BIOGEOCHEMICAL ASPECTS OF

AQUIFER THERMAL ENERGY STORAGE

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BIOGEOCHEMICAL ASPECTS OF AQUIFER THERMAL ENERGY STORAGE

Proefschrift

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STELLINGEN

1. Naast thermodynamische overwegingen kan stikstofoxide een rol spelen bij de ruimtelijke scheiding van denitrifikatie, ijzerreduktie en sulfaatreduktie in anaërobe aquifers en sedimenten.
   Dit proefschrift.

2. De vorming van ferro-nitrosyl complexen kan de kinetiek van chemodenitrifikatiereakties beïnvloeden.
   Dit proefschrift.

3. De door Unden en Trageser voorgestelde werking van molekulaire zuurstof op het *f*umaraat-*n*itraat-*r*egulator (FNR) eiwit in *E.coli* kan de derepressie van nitraatreduktase gedurende aërobe groei in een kontinue kultuur in onvoldoende mate verklaren.
   Unden G. and Trageser M. (1991) Oxygen regulated gene expression in Escherichia coli: Control of anaerobic respiration by the FNR protein. Antonie van Leeuwenhoek 59: 65-76. Dit proefschrift.

4. De bewering van Nealson en Myers dat *Shewanella putrefaciens* spp. 200 anaëroob kan groeien met driewaardig ijzer als elektronenacceptor blijkt niet uit de door deze auteurs geciteerde literatuur.
   Nealson K.H. and Myers G.R. (1992) Microbial reduction of manganese and iron: New approaches to carbon cycling. Appl. Environ. Microbiol. 58: 439-443.

5. In tegenstelling tot hetgeen Matěju *et al.* in hun overzichtsartikel beweren moet de eerste bakterie die in staat is tot denitrifikatie met tweewaardig ijzer als elektronendonor nog beschreven worden.
   Mateju V., Cizinská S., Krejcí J. and Janoch T. (1992) Biological water denitrification - A review. Enzyme Microb. Technol. 14: 170-183. Dit proefschrift.

6. Nog steeds wordt bij studies naar metaalcorrosie veroorzaakt door sulfaatreducerende bakteriën verwezen naar een artikel over kathodedepolarisatie dat in 1934 werd gepubliceerd door van Von Wolzogen Kühr en Van der Vlugt. Voor een van origine Nederlandstalig artikel is dit een opmerkelijke verdienste.
   Von Wolzogen Kühr C.A.H. en Van der Vlugt L.S. (1934) De grafiteering van gietijzer als electrobiochemisch proces in anaerobe gronden. Water 16: 147-165.

7. De promovendus wikt, maar de promotor beschikt; wetenschap is een tijdloos en universeel houvast.

8. Wetenschap geeft weliswaar geen antwoord op alle vragen, maar kan wel op een tot de verbeelding sprekende wijze de grenzen van het onzegbare aangeven.

9. Een proefschrift is uniek in de zin dat het zichzelf is en niet iets anders; in dat opzicht is het in feite allerminst uniek, want hetzelfde geldt voor al het andere.

10. Indien men de arbeidsparticipatie zou willen vergroten verdient het aanbeveling om de kostwinnersvoordelen in de belastingheffing geleidelijk af te schaffen.

11. De wettelijke verplichting van het emissie-arm uitrijden van mest met behulp van een bodeminjekteur is een stap naar een milieuvriendelijke landbouw die een groot gevaar inhoudt voor het reeds bedreigde grondwater.

12. Een personeelsparkeerplaats kan men opvatten als een visitekaartje en daardoor als een graadmeter voor de heersende bedrijfskultuur.

13. Naar analogie van de aardbevingsschaal van Richter is er iets voor te zeggen om de eerste wet van Murphy (als iets fout kàn gaan, dan gáát 't ook fout) te nuanceren met een logaritmisch opklimmende blunderschaal.

