these scarce substrates in competition with other species (Nedwell 1999). In Boreal lakes, phosphorus often limits microbial growth (Salonen *et al*. 1994).

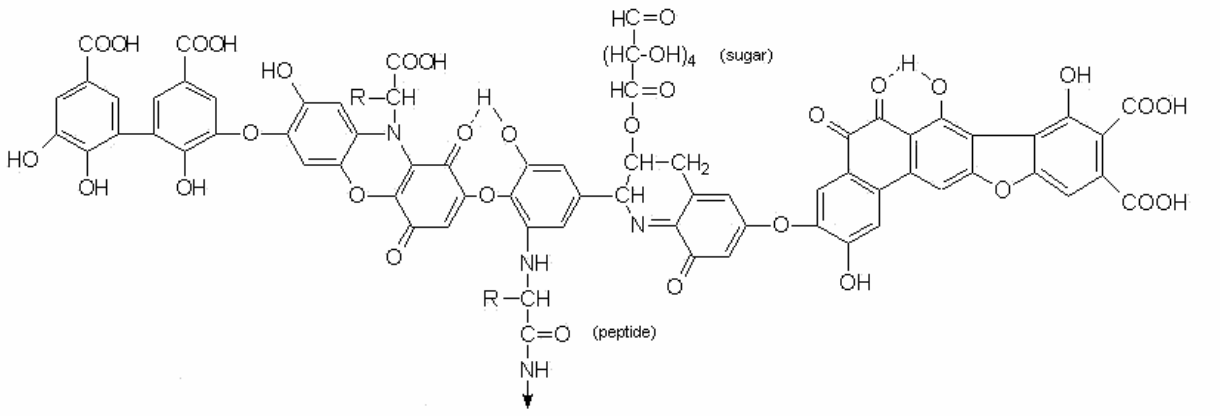


Figure 1.2. Proposed structure of humic acid according to Stevenson (1982).

1.4 NOM transformations along the aquifer flow path

NOM removal in AGR occurs through physical, chemical and biological mechanisms, as well as their interaction. Studies concerning NOM removal mechanisms, however, are scarce. The mechanisms depend on several regional and operational factors, such as the geological and hydrological characteristics of a given site, raw water quality, season, hydraulic retention time and infiltration rate. Helmisaari *et al*. (2003) found, at five Finnish AGR sites, that in order to produce water with 2 mg TOC l^{-1} the required retention time varied from 7 d to 80 d and the infiltration distance from 160 m to 1300 m, depending on site-specific factors. Sorption and biodegradation are considered to be the main NOM removal / transformation mechanisms in AGR and are briefly discussed below. Other mechanisms affecting NOM transformations include filtration, precipitation, mechanical straining, redox reactions, dissolution by natural groundwater and ion exchange. This thesis focuses on the role of biodegradation in NOM removal and the factors affecting this process.

NOM removal in AGR occurs in different zones encompassed with changing environmental gradients. Studies concerning NOM removal at different zones have revealed contradictory results. This is likely due to both regional differences and the lack of reliable sampling techniques to detect changes, especially in the vadose zone. In basin infiltration, NOM removal occurs in three zones: the bottom of the basin, the vadose zone and the saturated zone (Fig. 1.3). The first few centimetres of the basin sediments are covered by a biofilm layer that is composed of both live biomass (microorganisms, algae, protozoa etc.) and accumulated organic matter. This layer is responsible for the initial NOM transformations. Jacks (2001a) found that the filter sand at a Swedish AGR site efficiently removed mineral and organic suspended matter. In sprinkling infiltration, a net increase in DOC content within the first meter has been shown as the result of the leakage of organic matter from the forest floor (Lindroos *et al*. 2002). Helmisaari *et al*. (2003) found that no substantial decrease in DOC occurred during infiltration in the vadose zones at five Finnish AGR sites. Jacks (2001b), on the other hand, found that 50% of DOC removal occurred in the vadose zone of a Swedish AGR site. Frycklund (1995) found seasonal variation in TOC content (net decreases and increases) in the vadose zone.

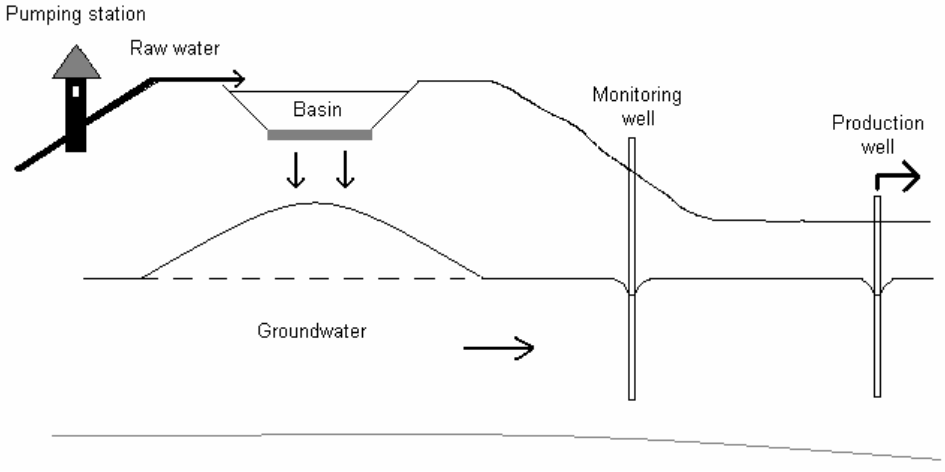


Figure 1.3. Schematic of an AGR that uses continuous infiltration through a basin and subsequent extraction through a production well. Monitoring wells are used for water quality evaluations along the flow path.

1.4.1 Sorption

Sorption (adsorption) onto the sand surface is thought to be one of the major NOM removal and transformation mechanisms in AGR. Sorption is a combination of physical and chemical processes resulting in the adherence or bonding of molecules and ions onto the mineral surface. Sorption mechanisms include hydrophobic sorption, hydrogen bonding, ligand exchange and ion exchange (Thurman 1985). In desorption, the molecule is released from the particle surface and this may result from changes in prevailing environmental conditions.

Sorption is influenced by the nature of sorbent (geologic matrix), the sorbate (NOM) and the solution (water) (Klavinš *et al*. 2000, Sollins *et al*. 1996). An increase in clay content increases the sorption efficiency of humic substances (Juhna *et al*. 2003). The influence of clay likely results from its large specific surface area. The solution pH also affects sorption by, e.g., affecting the surface charge of some clays (Sollins *et al*. 1996). Juhna *et al.* (2003) observed an increase in the sorption efficiency of HS with a decrease in pH.

Aromatic and large molecular size components of NOM have shown a preferential sorption on mineral surfaces (Namjesnik-Dejanovic *et al*. 2000, McKnight *et al*. 1992). Kalbitz *et al*. (2005) found that recalcitrant dissolved organic matter (DOM) fractions were sorbed to a larger extent on mineral soil compared to the labile fractions. Aufdenkampe *et al*. (2001) showed that the nitrogen rich components of organic matter were selectively partitioned onto mineral surfaces. Moreover, hydrophobic substances are sorbed more readily than hydrophilic (Jardine *et al*. 1989).

The sorption studies performed so far have been criticised for their use of artificially cleaned clays free of organomineral surfaces (Sollins *et al.* 1996). In addition to organic substances, the mineral particles in an aquifer may be coated with iron and manganese oxides as well as biofilms. Furthermore, the distinguishing of physical sorption from biodegradation is not straightforward due to the access of attached bacteria on sorbed NOM.

1.4.2 Biodegradation

The role of biodegradation on the fate of NOM in AGR is of great importance. Heterotrophic bacteria are the key trophic level in NOM decomposition, nutrient cycling, and the structure of the food webs (Chróst and Siuda 2002, Azam *et al*. 1983). This is mainly due to their large surface-to-volume ratio that allows nutrient absorption at very low concentrations. Bacteria use NOM as their energy and C source; i.e., for the generation of ATP and for the biosynthesis of new cell components (Madigan *et al*. 1997). Catabolism results in the release of CO_2, which is partly dissolved in water, partly evaporated and partly reutilised in microbial anabolism. The final products of biodegradation include inorganic carbon, water, new biomass, metabolites (broken NOM molecules, simple organic acids, polysaccharides and extracellular enzymes) and excreted cellular components (Fig. 1.4). Thus, in addition to decomposing NOM, bacteria produce particulate organic carbon (POC) through assimilation. Complete mineralisation; i.e., the conversion of NOM to CO_2, H_2O and other inorganic elements is, therefore, theoretical and always accompanied by the formation of metabolic products. In aquatic environments, NOM is further transported to higher trophic levels through grazing. The term bioavailability describes the potential of microorganisms for interacting with NOM (Marschner and Kalbitz 2003).

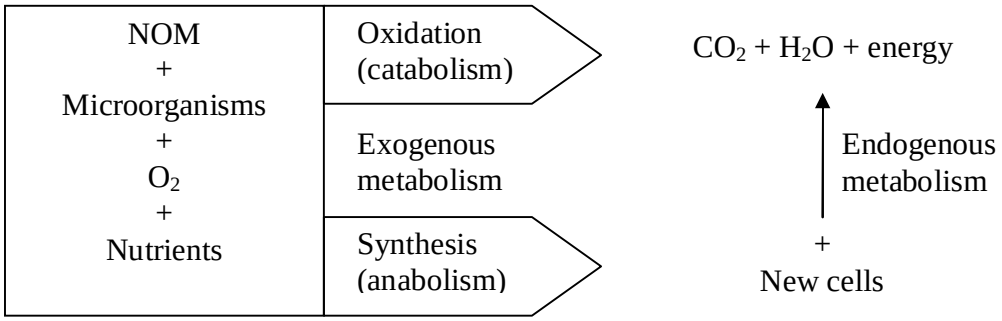


Figure 1.4. Schematic representation of NOM biodegradation by aquatic microorganisms (modified from Pitter and Chudoba, 1990).

In aerobic conditions, the changes in the average oxidation state of the carbon during mineralisation can be described by the simplified Eq. 1.1 and in anaerobic conditions by Eq. 1.2 (Thurman 1985):

$$CH_2O + O_2 = CO_2 + H_2O \quad (1.1)$$

$$CH_2O + A(Oxidized) = CO_2 + A(reduced) \quad (1.2)$$

where *A* is an electron acceptor other than oxygen, such as NO_3, SO_4 or an organic compound. In anaerobic mineralisation a series of reactions occur because the product of one organism can be the substrate for another.

Due to the macromolecular structure of NOM, it does not directly penetrate into the bacterial cells. Instead, heterotrophic bacteria produce extracellular enzymes to hydrolyse the macromolecules into smaller compounds (monomers and oligomers) so that they can be available for bacterial growth and nutrient cycles. This is the initial step in NOM biodegradation and is also important in the regeneration of inorganic nutrients from high-molecular weight compounds (Hoppe 1993, Chróst 1990). Several biochemical transformations can only be mediated by heterotrophic bacteria because the required enzyme systems are rarely found in other organisms (Hoppe 1993, Chróst 1990, Hollibaugh and Azam 1983). Therefore, the heterotrophic bacteria are the key trophic level in NOM decomposition, nutrient cycling, and structure of the food webs (Chróst and Siuda 2002, Münster 1999a). The composition, i.e., the availability of organic carbon as well as the physico-chemical conditions all affect the development and activity of heterotrophic bacteria (Chróst and Siuda 2002 and references therein).

Since the EEA is, in many cases, a limiting factor in NOM decomposition, it can be used for studying the overall hydrolytic capacity (Hoppe 1993) and thus the physiological state of the prevailing bacterial community. According to Münster *et al.* (1989), the most important microbial extracellular enzymes in humic surface waters are glucosidases, phosphatases and aminopeptidases. Glucosidases cleave the glucosidic bond between two glucose molecules, being specific for α- or β-configurations (depolymerisation of polysaccharides). The first are normally found in autochthonous (starch, algal biomass) and the latter in allochthonous (cellulose) organic matter. In addition to glucosidases, several other extracellular enzymes are also involved in the hydrolysis of dissolved carbohydrates. Phosphatases, on the other hand, remove the inorganic

phosphate group from organic molecules (dephosphorylation) for microbial uptake and use. Aminopeptidases catalyse the hydrolysis of the peptide bond in proteins thereby releasing oligopeptides and monomeric amino acids for nitrogen uptake by microorganisms. Esterases split esters into an acid and an alcohol. Their substrate specificity and biological functions vary but they can generally be used for the determination of general extracellular enzyme activity.

1.4.3 Factors affecting NOM biodegradation

The chemical and the physical environment greatly affect microbial activities thus affecting their ability to grow and perform metabolic reactions. Different microbial species respond differently to different factors. The four major environmental factors affecting biodegradation in general are considered to be temperature, pH, water availability and oxygen content (Madigan *et al.* 1997). In lakes, photochemical mineralisation plays a substantial role in NOM degradation and transformation (Münster *et al.* 1999a, Vähätalo *et al.* 1999, Granéli *et al.* 1996, Salonen and Vähätalo 1994). In AGR, photochemistry functions only in basins. Nevertheless, several other factors simultaneously affect the biodegradation of NOM in the aquifer. These include the intrinsic NOM quality parameters, soil and solution parameters and external factors, such as the seasonality of meteorological parameters (Marschner and Kalbitz 2003). Some of these are discussed below.

The quantity and quality of NOM are among the major factors affecting biodegradation, especially in oligotrophic environments, as supported by studies performed by Eiler *et al.* (2003). They found that the substrate concentration of a nutrient supplemented humic lake water only influenced bacterial growth efficiency at concentrations below 6.2 mg DOC l^{-1}. The authors suggested that this reflects either a shift in the prevailing bacterial population or a simultaneous change in the population and its physiology. In addition to concentration, mass transfer limitation, i.e., an inefficient supply of nutrients to the attached microorganisms in porous media, affects both microbial growth as well as NOM biodegradation. The concentration of substrates at the cell surface is likely to be lower than in the adjacent water due to biodegradation and thus the resupply of substrates requires mass transfer via different pathways (Bosma *et al.* 1997). Mass transfer is to be expected when the substrate consumption rates exceed the rates of supply. An increase in the flow rate does not necessarily result in enhanced mass transfer, as shown by Simoni *et al.* (2001). Instead, the authors found an increase in mass transfer with an increase in grain size.