14. De vaccinologie in Nederland is een typisch voorbeeld van een wetenschapsgebied waar onderzoek en toepassing ver uit elkaar liggen.
Met dank aan J.T. Poolman van het RIVM te Bilthoven.

15. Arbeidstijdverkorting (ATV) gedraagt zich volgens de tweede hoofdwet van de thermodynamica: arbeid, ongeacht de duur, vult de dag zoals natuurlijke processen verlopen in een richting van toenemende entropie.

Stellingen behorende bij het proefschrift 'Biogeochemical aspects of aquifer thermal energy storage'. H.J. Brons, Wageningen, 30 juni 1992.

Contents

CHAPTER 1

GENERAL INTRODUCTION

Brons H.J. and Zehnder A.J.B. (1990) Biogeochemical aspects of aquifer thermal energy storage. In: Hydrochemistry and energy storage in aquifers, TNO Committee on Hydrological Research, no.43: 73-81. (Hooghart J.C. and Posthumus C.W.S., editors) TNO, The Hague.

1.1 AQUIFER THERMAL ENERGY STORAGE

Economic- and environmental developments in the 1970's and 1980's have strongly augmented the need for efficient energy conversion and energy saving strategies. Since 1973 long term economical developments have been influenced by the continuous changes in oilprices. It became clear that the constant increase in fossil fuel burning can cause environmental problems on a global scale. The use of nuclear fission as a major future energy source is strongly debated after severe accidents and remaining doubts about a satisfactory solution of the nuclear waste problem within a reasonable time frame. Concerns about sufficient energy supply in the future have stimulated research for alternative energy sources and efficient energy saving strategies. Aquifer thermal energy storage (ATES) constitutes one of the savings strategies which is promising enough for future exploration. ATES is aimed at the utilization and storage of periodical surplus heat and solar energy on a large scale.
Aquifers are natural geological formations of porous rock material saturated with groundwater. Most aquifers in The Netherlands are covered with clay and peat deposits and overlie an impervious clay stratum. The aquifer itself consists of fairly homogeneous unconsolidated sand sediment with a median grain size varying between 0.2 and 1.0 mm. The groundwater temperature in The Netherlands reflects the annual average temperature of 10-11°C and generally no specific elements or gases are present in high concentrations (76).
Basically, ATES-systems have a cool and a warm well (54). As shown in Figure 1 groundwater is pumped from the cool well to a heat exchanger and after being heated reinjected into the aquifer through the warm well. The stored thermal

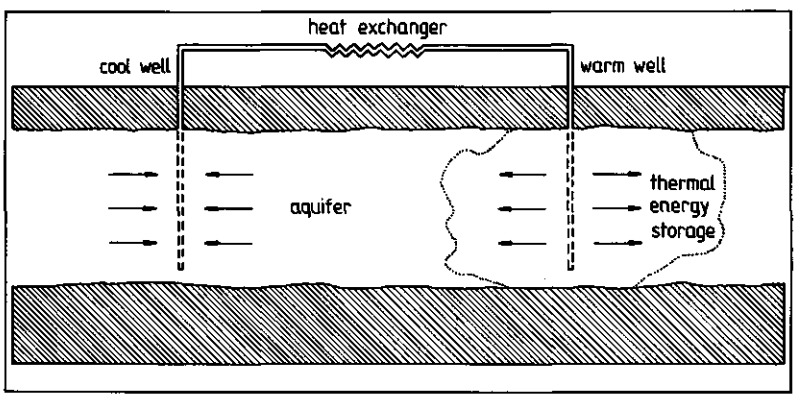

Fig. 1. Schematic representation of an aquifer thermal energy storage system.

energy is recovered in a reversed process. The warm water is pumped back to the heat exchanger and after cooling reinjected into the cool well.
Several ATES-systems have been investigated on pilot plant scale in a number of countries. The main objective of these investigations was to explore energy storage at varying depths and temperature ranges and in different kinds of geological formations. Until now it has been difficult to interpret the results that were obtained in these studies because detailed information on chemical and biogeochemical reactions in aquifers was lacking. In addition, ATES-research has been specifically related to particular projects in different countries and there was no attempt to undertake systematic investigations (30). Because of the promising results of ATES, both technically and economically, an international coordination of the various ATES-studies was regarded to be necessary. In 1987 the International Energy Agency (IEA) implemented a research and development program entitled: Environmental and Chemical Aspects of Thermal Energy Storage in Aquifers and Development of Water Treatment Methods (32). In this IEA-program the following subtasks have been defined:

A. To develop and validate a simulation model that calculates the space and time dependent mass transport of discrete chemical constituents in an aqueous medium in soil.

B. To undertake theoretical and experimental investigations of biogeochemical reactions and related environmental impacts caused by heat storage in different aquifers.

C. To classify and analyze different water treatment techniques for heat storage with respect to environmental impacts, long term efficiency and costs.

D. To undertake theoretical and experimental studies of the microbiological processes occurring during thermal energy storage in relation to sanitary aspects, bacterial clogging processes, and relevant treatment techniques.

E. To analyze scaling and corrosion problems in installations connected to aquifer heat stores (heat exchangers, mains and screens) in relation to water chemistry and water treatment.

F. To test the water treatment techniques selected in subtasks C and D and to evaluate their environmental effects in laboratory and field experiments.

G. To develop a generally applicable procedure for the choice of an optimized water treatment method to be used at future aquifer heat storage sites.

Currently, there are eight countries that participate in this IEA-program: Canada, Denmark, Finland, Germany, The Netherlands, Sweden, Switzerland and the United States of America. In The Netherlands research related to subtasks B and D has been carried out in the Institute of Earth Sciences of the Free University and the Department of Microbiology of the Wageningen Agricultural University.

1.2 BIOGEOCHEMICAL REACTIONS IN AQUIFERS

1.2.1 Thermodynamic considerations

Confined aquifers used for thermal energy storage are isolated from the soil's gas phase. Hence, oxygen consumed by chemical and biological processes cannot be replenished fast enough from the atmosphere. When the dissolved oxygen in groundwater is depleted the chemical- and biological reduction of inorganic

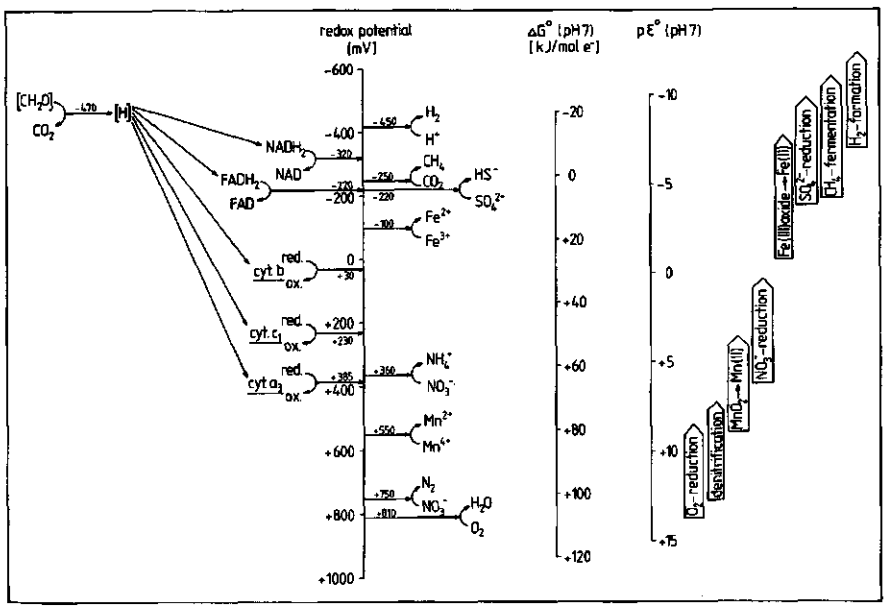

Fig. 2. Electron free-energy-level diagram for microbially mediated redox reactions with organic carbon (CH_2O) acting as an electron donor. (CH_2O) represents one-sixth of glucose (i.e.-153 kJ/mol). After Zehnder (92).

electron acceptors proceeds in accordance with their decreasing redox potential levels (73). Thermodynamically speaking more energy yielding reactions precede processes that are less energy yielding.