The structure of the NOM molecule affects its microbial utilisation. Sollins *et al.* (1996) listed three factors that affect organic carbon stability: 1) *Recalcitrance* which includes the molecular-level properties of the substances (elemental composition, functional groups, molecular conformation) and the influence on their degradation by microorganisms and enzymes, 2) *interactions* between organic substances and either inorganic substances or other organic substances that alter the degradation rates of the substances or the synthesis of new ones, and 3) *accessibility*, i.e., the location of organic substances with respect to microorganisms and enzymes. Kalbitz *et al.* (2003) found that the properties of soil-derived DOM, related to microbial stability, included UV absorbance, aromaticity, hydrophobicity, a degree of complexity, the condensation of molecules and the content of carbohydrates. Furthermore, carbohydrates were preferentially utilised by microorganisms whereas aromatic structures were highly stable.

Generally, the labile small molecular size NOM fractions are considered to be used most rapidly, followed by more refractory fractions (Coffin *et al*. 1993), however, results indicating the opposite also exist. Volk *et al*. (1997) demonstrated the importance of HS on microbial metabolism by showing that the measured biodegradable DOC (BDOC) in a plug-flow bioreactor contained 75% of HS. Moran and Hodson (1990) showed a difference in the bioavailability of the humic and non-humic DOC fractions between two different fresh water sources. They showed that bacterial utilisation of the humic fraction accounted for 22% to 53% of bacterial growth on DOC, depending on the water source. Långmark *et al*. (2004) found that the refractory humic acid fractions stimulated higher activity by microorganisms harvested from the sand matrix of sand columns compared to the microorganisms harvested from the raw water. When C is the limiting nutrient, the microorganisms are assumed to simultaneously utilise different C-sources, which gives them an advantage to grow in oligotrophic environments (Egli 2004). Another assumption is that microbial species are specialised at either using the labile (r-strategists) or refractory (K-strategists) fractions of organic matter (Fontaine *et al*. 2003, Paul and Clark 1989). The r-strategists grow fast and become substituted by the K-strategists that slowly degrade the refractory fractions. The K-strategists need to allocate more energy to extracellular enzyme production and to defending themselves from predation (Fontaine *et al*. 2003).

Temperature is one of the key factors affecting biodegradation since an increase of temperature accelerates chemical and enzymatic reactions that follow the laws of thermodynamics. This occurs within the optimal temperature range of a particular organism. In addition to affecting the physiological reaction rates, temperature has an influence on the physicochemical characteristics of the environment (Paul and Clark 1989). Microorganisms adapt at low temperatures through several adaptations in their cellular composition and physiology. These include conformational changes in the proteins and modifications of the membrane lipids (Russell 2008, Collins *et al*. 2008, Suutari and Laakso 1994). The conformational changes in the proteins result from the weakening of the bonds that control the tertiary structures. This results in an increased plasticity of the proteins. In order to maintain the cell membranes in a biologically functional fluid phase, the membrane lipids go through compositional changes as opposed to lowering temperatures (Nedwell 1999). The unsaturation of fatty acids as a response to decreasing temperatures is the most common change in the composition of membrane lipids (Suutari and Laakso 1994). Additionally, the proportion of branched chain lipids in the cell membranes is decreased at low temperatures. Männistö and Puhakka (2001) found substantial differences in the fatty acid composition of seven groundwater bacterial strains when grown at different temperatures (between 8 °C and 25 °C). Bacterial gene induction and expression as well as enzyme synthesis only requires a few minutes under normal growth temperature (Russell 2008). Thus, in addition to evolutionary adaptations, cellular adaptations to changing temperatures can be rapid.

The decrease in a membrane's fluidity results in a deterioration of the functioning of the membrane proteins (Nedwell 1999). This influences the active transport of substrates to the cells (Nedwell 1999, Tsukagoshi and Fox 1973). An increase in the substrate requirement has been shown to co-occur with a decrease in temperature (Wiebe *et al*. 1993). As Nedwell (1999) concludes, even a small change in temperature can substantially affect the affinity of microorganisms for the substrates. Peters *et al*. (1987) showed that temperature significantly affected organic carbon mineralisation by stream microorganisms associated with particulate matter. Furthermore, White *et al*. (1991) reported that an increase in water temperature had a positive influence on both bacterial production and specific growth rate in 57 studies conducted with fresh, marine and estuarine/coastal waters.

Microbial enzymes are pH dependent (Paul and Clark 1989). The tolerance for different pH conditions varies between bacterial species. The conditions of the Finnish lakes and aquifers are neutral or slightly acidic and relatively stable along the aquifer flow path (Helmisaari *et al*. 2003). However, as Paul and Clark (1989) emphasise, due to the cation layer around negatively charged clays and electronegative organic particles, the pH of water may be much higher compared to the charged surfaces.

Sorption of NOM onto mineral surfaces is likely to affect its bioavailability and thus the biodegradation, however, this phenomenon is not well understood. Jones and Edwards (1998) and van Hees *et al*. (2003) indicated that the sorption of simple organic compounds reduced their biodegradation. Kalbitz *et al*. (2005) assumed that the reasons for the decrease in sorbed DOM mineralisation could be the selective sorption of more recalcitrant components and the stabilisation of organic matter compounds as a result of sorption onto the mineral surface. One explanation could be the penetration of organic substances into micropores that are small enough to prevent the access of microorganisms (Ekschmitt *et al*. 2005). However, Sollins *et al*. (1996) remind us that in sorption experiments, several interactions may have contributed to the effects of sorption. Clay, for example, interacts with microorganisms, changes the solution properties (buffering of pH) and binds or inactivates extracellular enzymes. Thus, the influence of sorption on NOM biodegradation is difficult to determine.

The influence of microbial populations on NOM biodegradation is apparent, as discussed above, although difficult to detect due to changes in community composition along with changes in environmental conditions. Eiler *et al*. (2003), e.g., reported a gradual shift in the bacterial community composition of humic lake water along with a change in DOC concentration. This change was more apparent with low rather than high DOC concentrations (total range of 0.5 mg l^{-} to 30 mg l^{-1}). Van Hannen *et al*. (1999) indicated that the origin of the detritus affected the structure of a bacterial community in lake water. Microorganisms have a remarkable capacity to adapt to changing environments through structural and functional adaptations, such as natural selection, mutation, and non-genetic adaptation (Alexander 1965) as well as horizontal gene transfer (Wuertz *et al*. 2004, Tiirola *et al*. 2002).

Due to the changes in water quality along the aquifer flow path in AGR, different factors vary both temporarily and spatially. Furthermore, a change in one factor is often simultaneously accompanied by a change in another factor; e.g., seasonal temperature variation is accompanied by changes in raw water quality (chemical and microbiological) and nutrient concentration is related to its mass transfer. Fig. 1.5 summarizes NOM transformations in an AGR aquifer and lists the main site-specific and operational factors affecting these transformations.

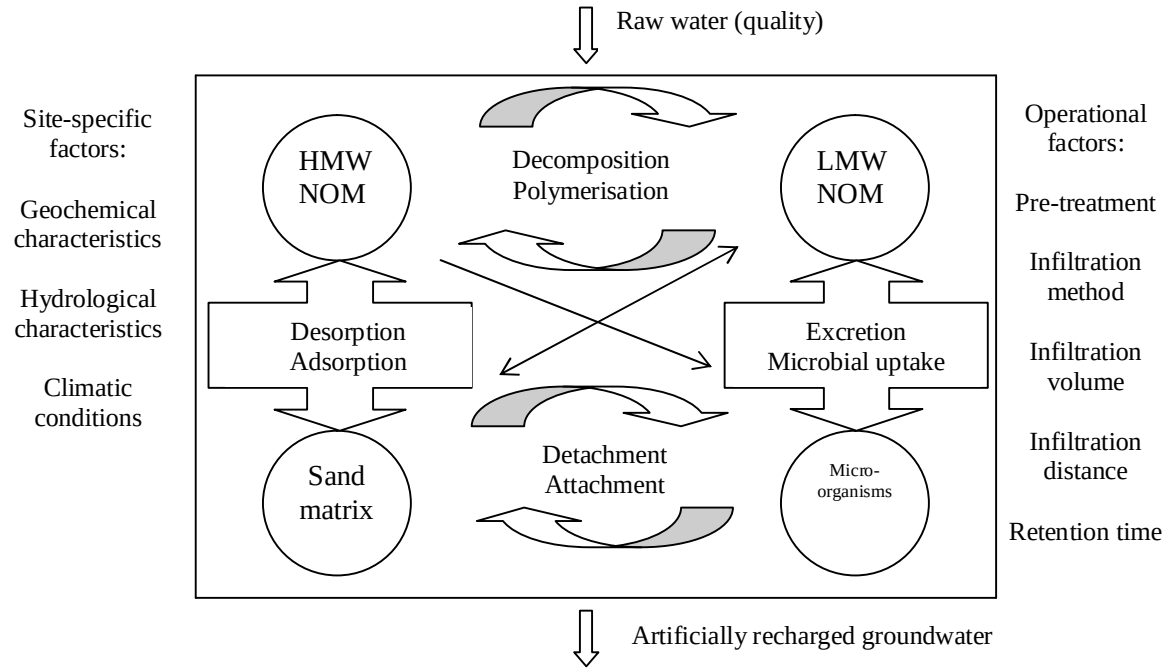


Figure 1.5. Transformation of NOM and the factors affecting the processes in an AGR aquifer. HMW = High Molecular Weight, LMW = Low Molecular Weight.

1.5 Microbial abundance and distribution in the subsurface

Groundwater habitat differs physically from freshwater habitats due to its solid matrix of variable porosity, variable degree of saturation, hydrological and geological stratification as well as the lack of sunlight (Madsen and Ghiorse 1993). Life forms other than microorganisms are mostly excluded. Bacterial abundance, distribution and species composition vary in different subsurface zones depending on factors such as the geochemical and hydrological conditions and water quality. In the unsaturated zone, the decline in nutrients is paralleled by a decrease in bacterial abundance (Madsen and Ghiorse 1993). However, the number of cells usually increases at the water table, which is likely due to the mixing of oxygen and recently recharged nutrients. In natural aquifers, the total number of bacteria ranges between 10^5 and 10^8 cells g^{-1} dw of sediment and 10^3 and 10^7 cells ml^{-1} groundwater (Männistö *et al*. 2001, Anderson and Lovley 1997, Hazen *et al*. 1991, Ghiorse and Wilson 1988).

Most of the microorganisms in the subsurface are attached to sand surfaces as biofilms. The structure of the biofilm is influenced by the hydrodynamic conditions, substrate loading and the type of organism (van Loosdrecht *et al*. 1995). The growth of biofilm is balanced by detachment, which results from, e.g., the physical characteristics (such as shear forces) of the surrounding environment and the type of organisms present. The exchange between attached and unattached bacterial communities is regulated by several factors that affect the growth, attachment and life cycles of the subsurface bacteria. Thus, the communities of attached and unattached bacteria overlap but differ from each other (Madsen and Ghiorse 1993). Furthermore, as Hirsch and Rades-Rohkohl (1983) proposed, the attached microorganisms are likely to have motile, unattached stages. To summarise, biofilms are dynamic microbial communities with continuous accumulation and degradation, which occurs upon immediate connection with the water phase.

1.6 Determination of NOM biodegradation

Several studies have confirmed the efficiency of AGR in removing NOM both in Finland (Hatva 2004, Helmisaari *et al*. 2003, Kivimäki 1992) and other countries but only a few attempts have been made to determine the mechanisms involved. Studying NOM dynamics in an AGR aquifer is a complex task due to the heterogeneous nature of NOM, analytical limitations as well as several simultaneous physico-chemical and biological transformation processes occurring during the flow path. The determination of NOM biodegradation is further complicated by the several products formed and released in the process (see Chapter 1.4.2). Thus, no generally accepted standard method exists for the determination of NOM biodegradation. Due to the differences between experimental approaches, the tests either identify different NOM pools (labile, refractory etc.) or quantify the DOC mineralisation or the DOC that remains after a certain time period. Many parameters, such as the origin of microorganisms, the type and duration of test systems (batch assays, continuous-flow bioreactors), nutrient supplementation, and prevailing temperature vary between different studies and make inter-study comparisons difficult.

The quantification of NOM biodegradation is based on either the measurement of microbial growth or a parameter affected by growth. The most common methods used by soil scientists, groundwater hydrologists, limnologists and oceanographers include the measurement of CO_2 production and DO consumption in batch tests and related kinetic calculations as well as DOC disappearance in batch tests or bioreactors (Marschner and Kalbitz 2003). Batch tests can either contain a pure culture or indigenous microorganisms.

The measurement of the reduction in DOC after incubation with a microbial inoculum (Servais *et al*. 1987), or after passage through a plug-flow reactor filled with a solid matrix (Lucena *et al*. 1990, Frias *et al*. 1992, Kaplan and Newbold 1995) are by far the most commonly used methods in biodegradation studies. The obtained BDOC is, however, a net value and does not take into account the excreted and incorporated proportion of DOC. Kaplan and Newbold (1995) found this error to be up to 32%. Bioreactors typically result in about 20% greater DOC utilisation compared to batch cultures, as reported by Søndergaard and Worm (2001). The authors assumed this to result from the enrichment of specialised bacterial species along the resource gradient in bioreactors. Trulleyová and Rulik (2004) found that BDOC determination using suspended bacteria as the inoculum compared to the attached bacteria resulted in 5% to 25% underestimation of the obtained BDOC value. The authors suggested that this results from a more diverse biofilm community that has a higher metabolic activity compared to the suspended population. As mentioned by Søndergaard and Worm (2001), batch cultures do not allow the development of bacterial communities with proper decomposition capabilities. Kaplan and Newbold (1995) used a plug-flow reactor and found that BDOC was affected by the hydraulic residence time, DOC concentration and water temperature.

The determination of the head-space CO_2 (e.g. Kalbitz *et al*. 2003) or the dissolved oxygen (DO) content over time allows for the kinetic evaluation of the decomposition rate constants of the labile and refractory NOM fractions. The determination of DO and/or CO_2 at a full-scale AGR site can be used for the preliminary evaluation of the contribution of biodegradation to NOM removal (Helmisaari *et al*. 2003, Jacks 2001b). However, the measurements involve several uncertainties such as the errors caused by pumping at the full-scale sites and the mixing of infiltration water with natural groundwater.

The determination of assimilable organic carbon (AOC) (van der Kooij 1982) is a method used to assess the biodegradability of organic matter. However, AOC only considers the assimilated part of the organic carbon used by the test organisms rather than the biodegradation and, therefore, addresses the likelihood of water samples to support microbial growth. As criticised by Servais *et al*. (1987), the measurement of microbial growth defines the biodegradability of DOM with respect to the metabolic capacities of the test organisms rather than the true biodegradability of NOM. Furthermore, nutrients other than organic carbon might limit microbial growth. Thus, the obtained AOC values are lower than the BDOC values and additionally, result in a higher standard deviation of the measured values (Frías *et al*. 1995). Långmark *et al*. (2004) used AOC determination in sand column studies and found that the sand columns could only remove 10±5% of TOC but 87±5% of assimilable organic carbon (AOC).

The isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) in water can be used as a tracer for the oxidative decomposition of organic matter and thereby for quantifying the mineralisation of NOM (Kortelainen and Karhu 2006). Photosynthesis, the basis of the organic matter formation in nature, causes considerable isotopic fractionation in carbon. Thus, in terrestrial high-latitude regions the $\delta^{13}C_{DOC}$ value of C_3-vegetation is typically around -27‰ (Deines 1980, Vogel 1993). This is in strong contrast to the $\delta^{13}C_{DIC}$ of lake water, which is close to that of the atmospheric CO_2 (-7‰). As a result of the microbial decomposition of NOM, the DIC with a distinctively negative $\delta^{13}C_{DIC}$ value is added to the DIC pool. The final $\delta^{13}C_{DIC}$(end) in water is a mixture of the initial $\delta^{13}C_{DIC}$(t=0) and the $\delta^{13}C_{DIC}$ emerging from the mineralisation of the DOC. The method was used for the quantification of the oxidative composition of NOM at a Finnish AGR site by Kortelainen and Karhu (2006).

1.7 Aims of the study

Artificial groundwater recharge is a widely applied method in drinking water production and in several other applications. In Finland, the proportion of AGR from drinking water production is likely to increase in the near future. Therefore, it is of great importance to understand the purification processes involved and to develop methods for monitoring the AGR process and its environmental impacts. Biodegradation plays a crucial role in the long-term sustainability of AGR. This study focused on the role of biodegradation in NOM removal, the influences of seasonal changes on that process, the microbial communities involved and the impact of changing nutritional conditions on the physiological state of microorganisms along the aquifer flow path.

The specific aims of the study were as follows:

- Evaluate NOM and bacterial cell removal, changes in NOM aromaticity and the removal of different NOM molecular size fractions along the aquifer flow path (Papers I, IV and V).

- Determine the influences of seasonal changes (especially the temperature), hydraulic load and incubation time on NOM biodegradation (Papers I and II).

- Develop a simplified experimental set-up to enrich a culture of NOM degrading microorganisms from lake water and to use the obtained enrichment culture for the determination of DO consumption rates in different seasons and to quantify NOM biodegradation at different temperatures (Paper II).

- Estimate the quantity of biomass accumulation in AGR and to determine the influence of temperature on the accumulation (Papers I, II and V).

- Determine the spatial and temporal changes of microbial communities in AGR using culture independent molecular biology methods (Papers III and IV).

- Assess the EEAs of α-D-glucosidase, β-D-glucosidase, phosphomonoesterase, leucine aminopeptidase and acetate esterase and nutrient availability for microorganisms in AGR (Paper V).

2 MATERIALS AND METHODS

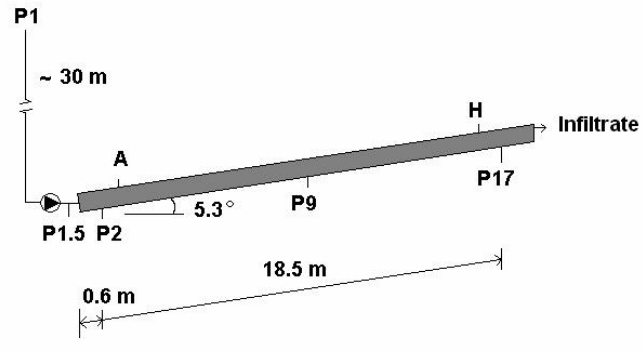
2.1 Experimental approach

This study comprised both experimental AGR simulation systems and full-scale investigations. Long-term studies with the experimental AGR systems were used in controlled conditions to predict the mechanisms of *in situ* applications. Full-scale studies provided performance and operational information on three Finnish AGR sites. A combination of physico-chemical, microbiological and molecular biology methods were used.

2.2 Sand column

A sand column (length: 18.5 m, Ø: 0.3 m, volume: 1.3 m^3) was used to simulate the artificial recharge of an aquifer as described in Papers I and III (Fig. 2.1). The column was located in the basement of Tampere waterworks (Rusko) and consisted of 16 sampling ports (P2-P17). The first sampling port of the sand column (P2) was located at 0.6 m distance from the inlet, followed by ports P3 to P17 about 1.2 m apart. The raw water originated from Lake Roine, 7 km from the water works, retrieved 4 to 5 m from the water level and 2 m from the bottom of the lake. The source water used in the study will serve as source water at an AGR site that is currently proposed, pending full-scale investigations and permits. The inlet for the raw water (P1) was located 30 m from the sand column and the water was directed to the column through plastic tubing. The sand originated from a vadose zone of Vehoniemi-Isokangas esker (~5 m from the surface), in the municipality of Kangasala, Finland, a proposed area for AGR. The column was set at a 5.3° angle and the water was directed in bottom-up direction to simulate the saturated zone of an aquifer. The temperature in the cellar (5 °C to 22 °C) followed seasonal temperature variations.

a)



b)

Figure 2.1. A schematic illustration (a) and a photograph (b) of the sand column (Papers I and III). Only selected sampling ports are shown. Symbols: P1: Lake water, P1.5: Inlet water, P2: Water sampling port at 0.6 m, P9: Water sampling port at 8.9 m, P17: Water sampling port at 18.5 m (outlet). A-H: Sampling ports for sand.

2.3 Fluidized-bed reactor

A glass-made FBR (height: 0.43 m, ID: 0.044 m, water volume with recirculation tubing: 0.535 dm^3), also placed in Tampere waterworks' basement, was used to enrich a culture of NOM degrading microorganisms from lake water (Fig. 2.2). The FBR was kept in a dark place. The enrichment culture thus obtained was used for kinetic studies on lake water NOM biodegradation at different ambient temperatures (Paper II) and to characterise the differences between liquid-phase and attached bacterial populations (Paper III). With regard to the sand column, the raw water for the FBR was taken from Lake Roine but supplemented with nitrate (final concentration of 6.5 mg KNO_3 l^{-1}) and phosphate (0.2 mg KH_2PO_4 l^{-1}) in a 25 l feed tank to promote biofilm accumulation and to keep organic C as the limiting nutrient in the system. Porous siliceous ceramic Celite R-633 (Celite Corporation) was used as the biocarrier with fluidization maintained at 10% (varied between 1% and 22%).

a) b)

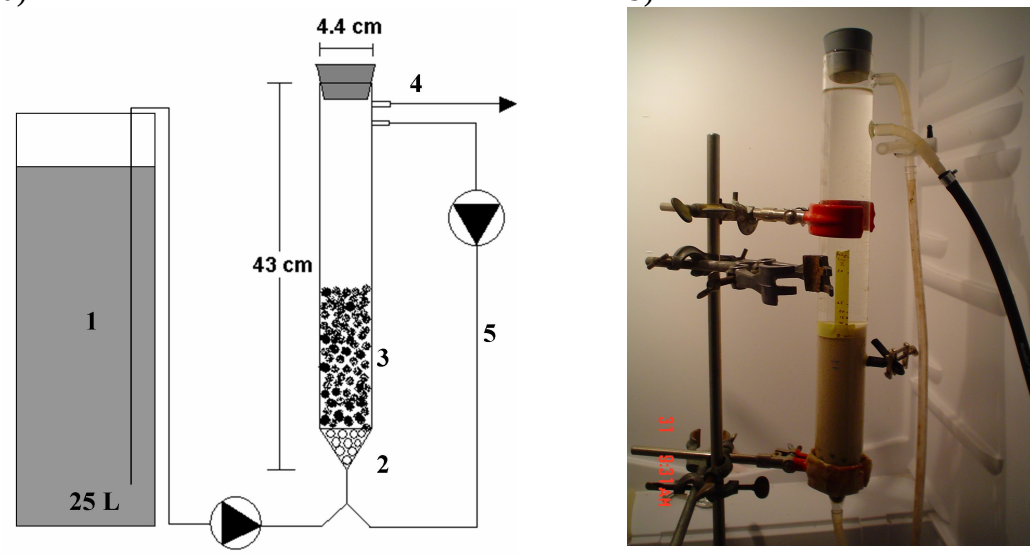


Figure 2.2. A schematic illustration (a) and a photograph (b) of the fluidized-bed reactor (Papers II and III).

2.4 Full-scale AGR sites

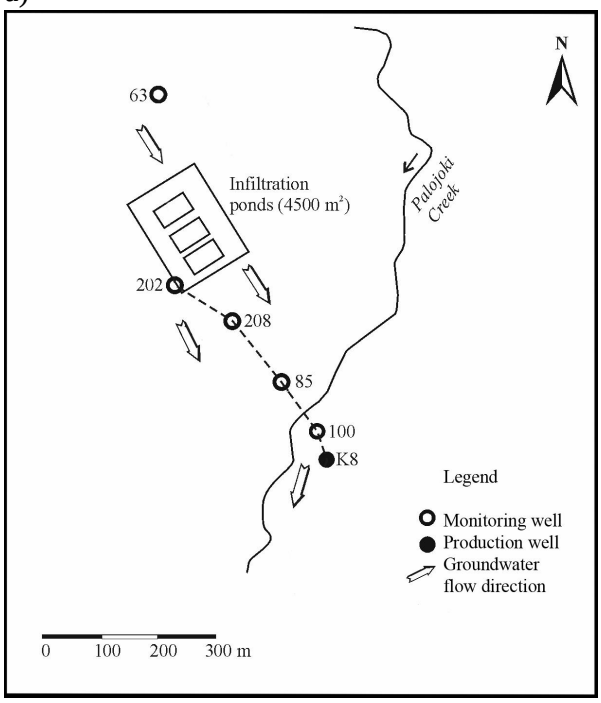
The AGR study sites were unconfined glacial aquifer formations, located in the public water works of Hämeenlinna (Ahvenisto) (site A), Jyväskylä (Vuontee) (site B) and Tuusula (Jäniksenlinna) (site C), Finland. The characteristics of the AGR sites are given in Table 2.1. NOM removal and structural changes in bacterial communities during AGR were studied in January 2005 and August-September, 2005, as described in Paper IV. Furthermore, the EEA and nutrient availability for bacteria during the AGR was evaluated at site C on October 10 and December 17, 2007 as described in Paper V. At site C, natural groundwater in the same area but outside the influence of the recharge was used as a reference. Fig. 2.3 shows the schematic of site C and sampling at sites B and C.

Table 2.1. Characteristics of the studied AGR sites (Papers IV and V).

Site	Symbol	Recharge method	Start of recharge	Recharge volume ($m^3\ d^{-1}$)	Infiltration distance (m)	Estimated retention time[1] (d)
Hämeenlinna (Ahvenisto)	A	Basin Sprinkling	1976 1995	20 000	1000-1300	90
Jyväskylä (Vuontee)	B	Sprinkling	2000	15 000	200-550	15-30
Tuusula (Jäniksenlinna)	C	Basin	1979	10 000	500-700	30-60

[1]Helmisaari *et al.* (2003)

a)



b)

c)

Figure 2.3. a) Horizontal map of site C (modified from Kortelainen and Karhu, 2006). Monitoring wells 63, 202, 208 and 85 and production well K8 were sampled in the study (Paper V). The dashed line illustrates the flow direction along the aquifer flow path. b) Sampling at site B in January, 2005 (Paper IV). c) Sampling at site C in October, 2007 (Paper V).

2.5 Quantification of NOM biodegradation

The role of biodegradation in NOM removal over the seasonal changes was studied in both the sand column and the FBR. The mineralisation of the DOC was quantified by using the stable inorganic carbon isotope ($\delta^{13}C$) method as described in Papers I and II. The analysis was performed at the Geological Survey of Finland. The fraction of carbon (f_{org}) in the DIC pool that resulted from the oxidative decomposition of DOC (f_{org}) was calculated as:

$$f_{org} = \frac{\delta^{13}C_{DIC}(end) - \delta^{13}C_{DIC}(t=0)}{\delta^{13}C_{DOC}(t=0) - \delta^{13}C_{DIC}(t=0)} \quad (2.1)$$

where *DIC(end)* = DIC in water at the end, *DOC(t=0)* = DOC in water at the beginning and *DIC(t=0)* = DIC in water at the beginning of the batch test. The $^{13}C/^{12}C$ ratio was expressed as $\delta^{13}C$ (‰) relative to the VPDB (Vienna Peedee Belemnite) standard.