Table 1. Equilibrium constants of redox processes pertinent in aquatic conditions at 25°C (73,92).

Reaction	$p\varepsilon^\circ$ ($=\log K$)	$p\varepsilon^\circ$ (pH)[a]
(1) $1/4\ O_2\ (g) + H^+ + e \rightleftarrows 1/2\ H_2O$	+ 20.75	+ 13.75
(2) $1/5\ NO_3^- + 6/5\ H^+ + e \rightleftarrows 1/10\ N_2(g) + 3/5\ H_2O$	+ 21.05	+ 12.65
(3) $1/2\ MnO_2\ (s) + 1/2\ HCO_3^-\ (10^{-3}) + 3/2\ H^+ + e \rightleftarrows 1/2\ MnCO_3\ (s) + H_2O$	-	+ 8.9[b]
(4) $1/8\ NO_3^- + 5/4\ H^+ + e \rightleftarrows 1/8\ NH_4^+ + 3/8\ H_2O$	+ 14.90	+ 6.15
(5) $FeOOH\ (s) + HCO_3^-\ (10^{-3}) + 2\ H^+ + e \rightleftarrows FeCO_3\ (s) + 2H_2O$	-	- 0.8[b]
(6) $1/8\ SO_4^{2-} + 9/8\ H^+ + e \rightleftarrows 1/8\ HS^- + 1/2\ H_2O$	+ 4.25	- 3.75
(7) $1/8\ CO_2\ (g) + H^+ + e \rightleftarrows 1/8\ CH_4\ (g) + 1/4\ H_2O$	+ 2.87	- 4.13
(8) $H^+ + e \rightleftarrows 1/2\ H_2\ (g)$	0.00	- 7.00

a) Values for $p\varepsilon^\circ$ (pH) apply to the electron activity for unit activities of oxidant and reductant in water of 25°C and pH = 7.

b) These data correspond to $(HCO_3^-) = 10^{-3}$ M rather than unity, so they are not exactly $p\varepsilon^\circ$ (pH); they represent typical aquatic conditions more nearly than $p\varepsilon^\circ$ (pH) values do

The elements that predominantly participate in natural redox processes are: carbon, hydrogen, nitrogen, oxygen, sulphur, iron and manganese. Table 1 and the right hand side of Figure 2 present equilibrium constants of natural redox processes. The equilibrium constants are expressed as $p\varepsilon^\circ$ units ($p\varepsilon = -\log[e^-]$; $[e^-]$ = electron activity) and have been calculated for neutral water of pH 7

and 25°C. The left hand side of Figure 2 lists the sequence of redox reactions from strong reductants derived from organic carbon (CH_2O) at the top to strong oxidants at the bottom. This ordinate readily shows that sulphate, e.g., can from a thermodynamic point of view, oxidize organic carbon to carbon dioxide but cannot oxidize ammonium to nitrate. Figure 2 may thus be interpreted as a free-energy-level diagram of which the ordinates measure the energy (expressible as kJ/mol e^- or $p\varepsilon^\circ$ units) that is required for the transfer of electrons from one free energy level to another. When reductants such as organic matter are added to a system that contains several redox couples, the lowest unoccupied electron levels will be filled first, followed sequentially by the higher levels. For example, during the biological mineralization of organic compounds in a confined anaerobic aquifer, the reduction of inorganic electron acceptors should from a thermodynamic point of view (but not necessarily a kinetic one) proceed in the order: nitrate, manganese (IV), ferric iron, sulphate and carbon dioxide. Consequently, when the rate at which these oxidants are depleted exceeds the rate of oxidant import the redox potential (mV) will decrease. The energy gained in such processes per mole of electrons transferred can be read from the central ordinate in Figure 2: ΔG° (pH 7) = 2.3 RT ($p\varepsilon_2$ - $p\varepsilon_1$); where R is the gas constant (kJ/mol.K) and T the absolute temperature (K). The $p\varepsilon$-values are related to equilibrium redox potential E_H (Volts, pH = 7 scale) by: $p\varepsilon = E_H/2.3RTF^{-1}$ (F is the Faraday constant in Coulomb/mol).