The δ-value is defined as:

$$\delta^{13}C = \left[\left(\frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{standard}} \right) - 1 \right] \cdot 1000 \quad (2.2)$$

The theoretical calculations of NOM mineralisation were based on Eq. 2.3 in which CH_2O represents a generalised concept for an organic carbon oxidation state in complex organic matter such as humus:

$$CH_2O + O_2 \rightarrow CO_2 + H_2O \quad (2.3)$$

2.6 Determination of dissolved oxygen consumption rates in the FBR

The NOM biodegradation kinetics at different ambient temperatures was studied by the FBR enrichment culture as described in Paper II. A series of FBR batch DO consumption tests were conducted during 609 days of operation in order to ascertain the impact of temperature on NOM biodegradation. The DO consumption rates (k, mg_{DO} $(g_{biomass}\ h)^{-1}$) were normalised to biomass quantity according to Eq. 2.4:

$$k = \frac{\Delta c_{DO} \cdot V}{t \cdot m_{biomass}} \quad (2.4)$$

where Δc_{DO} = DO consumed in time t (mg l^{-1}), V = total water volume in the reactor (l), t = duration of linear DO consumption (h), and $m_{biomass}$ = quantity of biomass on the carrier surface (g) determined as volatile solids (VS). The relationship between temperature and DO consumption rate k was determined using the Arrhenius equation:

$$\ln k = -\frac{E_a}{R}\left(\frac{1}{T}\right) + \ln A \quad (2.5)$$

where E_a = activation energy (mol J^{-1}) (energy needed for sufficient molecular collision), R = universal gas constant (8.314 J $(mol\ K)^{-1}$), A = Arrhenius constant (mg $(mg\ VS\ h)^{-1}$) (proportion of molecules that collide and react) and T = temperature (K).

2.7 Bacterial community characterisation

Changes in the bacterial community structure during infiltration at sites A, B and C were studied by PCR of 16S rRNA genes, which was followed by denaturing gradient gel electrophoresis (DGGE) fingerprinting (Paper IV). Furthermore, spatial and temporal changes in the feed lake water (Lake Roine), FBR, and sand column bacterial communities were determined by DGGE

and the length heterogeneity analysis of the amplified PCR products (LH-PCR) (Paper III). Two clone libraries were created to link the LH-PCR results to the dominant bacterial groups.

2.8 Extracellular enzyme activities in AGR

The extracellular enzyme activities (EEAs) of α-D-glucosidase (α-Glu), β-D-glucosidase (β-Glu), phosphomonoesterase (PME), leucine aminopeptidase (LAP) and acetate esterase (AEST) were determined at site C in order to assess the availability of nutrients (C, N, P) for bacteria in the AGR as described in Paper V. The specific EEAs were determined from the basin sediments as well as the water samples along the flow path (Fig. 2.3). Sediment samples were taken from different locations at the infiltration basin using a sediment core sampler (h: 50 cm, Ø: 5 cm) (Fig. 2.4). Several physicochemical parameters were measured along the flow path in order to ascertain associations between the EEAs and the prevailing environmental conditions.

a) b)

Figure 2.4. Sediment core sampler and the sampled sediment at site C's basin in October, 2007 (Paper V).

2.9 Physico-chemical and microbiological analyses

The physico-chemical and microbiological analyses performed in the study are summarised in Tables 2.2 and 2.3.

Table 2.2. Physico-chemical analyses performed in the study.

Physico-chemical analyses	Method	Reference	Paper
Temperature	Electrode	Papers I, II, V	I, II, V
pH	Electrode	Papers I, II, V	I, II, V
Dissolved oxygen	Electrode	Papers I, II, V	I, II, V
Conductivity	Electrode	Papers I, V	I, V
DOC[a]	Total organic carbon analyzer	APHA (1995)	I, II, IV, V
TOC[a]	Total organic carbon analyzer	APHA (1995)	I, II, IV, V
UVA_{254}[a]	Spectrophotometer	APHA (1995)	I, II, IV, V
UVA_{280}[a]	Spectrophotometer	APHA (1995)	IV
NOM molecular size distribution	HPSEC	Vartiainen *et al.* (1987), Peuravuori and Pihlaja (1997)	I, IV, V
COD_{Mn}	Oxidation with permanganate	SFS-3036	I
$\delta^{13}C$[b]	Mass spectrometer	Atekwana and Khrishnamurthy (1998), Kortelainen and Karhu (2006)	I, II
Alkalinity	Titration	SFS-EN ISO 9963-1	I
tot-P[a]		SFS 3026	V
tot-N[c]		SFS-EN ISO 11905-1	V
NH_4^+[c]		SFS-EN ISO 11732	V
NO_3^-[c]		SFS-EN ISO 13395	V
NO_2^-[c]		SFS-EN ISO 13395	V
Amino acids	HPLC	Lindroth and Mopper (1979), Münster (1999b), Langwaldt *et al.* (2005)	V
Volatilde solids (VS)	Furnace	APHA, 1995	I, II, V

[a]Analysis performed at Tampere waterworks laboratory.
[b]Analysis performed at the Geological Survey of Finland.
[c]Analysis performed at the Laboratory of the Water Protection Association of Kokemäenjoki River.

Table 2.3. Microbiological and molecular analyses performed in the study.

Microbiological and molecular analyses	Method	Reference	Paper
DAPI staining	Epifluorescence microscopy	Coleman (1980), Männistö *et al.* (2001)	I, II, IV, V
Phytoplankton biomass (chl-a)	Ethanol extraction	SFS-5772	V
Diversity and identification	DNA extraction and purification	Papers III and IV	III, IV
	PCR	Papers III and IV	III, IV
	DGGE	Muyzer *et al.* (1993)	III, IV
	LH-PCR[a]	Suzuki *et al.* (1998), Tiirola *et al.* (2003)	III
	Cloning[a]	Paper III	III
	Sequencing[a]	Papers III and IV	III, IV
Extracellular enzyme activities	Fluorometer	Hoppe (1983), Palmroth *et al.* (2005)	V

[a]Analysis performed at Jyväskylä University.

3 RESULTS AND DISCUSSION

This study aimed to examine the microbiological aspects of AGR, i.e., the role of biodegradation in NOM removal, the influence of seasonal changes on biodegradation, the spatial and temporal dynamics of the bacterial communities involved and the responses of EEAs regarding prevailing nutrient concentrations and thus the availability of nutrients for bacteria along the aquifer flow path. The study contributes to the scientific basis and understanding of water purification mechanisms in AGR.

3.1 NOM removal and nutritional conditions along the aquifer flow path

The removal of different NOM molecular size fractions and their conversion to total and dissolved organic carbon (DOC and TOC) was determined in the closed sand column (Paper I) and at three full-scale Finnish AGR sites (Papers IV and V). In addition, tot-P, tot-N, NO_2^-+NO_3^- and NH_4^+ were determined at site C (Paper V). The concentrations of DOC in raw waters at the three sites varied between 5.1 mg l^{-1} and 11.2 mg l^{-1} (n=8) (Table 3.1). All the full-scale AGR sites revealed an efficient removal of organic carbon by fulfilling the national recommendation of 2 mg TOC l^{-1} in drinking water. Reductions of TOC at sites A, B and C were 91%, 84% and 74%, respectively, in the winter, and 88%, 77% and 73%, respectively, in the summer (Paper IV). Similar TOC reductions at the same AGR sites were reported in earlier studies by Helmisaari *et al*. (2003). At site C, DOC content correlated equally with both the infiltration distance and the estimated retention time (r~0.9, P<0.05) (Paper V). Helmisaari *et al*. (2003) concluded that retention time played a greater role in organic carbon removal than the recharge distance.

In Lake Roine water that was directed to the sand column the DOC varied between 4.4 mg l^{-1} and 7.9 mg l^{-1} (average 5.4 mg l^{-1}, n=27) (Table 3.1) and the TOC between 5.2 mg l^{-1} and 8.1 mg l^{-1} (average 6.0 mg l^{-1}, n=27) during the experimental period of 941 d. In the saturated sand column, on average 76% and 81% of TOC was removed within the first 0.6 m and the entire length of 18.5 m, respectively (Fig. 3.1). No detectable accumulation of NOM was detected, despite the long recharge period. Increasing the hydraulic load from 0.3 $m^3(m^2d)^{-1}$ to 3.1 $m^3(m^2d)^{-1}$ did not affect the TOC removal. This was, however, likely due to the washout of NOM from the sand matrix due to the sudden increase in the flow rate. The results indicate the washout of TOC from the sand matrix along the flow path throughout the experimental period. It is likely that washout TOC included microbial cells sloughed off from the sand matrix. No significant NOM removal occurred after the first sampling port at 0.6 m. This indicates the presence of refractory NOM fractions and/or the continuous washout of NOM from the sand matrix.

Table 3.1. Average DOC concentrations [mg l^{-1}] in raw waters and extracted groundwaters at the studied AGR Sites (Papers IV and V) and the sand column (Paper I). Site A = Hämeenlinna; site B = Jyväskylä; site C = Tuusula.

Date	Site A	Site B	Site C	Sand column
Jan 2005	11.3 – 1.1	7.0 – 1.4	5.1 – 1.6	-
Aug & Sep 2005	9.2 – 1.5	7.2 – 1.7	7.7 – 1.7	-
Oct 2007	-	-	6.1 – 1.9	-
Dec 2007	-	-	6.3 – 1.9	-
Sept 2004-Apr 2007 (941 d)	-	-	-	5.4 – 1.2

As with organic carbon, total P decreased along the flow path down to the detection limit of 2 µg P l^{-1} (see Table 3.2 for site C) (Paper V). The nutritional conditions of water thus became gradually oligotrophic along the flow path. The proportion of organic N to total N decreased from 45% in the infiltration basin to 4-15% in the production well, which was most likely the result of microbial catabolism. The average ratios of C:N:P indicated P limitation in all samples, based on Redfield's stoichiometric make-up of a microbial cell, i.e., a C:N:P ratio of 106:16:1 (Cleveland and Liptzin 2007, Redfield 1958). However, this nutrient ratio does not consider the bioavailability of DOC that also contains refractory humic substances. The nutritional conditions in the natural groundwater, which was used as a control, were even more oligotrophic. Amino acid analysis illustrated a great variation in the different fractions between the two sampling dates and along the aquifer flow path indicating that amino acids were a relatively labile N-source for the microorganisms.

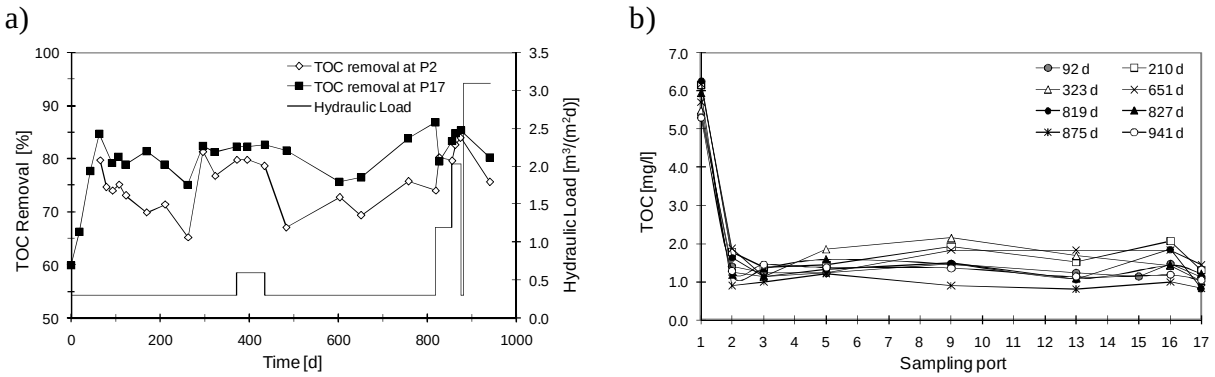


Figure 3.1. Sand column performance during the experimental period (Paper I). a) TOC removal percentages at ports P2 (0.6 m) and P17 (18.5 m) and the hydraulic load during the experimental period. b) TOC along the column.

The determination of different molecular size fractions of NOM by HPSEC illustrated that larger molecular size fractions (fractions I and II) of NOM were removed more efficiently than the smaller ones (fractions III-V) along the flow path (Fig. 3.2) (Papers I, IV and V). In natural groundwater (at site C), only the three smallest fractions existed. This is in accordance with studies made of 34 water samples by Nissinen *et al*. (2001) who showed that raw waters generally comprised 6 NOM fractions of large and intermediate size whereas artificially recharged and natural groundwaters comprised 4 to 6 and 2 to 5 intermediate and small fractions, respectively. While showing a preferential removal of large NOM fractions in the AGR, no accumulation of smaller fractions was observed. This could be interpreted as the sorption or entrapment of the larger fractions onto the sand matrix rather than their mineralisation. However, the results of this study give an indication that NOM macromolecules included fractions that could be utilised by the indigenous bacteria (Paper V).

NOM aromaticity, determined as $SUVA_{254}$, decreased along the flow path in both the full-scale AGR sites and the sand column (Papers I, IV, V). However, slightly higher aromaticities were seen in the infiltration basins and the sprinkling networks of sites A, B and C compared to the raw waters. The decrease in aromaticities along the flow path may be due to the preferential sorption of the aromatic NOM moieties on the sand surfaces rather than their mineralisation. Support for this finding has been reported by McKnight *et al*. (1992), who observed that the aromatic components of aquatic NOM were preferentially sorbed onto hydrous aluminium and iron oxides.

Table 3.2. Physico-chemical water quality at each sampling location of site C in October (a) and December (b), 2007 (Paper V). The distance from the infiltration basin and the approximate hydrological retention time of each well are taken from Helmisaari *et al*. (2003). MW and PW refer to a monitoring well and a production well, respectively.

Parameter / Sampling location (distance from the basin, retention time)	Date	Päijänne-tunnel	Basin	MW202 (10 m, 4 d)	MW208 (120 m, 18 d)	MW85 (330 m, 18-36 d)	PW8 (480 m, 36-43 d)	MW63 Natural groundwater
Temp. [^{o}C]	a	10.3	8.7	11.2	10.3	8.9	6.9	7.8
	b	3.2	2.6	5.5	6.7	8.0	7.5	6.6
pH	a	6.4	6.1	6.3	6.9	6.9	6.8	6.7
	b	6.5	5.4	4.7	6.2	6.3	6.3	5.9
DO[a] [mg l^{-1}]	a	9.1	9.7	9.4	7.0	6.1	6.4	7.1
	b	12.0	12.2	10.0	8.2	6.4	4.4	7.1
Conductivity [µS cm^{-1}]	a	49	65	68	75	81	83	216
	b	69	69	70	76	79	82	215
DOC [mg l^{-1}]	a	6.0	6.1	4.1	4.1	3.4	1.9	0.9
	b	6.3	6.3	5.2	4.0	3.4	1.9	0.9
TOC [mg l^{-1}]	a	6.1	6.1	4.3	3.6	3.3	2.1	1.1
	b	7.6	6.2	4.7	4.2	3.6	2.1	0.7
$SUVA_{254}$ [l $(mg\ m)^{-1}$]	a	0.027	0.027	0.023	0.039	0.020	0.014	0.010
	b	0.026	0.027	0.020	0.021	0.019	0.014	0.010
tot-P [µg l^{-1} P]	a	7.1	7.8	4.7	3.7	3.2	3.1	2.4
	b	5.7	5.3	2.8	3.2	<2.0 (1.9)	<2.0 (1.0)	<2.0 (1.1)
tot-N [µg l^{-1}]	a	420	410	590	420	510	370	1730
	b	520	860	460	440	480	480	1150
NH_4^+ [µg l^{-1}]	a	<7	<7	<7	<7	<7	<7	<7
	b	<7	<7	<7	<7	<7	<7	<7
NO_2^-+ NO_3^- [µg l^{-1} N]	a	230	220	400	340	430	350	1700
	b	270	470	300	290	350	400	1100
NO_2^- [µg l^{-1} N]	a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	b	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5

n.d. = not detected
[a]Dissolved oxygen

The preferential sorption of large molecular size and more aromatic components of aquatic NOM on mineral surfaces have been shown (Namjesnik-Dejanovic *et al.* 2000). Other factors, such as N-content, may also influence the sorption of NOM (Aufdenkampe *et al.* 2001). Furthermore, the ability of microorganisms to utilise different molecular size fractions as well as the labile and refractory fractions of NOM varies between different studies (Kaplan *et al.* 2008, Bano *et al.* 1997, Volk *et al.* 1997, Coffin *et al.* 1993, Moran and Hodson 1990, Tranvik 1990, Meyer *et al.* 1987). Thus, no unambiguous conclusion regarding the removal mechanisms of different NOM fractions in this study can be given.

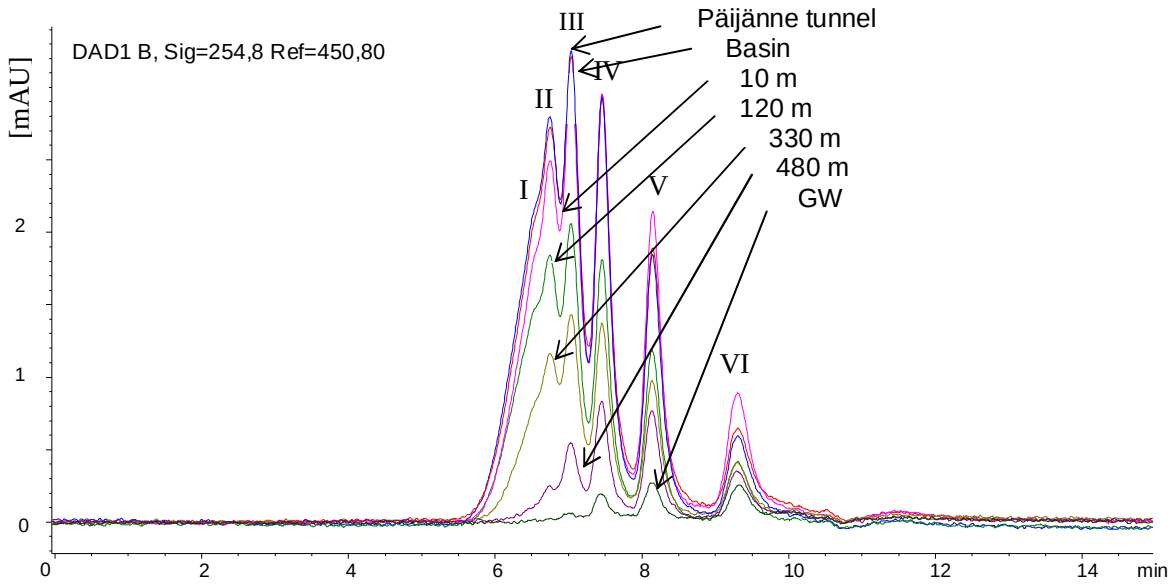


Figure 3.2. HPSEC chromatograms of water samples along the flow path at site C and in natural groundwater on December 17, 2007 (Paper V). The molecular weight approximates of each fraction were as follows: I = 3000 Da, II = 2600 Da, III = 2100 Da, IV = 1500 Da, V = 900 Da and VI = 400 Da.

3.2 Biodegradation of NOM over seasonal changes

The biodegradation of NOM over seasonal changes was determined in the sand column (Paper I) and in the FBR (Paper II). The $\delta^{13}C_{DIC}$ analysis was used to quantify the proportion of mineralisation from DOC removal at two different temperatures in both test systems and with two different loading rates in the sand column. Additionally, DO consumption rates over the seasonal temperature range were determined in the FBR by continuous-flow and batch test experiments to delineate the influence of temperature on NOM biodegradation.

In the sand column, DOC mineralisation varied between 32% and 52%, depending on the temperature and hydraulic load (Paper I). The temperature only slightly affected the mineralisation (32% and 38% at 5.9 °C and 22.7 °C, respectively). However, other factors may also have affected the biodegradation due to constant seasonal changes in lake water quality. The greatest proportion of mineralisation occurred on day 883 when the hydraulic load had been increased tenfold. The influence of increasing the hydraulic load, i.e., reducing the hydraulic retention time, can be explained by the greater mass flux of biodegradable material stimulating

microbial activity (Fontaine *et al*. 2003). Additionally, a sudden change in the hydraulic load may have caused NOM detachment from the sand matrix since the column had only been subjected to the higher hydraulic loading for 2 days.

Kortelainen and Karhu (2006) reported that a DOC drop of 28% occurred within 10 m of recharge relative to the raw source water (4 days residence time) at a full-scale AGR site using basin infiltration (site C). Within 350 m of recharge (of a total of 700 m), 44% of the DOC was removed. This was through mineralisation. A further 23% removal was due to adsorption and a final 14% removal was due to dilution with local groundwater. Based on dissolved CO_2 concentrations, Helmisaari *et al*. (2003) found, at the same site, that about 30% of organic carbon removal within the 480 m flow path was due to biodegradation. Jacks (2001b) reported that 50% of DOC retention occurred in the unsaturated zone of a Swedish full-scale AGR site and based on DO consumption, 25% of this was biologically degraded.

In the FBR, temperature significantly influenced NOM biodegradation, as shown by both the continuous-flow and batch test experiments (Paper II). In the batch tests, a Q_{10} of 2.3 was found, showing the strong temperature dependency of NOM biodegradation (Fig. 3.3a). This is very close to factor 2, which is generally reported for chemical reactions. However, for biological reactions, higher Q_{10} values have also been reported (e.g., Melin *et al*. 1998). The analysis of $\delta^{13}C_{DIC}$ revealed 27% and 69% mineralisation of DOC at 23 °C and 6 °C over 65 min and 630 min, respectively. Increasing the hydraulic loading to the FBR resulted in an increase in the DO consumption rates, suggesting that the faster flux of influent organic matter enhanced the oxygen uptake activity of the indigenous microorganisms in the FBR (Fig. 3.3b). After the initial increase the DO consumption rates declined in the second phase.

In both the continuous-flow mode and during the batch tests, the highest average DO consumption rate in the FBR was reached in the summer (June-August) when lake water temperature was at its highest, followed by the fall, spring and winter (if the high rate in the first fall season is neglected) (Fig. 3.3c-d). The high DO consumption rate at the beginning of the experiment cannot be compared with the rates obtained later on due to the low initial biomass content in the FBR (1.6 mg VS g^{-1} dw carrier). The slightly higher DO consumption rates obtained in the continuous-flow mode as opposed to the batch tests can be explained by the continuous flow of fresh lake water to the FBR in the first case, thus providing a continuous flow of NOM to the microorganisms.

In the FBR batch tests, the DO consumption at low temperatures followed a typical kinetic curve for NOM biodegradation; i.e., a sharp initial decrease followed by a more gradual decrease (Fig. 3.4). This illustrates the difference between rapidly and slowly biodegradable and non-biodegradable fractions (Yavich *et al*. 2004, Marschner and Kalbitz 2003). At higher temperatures this tendency was not apparent.

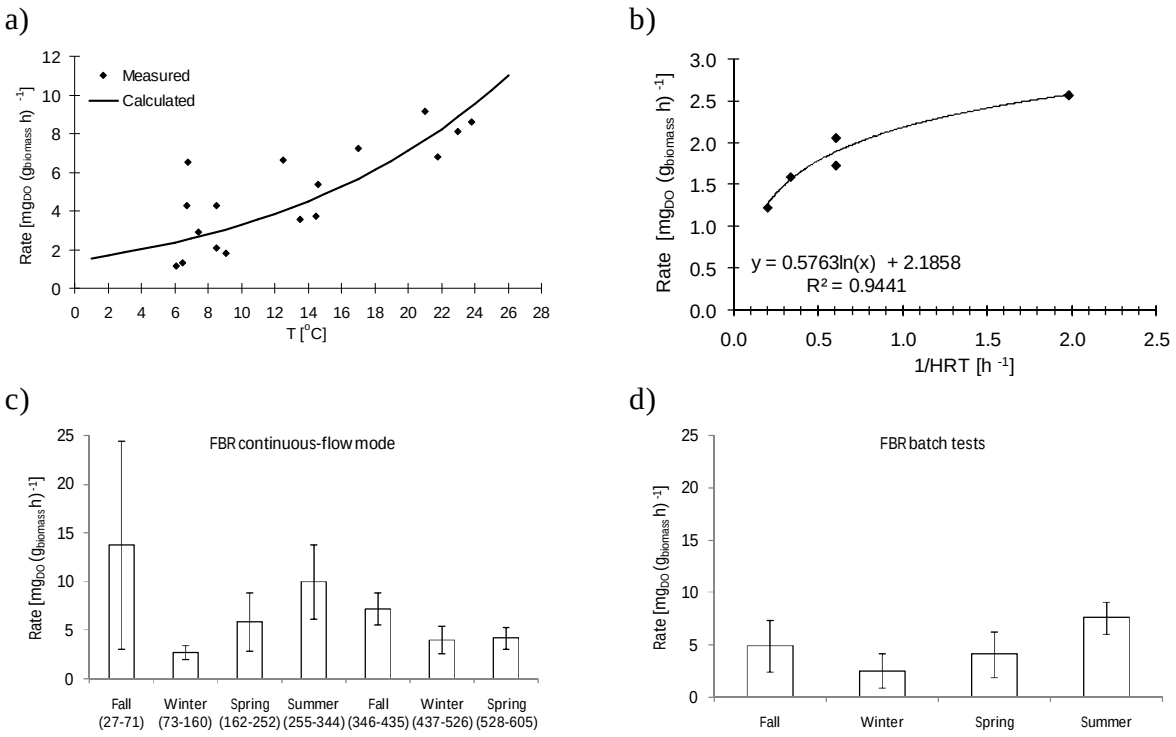


Figure 3.3. DO consumption rates in the FBR (Paper II): a) Measured and calculated rates in the batch tests. b) Influence of 1/HRT; i.e., the load of NOM, on the rates during days 547 to 554. c) Average of the seasonal rates in the continuous-flow mode. D) Average of the seasonal rates in the FBR batch tests.

The influence of other temporal factors in addition to temperature, such as NOM quality, could not be separately determined in this study. However, because microbial communities change in response to changes in their environment (Hahn 2006, Crump *et al*. 2003) it would not be reasonable to separate a community from its natural environmental conditions.

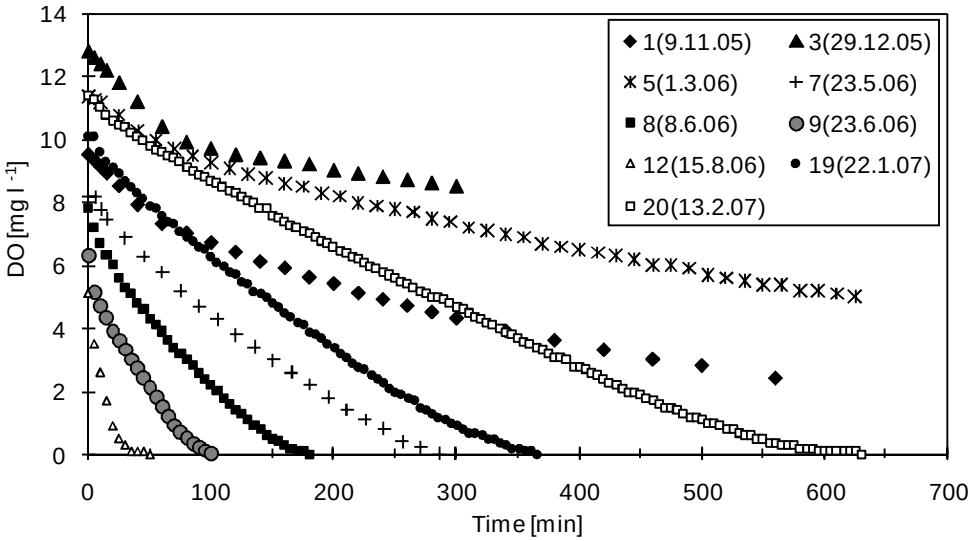


Figure 3.4. DO consumption over time in selected batch tests (Paper II). The first linear phase (35 min or 60 min) of the curves was used to determine the DO consumption rates according to Eq. 2.4.