The data given in Table 1 and Figure 2 can be used to predict the thermodynamic possibility and sequence of microbially mediated redox reactions under equilibrium conditions. For example Table 1 and Figure 2 show that ferrous iron can, from a thermodynamic point of view serve as a reductant in the process of chemodenitrification. In fact, it has been reported that ferrous iron is oxidized during denitrification in anaerobic waterlogged soils (39) and in anaerobic lake sediments to which nitrate was added (19,34). No pure cultures of bacteria capable of denitrification at the expense of ferrous iron have been obtained thusfar (21,46). Under equilibrium conditions in soils and sediments ferrous iron is not found in the presence of nitrate (28,40) and as far as we know denitrification and biological iron reduction are spatially separated (36,69). With regard to non-equilibrium conditions the data in Table 1 and Figure 2 are not necessarily applicable. For instance, in microbial chemostat cultures it has been well established that aerobic denitrification can occur

(45,71). Many examples given in the literature indicate that denitrification is possible in perfectly well aerated systems. *Klebsiella* (18) and *Hyphomicrobium* (49) still contain considerable nitrate reductase activity when grown in a chemostat culture at 10 and 35% air saturation, respectively. *Zoogloea ramigera* reduces nitrate at considerable rate at 8 mg oxygen per liter (72). *Thiosphaera pantotropha*, a denitrifying mixotroph, could use oxygen and nitrate concomitantly as terminal electron acceptors at 90% air saturation (64).
The thermodynamic data in Table 1 and Figure 2 only provide evidence about the direction and extent of chemical equilibria rather than actual reaction rates. Therefore, it should be emphasized that general conclusions regarding chemical dynamics of biogeochemical reactions cannot be drawn. For example, the spontaneous oxidation of both iron and sulphide is thermodynamically possible but can actually be prevented by slow kinetics in the environment (37,67) which enables chemolithotrophic bacteria to act as redox catalysts and use these compounds as energy sources for their growth. In marine sediments for instance, *Beggiatoa* can successfully compete with the spontaneous oxidation of sulphide to elemental sulphur in sulphide-oxygen gradients that are present at redox potentials between -100 and +100 mV and oxygen concentrations between 10 and 35% air saturation (79). Likewise, in aquifers *Gallionella ferruginea* can compete with spontaneous ferrous iron oxidation to ferric iron in the redox potential range between +200 and +320 mV and oxygen concentrations varying from 0.1 to 1.0 mg/l (26).

1.2.2 General aspects of biogeochemical reactions and groundwater characteristics during aquifer thermal energy storage.

During the process of aquifer thermal energy storage considerable fluctuations of the *in situ* groundwater temperature occur. This will influence the activity of chemoheterotrophic- and chemolithotrophic bacteria in the aquifer. As a consequence, the biogeochemical reactions mentioned in Table 2 will affect the groundwater characteristics which in turn may have a negative influence on the operational performance of wells, pipes and heat exchangers. For instance, in Table 2 it is shown that aerobic chemolithotrophic bacteria can use sulphide and ferrous iron as a substrate. Clogging problems in groundwater heat pump systems resulting from the growth of sulphide oxidizing *Beggiatoa* have actually been reported (44). The same holds for the ferrous iron oxidizing *Gallionella ferruginea*, which is a notable bacterium for the clogging of groundwater wells (67). The most important biogeochemical reactions during aquifer thermal energy

Table 2. Changes in groundwater characteristics as a result of biogeochemical reactions during aquifer thermal energy storage (93).