3.3 VS accumulation and cell retainment

Biomass accumulation was determined as VS at site C basin sediments (down to 10 cm depth) (Paper V), the sand column (at 1.2 m distance from the inlet) (Paper I) and the FBR carrier (Paper II). Bacterial numbers in water samples were determined by DAPI counts at the three full-scale AGR sites (Papers IV and V), the sand column (Paper I) and the FBR water (Paper II).

In the basin sediments, biomass content was clearly higher in the surface layer as compared to the bottom sediments: 12.5±1.5 mg VS g^{-1} (n=2) and 10.5±2.7 mg VS g^{-1} (n=5) in the surface and 6.0±1.0 mg VS g^{-1} (n=6) and 6.7±2.0 mg VS g^{-1} (n=6) in the bottom sediments in October and December, respectively. The chlorophyll-a (chl-a) content in the surface sediments was 50.6±39.0 mg g^{-1} (n=6) and 5.1±3.2 mg g^{-1} (n=5) and in the bottom sediments it was 1.4±1.0 mg g^{-1} (n=6) and 0.6±0.2 mg g^{-1} (n=6) in October and December, respectively. Thus, higher biomass content likely exists within the top layers of a filter bed compared to the lower layers.

A substantial decrease in total cell counts occurred in aquifers at sites A, B and C, the average cell counts in raw waters varying from 7.4 × 10^5 cells ml^{-1} to 24.0 × 10^5 cells ml^{-1} and in extracted groundwaters from 0.5 × 10^5 cells ml^{-1} to 1.0 × 10^5 cells ml^{-1} (Papers IV and V). At site C, where more intense sampling was performed, a substantial decrease occurred already within a 10 m distance from 18 × 10^5 cells ml^{-1} to 2 × 10^5 cells ml^{-1} (Fig. 3.5a) (Paper V). In the experimental sand column, the average decrease in the cell counts within 0.6 m distance was from 20.2±5.7 × 10^5 cells ml^{-1} (n=23) at P1 to 4.3±1.1 × 10^5 cells ml^{-1} (n=3) at P2 (Paper I). The decrease in cell counts was likely due to the entrapment and adsorption of cells in the sand matrix and only to a minor extent due to decay (Pang *et al.* 2004, Yavuz Corapcioglu and Haridas 1984). A slight variation in the cell counts occurred along the aquifer flow path, but more cells were present in the extracted groundwater compared to the natural groundwater (Paper V). For comparison, Lehtola *et al.* (2002) reported that total bacterial numbers after chemical treatment (before final disinfection) at 9 Finnish surface water works varied between 1.6 × 10^3 cells ml^{-1} and 3.1 × 10^5 cells ml^{-1}. This indicates that the bacterial numbers in extracted groundwaters at AGR sites fell within the upper limit of the chemically produced drinking waters. In the FBR, on average 35±5% fewer cells were in the outlet water compared to the inlet water. However, it is impossible to conclude what proportion of the cells in the FBR outlet water originated from the inlet water and what proportion from the attached biofilm.

Biomass content in the sand column and the FBR were determined as VS and thus included both bacterial cells and non-living organic material. In the sand column, the biomass showed continuous adsorption and desorption at 1.2 m distance (Paper I). The average VS quantity was 6.4±0.8 mg VS g^{-1} dw sand (n=9). Most of the NOM was removed by this distance. In the FBR, on the other hand, the biomass accumulation on the carrier continued until day 489 (Fig. 3.5b) (Paper II). Thereafter, the amount of biofilm was fairly stable, at about 10 mg g^{-1} dw of carrier. The maximum biomass quantity in the FBR was 13.1 mg VS g^{-1} dw carrier and the net biomass yield at the end of the experimental period was 0.045 mg VS mg^{-1} DOC. In the FBR, biomass enrichment was accelerated due to an increase in temperature (from 7 °C to 24 °C). However, the response to further temperature decreases was slow. The carrier matrix of the FBR was porous and thus improved biofilm formation when compared to aquifer sand but, on the other hand, the FBR was run under high-shear conditions due to the high recycle flow. However, no sampling could be performed prior to 1.2 m distance from the beginning of the sand column where the greatest amount of biomass was likely to be accumulated.

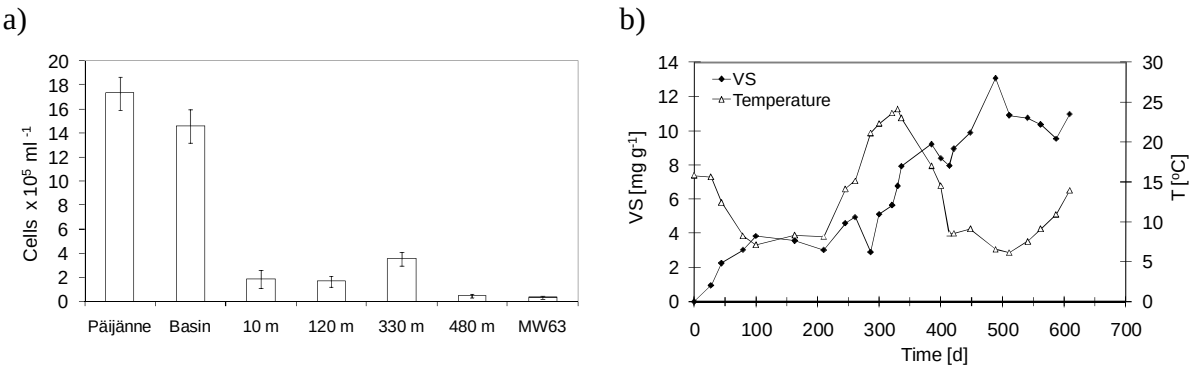


Figure 3.5. a) Direct cell count of water samples (average of two replicates) along the flow path at site C on October 10, 2007 (Paper V). b) Biomass accumulation as VS (average of two replicates) and temperature fluctuations in the FBR during the study (Paper II).

Clogging of the aquifer; i.e., the increase in flow resistance due to the accumulation of colloidal and particulate matter in the pores, is often considered a potential risk in AGR. In basin infiltration, clogging generally occurs within the top layers of the basin sediments. This is mainly caused by the algal growth and organic matter accumulation and is controlled by annual scraping of the top layer. In this study, higher chl-a content existed in October compared to December and more biomass (as both VS and chl-a) was found in the top than the bottom sediments (down to 10 cm). Also, the cell counts drastically decreased at the beginning of the flow path. Thus, a substantial proportion of biomass is likely retained already within the first few centimetres of the basin sediments. Albrechtsen *et al.* (2001) reported that microbial populations in a sand column postponed the clogging when compared to a column where formaldehyde treated water was infiltrated. This study did not show a major biomass development over the course of time the experimental systems were in use. Ideally the accumulation of biomass should sustain biomineralisation but not lead to the clogging of pore spaces.

3.4 Spatial and temporal changes in bacterial community dynamics

The spatial and temporal changes in bacterial communities were determined at the full-scale AGR sites (Paper IV) and by using the experimental setups (Paper III). At sites A, B and C, the bacterial communities in raw waters and extracted groundwaters were diverse and changes occurred during infiltration, which was shown by DGGE fingerprinting. While the natural groundwater microbial community at site C was diverse, it was different from that of the extracted groundwater in the AGR area.

In the experimental systems, the diversity of the microbial communities was profiled using both the DGGE and LH-PCR. The bacterial community of Lake Roine water was relatively stable with three dominant LH-PCR fragments (Fig. 3.6). The most dominant fragment was derived from Actinobacteria and accounted for up to 72% of all bacterial groups. The gram-positive Actinobacteria with high genomic G+C content can be recovered from a wide variety of soil, water and other environments and is a physiologically diverse group that includes potential species capable of utilising refractory NOM fractions (Haukka *et al*. 2005). The potential reasons for the slight temporal changes in the microbial community composition of Lake Roine water include changes in pH, temperature, NOM quality and quantity, predation, phytoplankton composition and ultraviolet radiation (Hahn 2006, Lindström *et al*. 2005, Crump *et al.* 2003).

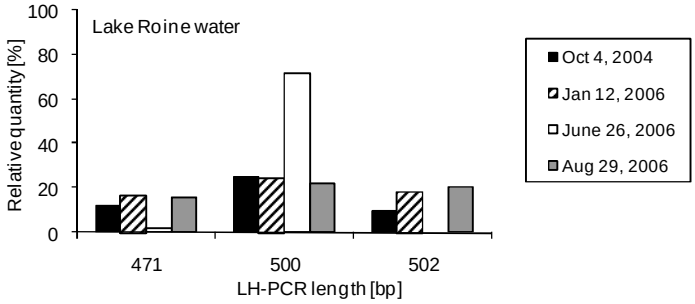


Figure 3.6. Relative quantity of the three most dominant LH-PCR fragments in Lake Roine water at different sampling dates (Paper III). LH-PCR lengths were assigned to α-Proteobacteria (471 bp), Actinobacteria (500 bp) and Planctomycetes (502 bp) based on the cloning and sequencing analysis performed in this study.

The original lake water community changed overnight in the FBR feed tank that was amended with phosphate and nitrate (Fig. 3.7). Furthermore, the feed tank community differed from the FBR outlet water community. While the water phase was dominated by Actinobacteria, Proteobacterial groups dominated in the biofilm. However, the dominance of the specific groups was not constant. This illustrates the dynamic nature of both communities. These differences illustrated attachment and detachment between the liquid-phase and the carrier during the flow-through and recirculation.

a)

b)

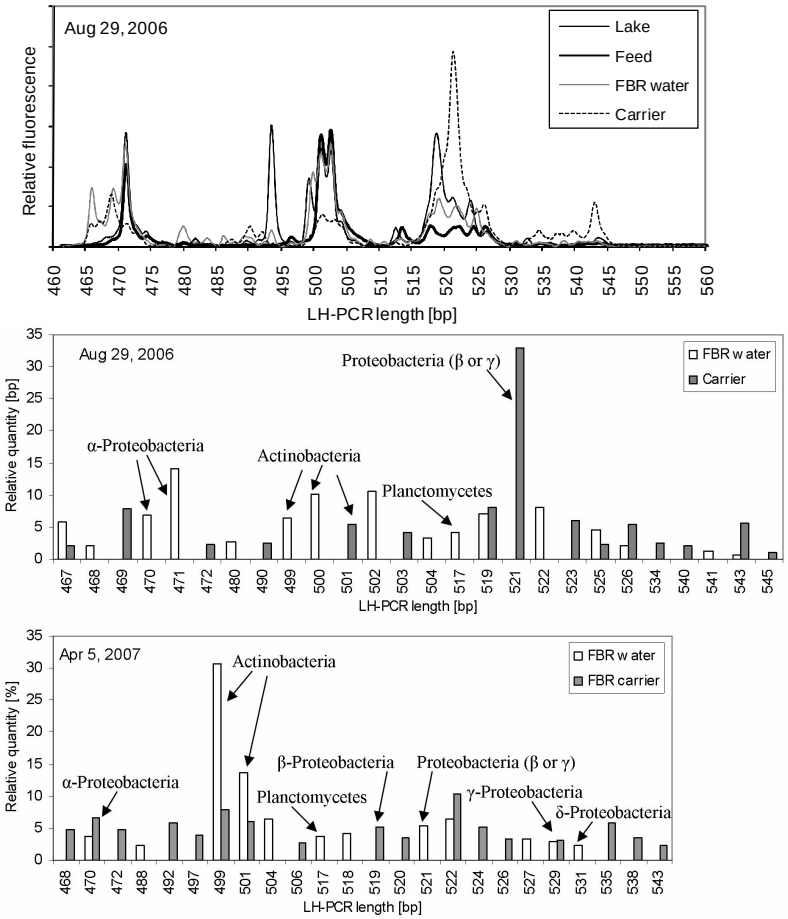


Figure 3.7. LH-PCR analysis of 16S rRNA gene fragments from the FBR (Paper III). a) Electropherogram of lake, feed tank, FBR water and carrier (August 29, 2006). b) Comparison of planktonic and sessile FBR communities as relative fragment quantities (August 29, 2006 and April 5, 2007). Fragments comprising at least 2% are shown.

In the sand column, a substantial change in the bacterial community had already occurred by the first sampling port at 0.6 m distance (Fig. 3.8). This change co-occurred with the decreases in cell counts and DOC (Paper I). The dominant fragment assigned to Actinobacteria, that was present in lake water on both sampling dates, disappeared by the 0.6 m distance. At the beginning of the simulation (day 21), however, another fragment also assigned to Actinobacteria dominated in the column (at P9 and P17). No similar dominance of Actinobacteria occurred later on (day 651), and other bacterial groups were found to be more dominant. Thus, a major change in the microbial community occurred within a relatively short distance of the flow path, accompanied with the substantial mineralisation of DOC (32% to 52%). This suggests an interplay between aquifer microorganisms and environmental determinants. Szewzyk *et al.* (1998) demonstrated shifts in a microbial community within a few centimetres of lake water infiltration through a sand column by using Proteobacterial subgroup-specific FISH probes.