Bacteria	Substrates	Respiration	Products	Groundwater reactions	
				biological	chemical
chemo-hetero trophic bacteria	organic compounds, NH_4^+, NO_3^-, SO_4^{2-}, Fe^{3+}	aerobic	biomass + organic compounds, SO_4^{2-}, H_2O, NO_3^-, CO_2	increase: CO_2; decrease: pH, pε, O_2	$CaCO_3 + H_2CO_3 \rightarrow Ca^{2+} + 2\,HCO_3^-$
		anaerobic	biomass + organic compounds, H_2, H_2O, NH_4^+, CO_2, Fe^{2+}, N_2, HS^-	increase: CO_2; decrease: pH, pε	$Fe^{3+} \rightarrow Fe^{2+}$; $Fe^{2+} \rightarrow FeS$; $CaCO_3 + H_2CO_3 \rightarrow Ca^{2+} + 2HCO_3^-$
chemo-litho trophic bacteria	NH_4^+, NO_2^-, H_2, HS^-, S^0, CO_2, Fe^{2+}	aerobic	biomass + organic compounds, NO_3^-, H_2O, SO_4^{2-}, CO_2, S^0, Fe^{3+}	increase: pε; decrease: CO_2, pH, O_2	$Fe^{3+} \rightarrow Fe(OH)_3 \rightarrow Fe_2O_3$
	H_2, CO_2, NO_3^-, SO_4^{2-}	anaerobic	biomass + organic compounds, NH_4^+, H_2O, HS^-, CO_2, CH_4	increase: pH; decrease: pε, CO_2	$Fe^{2+} \rightarrow FeS$

storage are probably catalyzed by the large and diverse group of chemoheterotrophic bacteria. The group of chemoheterotrophic bacteria mentioned in Table 2 are able to convert organic compounds into organic products such as biomass and slime deposits, which may eventually cause well clogging. Chemoheterotrophs can also produce ferrous iron and sulphide which may lead to iron precipitation near wells and anaerobic metal corrosion in pipes and heat exchangers. Table 2 also shows that during the biomineralization of organic matter the production of carbon dioxide will affect the calcium carbonate solubility, which may result in precipitation reactions and scaling of pipes and heat exchangers. The changes in groundwater characteristics that result from the discussed biogeochemical reactions will be outlined in more detail in the following chapters.

1.2.3 Bacterial growth and slime formation

The growth of bacteria in groundwater is in general determined by the presence of biodegradable dissolved organic carbon (8). The major source of dissolved organic carbon (DOC) in aquifers are humic substances derived from lignin, polysaccharides and protein degradation processes (20,75). In oligotrophic aquifers (DOC < 10 mgC/l) the amount of biodegradable dissolved organic carbon can actually be measured with the microbially available organic carbon (AOC) assay (55,78). In this assay the number of colony forming units of *Pseudomonas fluorescens* (CFU/ml) is compared with the growth yield of this bacterium on acetate as a standard source of carbon and energy. The available organic carbon content of a given water sample is usually expressed as mg acetate-carbon equivalents per milliliter (78). In general, the amount of groundwater AOC is less than 1% of the DOC in oligotrophic aquifers that are used for thermal energy storage. The AOC concept enables to quantitatively describe a well clogging process as a result of bacterial growth. The contribution of bacterial growth to well clogging depends on the so called AOC-*charge* (gram $C/m^2.h$) near a groundwater well (29). The AOC-*charge* results from the product of the AOC concentration (gram C/m^3) and the groundwater flux ($m^3/m^2.h$) near the aquifer-well interface. From the AOC concentration the potential bacterial growth near a groundwater well can be estimated as follows:

$$X(t) = X(o) + \exp\left(\frac{\mu V}{F} \right)$$

With:

$$\mu = \mu_{max} \frac{[AOC]}{[AOC] + K_{AOC}} - b$$

Where,

μ and μ_{max}	: specific growth rate and specific maximum growth rate	(h^{-1})
K_{AOC}	: AOC concentration at which $\mu = \mu_{max}/2$	($g.m^{-3}$)
[AOC]	: AOC concentration	($g.m^{-3}$)
b	: biomass decay rate	(h^{-1})
X(t) and X(o)	: biomass present at time (t) and at time (o)	(g)
V	: sediment volume located near the aquifer-well interface	(m^3)
F	: groundwater flow rate through the sediment volume denoted V	($m^3.h^{-1}$)