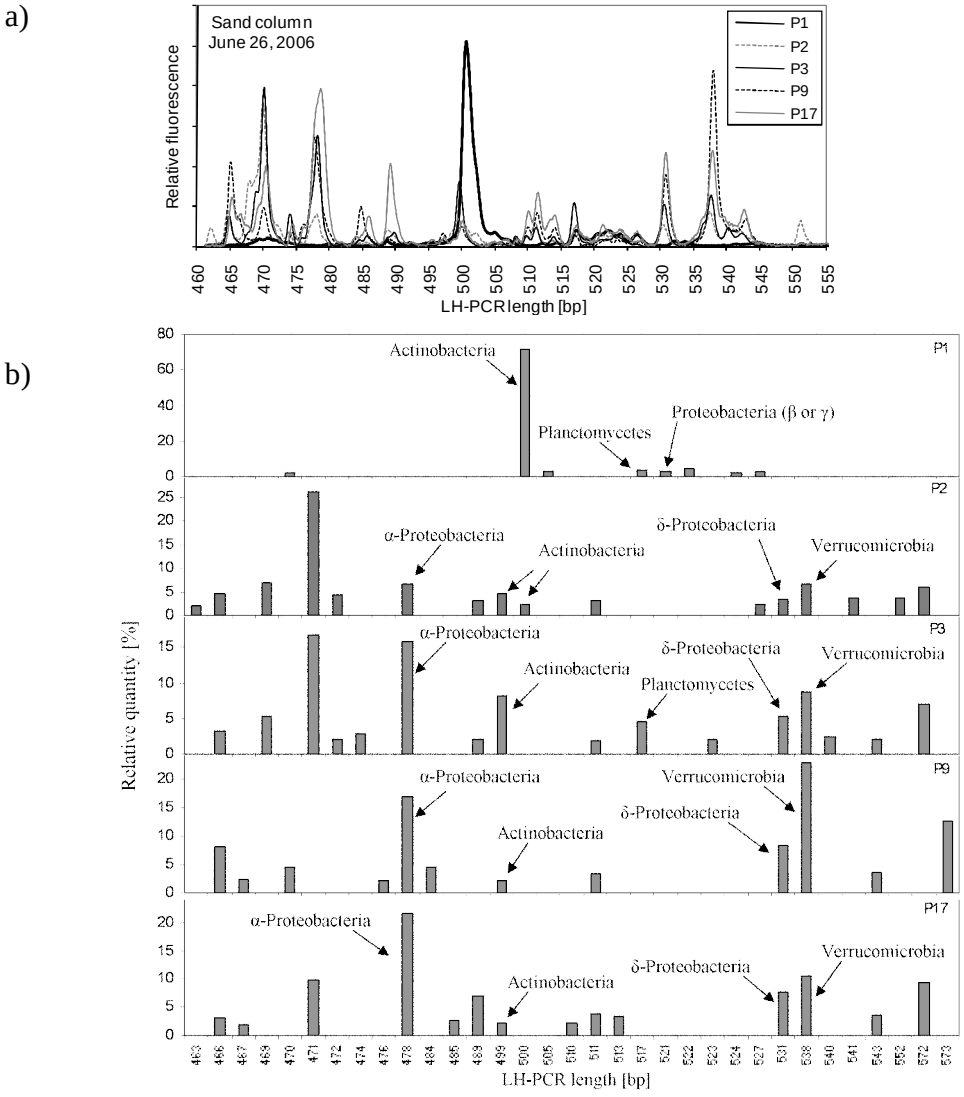


Figure 3.8. LH-PCR analysis of 16S rRNA gene fragments from water samples along the sand column after almost 2 years operation (June 26, 2006, day 651) (Paper III). Electropherograms of the samples at different sampling ports (a) and the relative quantities of different LH-PCR fragments comprising at least 2% of all fragments (b).

3.5 Extracellular enzyme activities and nutrient availability in AGR

The extracellular enzymatic activities of α-Glu, β-Glu, PME, LAP and AEST were determined from the basin sediments and the water samples along the flow path at site C (Paper V). These results show that the maximum hydrolysis rates were reached between 5 min and 30 min incubation, depending on the measured enzyme. The measured EEAs were in the range 10^{-22} - 10^{-17} mol (h cell)$^{-1}$. Substantial increases were detected in the specific EEAs of α-Glu, β-Glu, PME, LAP and AEST when measured in the AGR aquifer (Fig. 3.9). This co-occurred with decreasing nutrient concentrations (Table 3.2) shown by strongly negative correlations between the measured EEAs and the nutrient pools. The trend of increasing EEA along the flow path thus indicates a decrease in the availability of nutrients for bacteria.

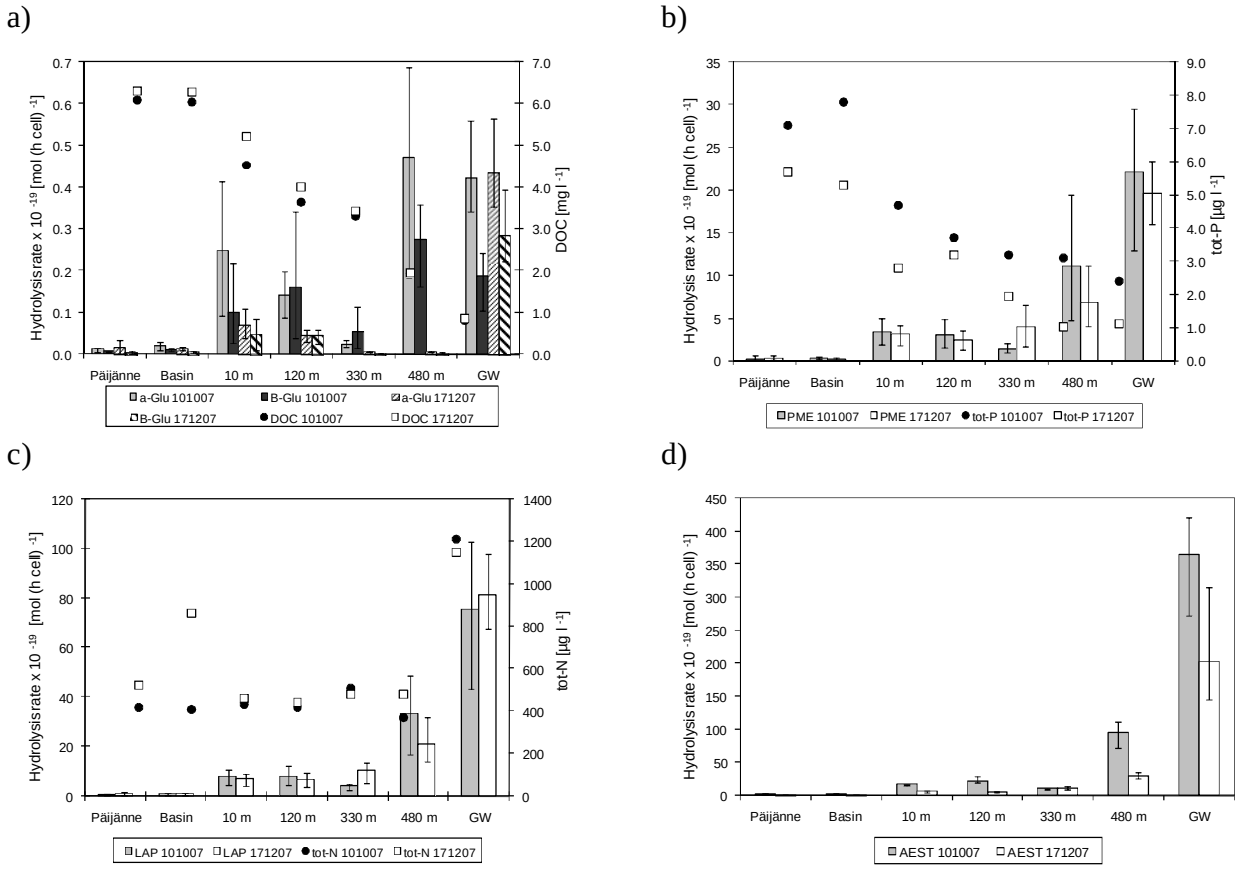


Figure 3.9. Hydrolysis rates of α-D-glucosidase (α-Glu) and β-D-Glucosidase (β-Glu) (a), phosphomonoesterase (PME) (b), leucine aminopeptidase (LAP) (c) and acetate esterase (AEST) (d) in water along the flow path and in natural groundwater (Paper V).

The EEAs in the basin sediment and the pore water samples (down to 10 cm) were of the same order of magnitude as the basin water, indicating similar nutritional conditions. This is in contrast to many studies in aquatic ecosystems that have shown higher cell specific activities for attached rather than for free-living bacteria (Riemann *et al.* 2000, Grossart and Simon 1998, Middelboe *et al*. 1995, Chróst 1990). This difference is likely due to the constant penetration of fresh NOM through the infiltration basin in the AGR as opposed to the slow sedimentation process in lakes.

The results were in accordance with the general understanding that the synthesis of virtually all extracellular enzymes in aquatic environments is repressed when readily utilisable dissolved organic matter is present (Chróst 1990). The EEAs had strong positive correlations with each other, suggesting synergistic and cooperative functions (Münster and de Haan 1998).

3.6 Implications of the study for AGR: improved process management

This study contributed to the microbiological aspects of AGR. Based on this and earlier studies, the following implications and recommendations can be drawn in order to further improve the understanding of subsurface processes and the management of the AGR process:

1. The contribution of biodegradation to NOM removal is most significant at the beginning of the aquifer flow path (Papers I and V, Kortelainen *et al.* 2006). In addition to biodegradation, other NOM removal mechanisms take place within the initial flow path. Therefore, installing the monitoring wells at the beginning of the aquifer flow path would allow for the close monitoring of the process. The first monitoring well should be located in the middle of (or next to) the infiltration basin or the sprinkling area followed by multiple monitoring wells along the flow path. An additional monitoring well, or wells, should be placed outside the influence of the infiltration as a reference for natural conditions in the aquifer.

2. Microorganisms respond promptly to changes in their environment (Papers I-V). Therefore, precisely monitoring the start-up phase of a new AGR site will provide information concerning the different biological and physico-chemical phenomena occurring in the aquifer and the responses of microorganisms to the changing environmental conditions. The physicochemical and microbiological characterisation of the natural groundwater should be performed already before start-up of a new site. The initial monitoring of a new AGR site is also recommended due to the wash-out of NOM from the sand matrix during the start-up of infiltration (Paper I).

3. The study demonstrates that temperature greatly influences NOM biodegradation (Papers I and II). In AGR, the temperature is equalised along the flow path, depending on the site specific conditions such as the porosity of the sand material and the infiltration distance. The extent of biodegradation from NOM removal is likely to be greater during the warmer seasons. The temperature in the AGR process cannot be adjusted. Therefore, the major consequence of the finding is that temperature should be considered as an input parameter in modelling applications concerning NOM removal in AGR. In addition to NOM biodegradation, temperature has a substantial influence on the formation of biomass as shown by the accumulation of biomass in the FBR (Paper II).

4. The decrease in total cell counts in the studied AGR sites varied from 75% to 91% resulting from 0.6×10^5 cells ml^{-1} to 1.9×10^5 cells ml^{-1} in extracted groundwater (Paper IV). When compared to bacterial numbers after chemical treatment at Finnish surface water works (from 1.6×10^3 cells ml^{-1} to 3.1×10^5 cells ml^{-1}, Lehtola *et al.* 2002), the bacterial numbers in the extracted groundwaters at the AGR sites fell within the upper limit of the chemically produced drinking waters. However, the nutrient content in extracted groundwaters is generally low thus reducing the potential for biomass re-growth in the distribution system. Therefore, a need for disinfection as a final treatment step should be considered on a site specific basis.

5. Due to the differences between sessile and planktonic bacterial populations and due to the difficulties in the sampling of the sand matrix, methods for the determination of aquifer biofilms should be developed. Of the existing methods, biofilm collectors immersed directly into the monitoring wells are recommended for use.

6. Lengthening the infiltration distance after reaching the goal of 2 mg TOC l^{-1} is not likely to further enhance NOM removal (Paper I). This is due to the presence of refractory NOM fractions, the highly oligotrophic conditions that lower microbial activities, and the continuous washout of NOM from the sand matrix.

7. *In situ* sampling is laborious and generally irregularly performed. Additionally, sampling by pumping may change some physico-chemical parameters. Thus, on-line measurements should be utilised when possible in order to produce continuous data. This will result in the detection of sudden changes or failures in the AGR operation.

8. Laboratory-scale experiments are needed for studying individual NOM removal processes under controlled conditions before performing on-site evaluations in a heterogeneous aquifer environment (Papers I, II and III). The obtained results can be used in modelling applications when aiming for the improved management and monitoring of the process. Full-scale studies, on the other hand, are also needed in order to determine the site-specific factors and to consider the synergy of different mechanisms responsible for NOM transformations.

9. NOM removal in AGR is site-specific (Paper IV and several earlier studies). Therefore, generalisations should be made cautiously concerning the role of different processes in AGR. Thus, studies should be performed at every AGR site individually in order to understand the mechanisms involved and to manage the process at each AGR site.

4 CONCLUSIONS

Both in Finland and elsewhere, the production of artificially recharged groundwater is likely to increase in the near future in both the drinking water sector as well as other applications. This is due to increasing demands regarding quality expectations and/or the scarcity of available clean water. This study demonstrated that microorganisms substantially contribute to the removal of NOM in AGR. This is of great importance from the perspective of the sustainability of drinking water production. Biodegradation is influenced by seasonal changes, especially the temperature. The study also illustrated that a change in environmental conditions is reflected by changes in the composition and the physiological functioning of the microbial community. Based on this study, the following conclusions can be drawn:

- The removal of NOM in the conditions of the studied Finnish AGR sites is efficient and the target of 2 mg TOC l^{-1} is easily reached (Papers IV and V). While a preferential removal of high molecular weight NOM fractions in AGR occurs, no accumulation of low molecular weight fractions is likely. This can be interpreted as the sorption or entrapment of the larger NOM fractions on the sand matrix rather than their mineralisation. However, the results of this study give an indication that NOM macromolecules include fractions that could be utilised by the indigenous bacteria (Paper V). Furthermore, the highest biological activity is likely to be located at the beginning of the flow path (Papers I and V).

- Due to the refractory nature of NOM and its continuous washout from the sand matrix, lengthening the infiltration distance after reaching the goal of 2 mg TOC l^{-1} is not likely to further enhance NOM removal (Paper I). The aromaticity of NOM decreases along the flow path most probably due to sorption (Papers I, IV and V).

- A substantial proportion of NOM removal in AGR is due to biodegradation. In the sand column, DOC mineralisation varied from 32% to 52%, depending on the temperature and hydraulic load (Paper I). Up to 69% extent of mineralisation occurred in the FBR (Paper II). In the FBR, the HRT of the continuous-flow mode and the duration of the batch test substantially affect NOM biodegradation. The labile NOM fractions are used rapidly and are then followed by the refractory fractions. This trend is more apparent at low temperatures.

- An FBR is a suitable experimental setup for the enrichment of NOM degrading microorganisms from lake water (Paper II). The obtained enrichment culture can be used for the determination of DO consumption rates at different temperatures and to quantify NOM biodegradation according to seasonal variations. In Lake Roine, the average temperature coefficient (Q_{10}) is 2.3 thus demonstrating the strong temperature dependency of NOM biodegradation. Furthermore, the highest DO consumption rate occurs in the summer (June-August), followed by the fall, spring and winter.

- Biomass accumulation in the basin sediments depends on the season and especially the period of high primary production (Paper V). In general, biomass development in an AGR aquifer is accelerated due to an increase in temperature (Paper II). The continuous detachment of the biomass occurs and thus no constant level is to be expected (Papers I

and II). The average biomass quantity in the sand column at 1.2 m distance from the inlet was 6.4±0.8 mg VS g^{-1} dw sand (Paper I). The maximum biomass amount in the FBR was 13.1 mg VS g^{-1} dw carrier and the net biomass yield at the end of the experimental period was 0.045 mg VS mg^{-1} DOC (Paper II). Ideally the accumulation of biomass should sustain biomineralisation but not lead to the clogging of pore spaces.

- Despite the substantial removal of bacterial cells along the aquifer flow path, the remaining cell counts in extracted groundwaters are similar to or higher than those of chemically produced drinking waters (Papers I, IV and V).

- Bacterial communities in both raw and extracted groundwaters at the Finnish AGR sites are diverse and differ from each other and the natural groundwater in the same area (Paper IV). Infiltration through a sand matrix results in a substantial change in the bacterial communities, which is most likely the result of changing environmental conditions (Papers III and IV). AGR demonstrated the potential to efficiently remove Actinobacteria from raw water. Planktonic and attached bacterial communities are different. However, the dominance of a specific planktonic or a sessile bacterial group is not constant, demonstrating the dynamics of both communities.

- Substantial increases occur in the specific EEAs of α-Glu, β-Glu, PME, LAP and AEST when measured in the AGR aquifer. This co-occurs with decreasing nutrient concentrations. The trend of increasing EEA along the flow path indicates a decrease in the availability of nutrients for bacteria. The EEAs in the basin sediment and the pore water samples (down to 10 cm) are of the same order of magnitude as the basin water, which indicates similar nutritional conditions that result from the constant penetration of fresh NOM through the infiltration basin.

5 RECOMMENDATIONS FOR FUTURE RESEARCH

The removal of NOM in the saturated sand column mostly occurred within the first 0.6 m distance. Within the same distance, a substantial change occurred in the cell counts and the structure of the bacterial population of the water phase. However, the exact point of change or the gradual changes could not be detected due to the lack of sampling ports. Thus, a similar but substantially shorter column with more sampling ports would allow a more precise detection of NOM removal in relation to changes in other parameters/factors including bacterial populations. This would add to the understanding of the responses of the microbial populations to changes in the AGR environment.

The biodegradation of NOM in this study was revealed in two experimental setups. Kortelainen and Karhu (2006) quantified the extent of DOC mineralisation at a Finnish AGR site in August, 2001. The results of their study and this one are supportive of each other. However, further studies should be carried out at other Finnish AGR sites in order to determine the site-specificity as well as the temporarility of biodegradation. For this a sampling of the vadose zone is recommended. In order to prevent deep aquifer clogging, the potential for removing a substantial part of the NOM already in the infiltration basin sediments should be determined. Potential materials for this purpose should be tested. This layer would need regular regeneration due to biomass and NOM accumulation.

The FBR showed up to 69% DOC mineralisation. This shows the potential to reach even higher proportions of biodegradation than were obtained in the sand column (up to 52%) and in studies by Kortelainen and Karhu (2006) (44%). This could be achieved by engineering design and operational control. Since the FBR was nutrient-amended, the influence of adding phosphate to the biodegradation process should be evaluated by laboratory experiments. However, the prerequisites for this addition at full-scale AGR sites would require acceptance from relevant authorities and the removal of the supplemented phosphate within the flow path, in order to prevent microbial re-growth in the distribution network (Miettinen *et al.* 1996). Furthermore, both the sand column and the FBR showed an increase in biodegradation as a result of increasing the hydraulic load. This was likely due to the faster flux of influent organic matter for the microorganisms. However, in the case of the sand column, the washout of NOM was also likely to co-occur as a result of a sudden increase in the loading and thus no unambiguous conclusion could be made. Thus, it is recommended to further evaluate the influence of increasing the hydraulic load on biodegradation and NOM removal as a whole in both laboratory and full-scale experiments.

High-molecular weight NOM fractions were preferentially removed and the aromaticity of NOM decreased along the aquifer flow path. There was no increase in the smaller fractions along the flow path. Thus, the retention of the largest fractions was suggested as being the result of entrapment or sorption onto the sand particles rather than the result of them being degraded into smaller fractions. However, the results of this study give an indication that NOM macromolecules included fractions that could be utilised by the indigenous bacteria. No interpretation on the removal mechanisms of different size fractions of NOM could be made based on this study and thus this aspect requires further investigation in order to delineate the removal mechanisms of different NOM fractions.

This study evaluated the influence of lake water temperature on NOM biodegradation and concluded that temperature substantially affects the process. However, since the operational temperatures followed seasonal lake water temperatures, other physico-chemical and microbiological parameters/factors in the lake water were also subject to seasonal changes and may have affected the result. Furthermore, due to a constant change in temperature, the determination of the influence of other factors (such as HRT) is challenging. Further studies should be performed to determine the influence of other factors, in addition to temperature, on biodegradation in AGR. This should be done by the determination of other water quality parameters in parallel with temperature rather than by maintaining one factor as a constant and allowing the others to follow the seasonal changes.

In this study, each AGR site was sampled up to four times and irregular analyses were performed for the experimental systems. Since microbial populations in the subsurface respond rapidly to a change in their environment and due to the laborious nature of sampling *in situ*, the usefulness of on-line monitoring in detecting their responses should be evaluated. One question to address is, whether the on-line analysis of stagnant water in a monitoring well gives similar results to the laboratory analysis of pumped water samples.

Microbial populations were shown to change along the flow path at the studied AGR sites and to differ from those of the natural groundwater. However, due to the poor separation of the numerous DGGE bands, only a limited number of bands were amenable for sequencing. Thus, the conclusion was primarily based on a visual evaluation accompanied by a cluster analysis. Furthermore, most of the sequenced bands showed a close relation with an uncultured bacterial species. The LH-PCR of the samples derived from the experimental setups showed the potential to detect diverse populations in long-term studies. Thus, LH-PCR is a useful tool for monitoring bacterial community dynamics at full-scale AGR sites. The samples should include attached biofilms since the FBR experiment showed a difference between the planktonic and sessile bacterial groups. Furthermore, changes in microbial community dynamics should be linked with changes in environmental conditions at full-scale AGR sites. Other phylogenetic chronometers than 16S rRNA genes should be used in order to evaluate the functionality of the microbial communities involved with water purification.

Actinobacteria were removed in the sand column after the column had been stabilised (day 651). This is a desired trend since Actinobacteria have been related to drinking water taste and odour problems (Zaitlin and Watson 2006). The removal of Actinobacteria in AGR has another potential advantage as some Actinobacteria are pathogenic (Goodfellow and Williams 1983). Further work could include the development of more specific molecular methodologies for AGR monitoring and the screening of pathogenic Actinobacteria as well as other pathogenic bacteria.

The study demonstrated the efficient removal of NOM by AGR, although the remaining cell counts were similar or higher than those of chemically produced drinking water. This study did not focus on the microbiological stability of the produced water. Further studies should be performed in order to determine the need for a final disinfection after AGR.

This study provided information on the biodegradation of NOM and nutrient availability for bacteria in AGR and illustrated that extracellular enzymes in aquatic environments are bottom-up and top-down regulated by nutrient availability. This kind of information is essential for the understanding of the biological factors involved in the attenuation of NOM. Further studies

should focus on the link between EEA, the composition of NOM pools and biomass growth in order to assess the limitations of biodegradation in the overall infiltration process. An understanding of these coupled processes and limiting factors may yield tools to enhance indigenous microbial biodegradation activities in concert with sustainable drinking water production.

In this study, a number of chemical, microbiological and molecular biology methods were used. For future research, a multidisciplinary approach is recommended. This requires co-operation between specialists with different backgrounds and resources. This information could also be used in modelling applications concerning NOM removal mechanisms. Modelling would provide tools for improved process management and optimisation.

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I

Biodegradation of natural organic matter in long-term continuous-flow experiments simulating artificial groundwater recharge for drinking water production

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TECHNICAL REPORTS

Bioremediation and Biodegradation

Biodegradation of Natural Organic Matter in Long-Term, Continuous-Flow Experiments Simulating Artificial Ground Water Recharge for Drinking Water Production

Reija E. Kolehmainen[a,*], Nina M. Kortelainen[b], Jörg H. Langwaldt[c] and Jaakko A. Puhakka[a]

[a] Dep. of Chemistry and Bioengineering, Tampere Univ. of Technology, P.O. Box 541, 33101 Tampere, Finland
[b] Geological Survey of Finland, P.O. Box 96, 02150 Espoo, Finland
[c] Geological Survey of Finland, Tutkijankatu 1, 83500 Outokumpu, Finland

[*] Corresponding author (reija.kolehmainen@tut.fi).

Received for publication January 30, 2008. The role of biodegradation in the attenuation of natural organic matter (NOM) was investigated in long-term experiments that simulate artificial ground water recharge (AGR) for drinking water production. Lake water containing 5.8 mg L^{-1} total organic carbon (TOC) was continuously fed into an 18.5-m-long sand column. During the 941 d of operation, on average 76 and 81% of TOC was removed within the first 0.6 m and the entire column length, respectively. Large molecular size fractions (approximately 1800–2200 Da) of NOM were removed more efficiently than smaller ones (approximately 250–1400 Da). The biodegradation of dissolved organic carbon (DOC) within the first 0.6 m, measured by the stable inorganic carbon isotope ($\delta^{13}C$) method, depended on temperature and hydraulic load: The extent of mineralization was 32% at 6°C (Day 442) and 38% at 23°C (Day 708) with a 0.3 m^3 $(m^2d)^{-1}$ hydraulic load and 52% at 5.5°C (Day 883) with a 3.1 m^3 $(m^2d)^{-1}$ hydraulic load. The rest of the DOC removal was likely due to entrapment or sorption onto the sand particles. Decreases in DOC and the total cell counts in the water along the column were positively correlated (r = 0.99; P = 0.001). The accumulation of biomass was minor, with the highest concentration amounting to 7.2 mg g^{-1} dw of sand. In summary, this study demonstrated that biodegradation has a key role in NOM removal in AGR and is dependent on temperature.

Abbreviations: AGR, artificial ground water recharge • COD, chemical oxygen demand • DAPI, 4',6-diamidino-2-phenylindole • $\delta^{13}C$, the isotopic ratio of carbon as a per mil (‰) difference relative to the international VPDB standard • DO, dissolved oxygen • DOC, dissolved organic carbon • HPSEC, high-performance size-exclusion chromatography • HRT, hydraulic retention time • NOM, natural organic matter • SUVA, specific ultraviolet absorbance • TOC, total organic carbon • UVA, ultraviolet absorbance • VPDP, Vienna Peedee Belemnite • VS, volatile solids

II

Biodegradation of aqueous organic matter over seasonal changes: Bioreactor experiments with indigenous lake water bacteria

Kolehmainen, R.E., Crochet, L.M., Kortelainen, N.M., Langwaldt, J.H., Puhakka, J.A.

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Biodegradation of aqueous organic matter over seasonal changes: Bioreactor experiments with indigenous lake water bacteria

Reija E. Kolehmainen[1]; Ludivine M. Crochet[2]; Nina M. Kortelainen[3]; Jörg H. Langwaldt[4] and Jaakko A. Puhakka[5]

Abstract: Artificial groundwater recharge for drinking water production involves infiltration of surface water through sandy soil and its capture into a groundwater aquifer. The transformation of aqueous organic matter is one of the central issues in this process. The purpose of this work was to assess the potential of indigenous microorganisms in the source water to contribute in the aqueous organic matter biodegradation. For this purpose, microorganisms were enriched from the source water in a fluidized-bed reactor (FBR) and used for kinetic studies on biodegradation of organic matter at ambient temperature range. Lake water (total organic carbon 5.8 mg l^{-1}) was continuously fed to the FBR containing porous carrier material to support biomass retention. In the inlet and outlet water there were on average 21±6 and 13±5·10^5cells ml^{-1}, respectively. Biofilm accumulation (as volatile solids) reached 13.1 mg g^{-1} dw carrier. In the continuous-flow mode and the batch tests, the highest oxygen consumption rate appeared in the summer, followed by the fall, spring and winter. At low temperatures, the biodegradation of aqueous organic matter was relatively rapid initially for labile fractions followed by a slower phase for refractory fractions. The average temperature coefficient (Q_{10}) in the system was 2.3 illustrating a strong temperature dependency of oxygen consumption. The isotopic analysis of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) analysis revealed 27 and 69% mineralization of dissolved organic carbon (DOC) at 23 and 6 °C over 65 and 630 minutes, respectively. These results can be used to construct additional input parameters in modelling applications of artificial groundwater recharge process. The biological component especially, i.e., the biodegradation, is difficult to predict for on-site applications without experimental proof and thus the interpretation in this study will help formulate design predictions for the process.

CE Database subject headings: Organic carbon; Ground-water recharge; Fluidized beds; Biomass; Biodegradation; Aquifers

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[1] D.Tech., Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland, reija.kolehmainen@tut.fi

[2] M.Sc., Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

[3] Ph.D., Research Scientist, Laboratory for Isotope Geology, Geological Survey of Finland GTK, P.O. Box 96, FI-02150 Espoo, Finland, nina.kortelainen@gtk.fi

[4] Ph.D., Research Scientist, Geological Survey of Finland GTK, Tutkijankatu 1, FI-83500 Outokumpu, Finland, jorg.langwaldt@gtk.fi

[5] Professor, Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland, jaakko.puhakka@tut.fi

III

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