During ATES the bacterial growth rate is not only dependent on the availability of organic carbon but also on the temperature and on the presence and concentration of inorganic electron acceptors. Bacterial growth rates generally increase with temperature, as is true for most chemical reaction rates. Ideally, the temperature dependency of chemical reaction rates can be described with the Arrhenius equation (73). In aquifers with poorly defined mixed bacterial populations the relation between growth rates and temperature increase of e.g. 10 K can be characterized with an empirical constant termed factor Q_{10}. For example, studies with mineralization rates in lake sediments have shown an average Q_{10} of 1.5 after an adaptation period of two weeks at 55°C (93). Dutch aquifers used for ATES are anaerobic and contain only little amounts of nitrate. Therefore only sulphate reduction and methanogenesis are likely to contribute to biomass increase. As shown in Table 1 and Figure 2 sulphate reduction and methanogenesis are less energy yielding than oxygen respiration. In anaerobic habitats the bacterial growth yield will thus be lower than in other natural aerobic systems (76). Despite the low bio-availability of organic carbon and low growth yields it appears from field studies that small amounts of AOC nonetheless can cause microbiological clogging problems in groundwater wells that are used for drinking water production (29). Both under aerobic and anaerobic conditions microbiological well clogging is associated with slime formation and the development of organic deposits (57,81). Under aerobic conditions these organic deposits are located

in and around screen slots (77). Clogging material under anaerobic conditions is located more towards the gravel pack-aquifer interface (77). Bacterial slime found in organic deposits around groundwater wells consist to a large extent (98%) of water and only small amounts of bacterial extracellular polymeric carbon compounds (50). Generally, bacterial extracellular polymeric carbon compounds occur in two forms: capsular polysaccharides (CPS), and exopolysaccharides (EPS) (94). Capsular polysaccharides are intimately associated with or covalently bound to the bacterial cell surface (87). Such in contrast to exopolysaccharides which can be readily found free in the growth medium. Among the structural elements that can be found in EPS or slime polysaccharides are D-glucose, D-galactose, D-glucuronic acid, pyruvic acid and various amounts of succinyl and acetyl substituents (94). With regard to the regulation of polysaccharide excretion in bacterial pure cultures both the carbon to nitrogen ratio and the dissolved oxygen concentration are considered as important parameters (27). Currently little is known about the mechanisms that control slime formation in natural environments.

1.2.4 Carbon dioxide production and calcium carbonate precipitation

During the biological and chemical mineralization of organic matter in aquifers carbon dioxide will be produced. The amount and rate is a function of groundwater temperature and the presence and concentration of specific inorganic electron acceptors. In a given constant aquifer environment, an increase in the carbon dioxide partial pressure will affect the solubility of carbonates (73). The solubility of calcium carbonate (calcite) as influenced by the carbon dioxide partial pressure is given by:

$$CaCO_3 + CO_2 + H_2O \rightleftarrows Ca^{2+} + 2HCO_3^-$$

In ATES-technology it is important to know whether groundwater is in equilibrium with calcium carbonate and whether or not water softening is required to prevent carbonate precipitation (89). The tendency of groundwater to deposit calcium carbonate can be accurately determined by solving chemical equilibrium equations with geochemical computer models. For such purposes a modified version of the computer model WATEQ (60,61) can be used for natural waters in the temperature range between 0 and 95°C, pH-values between 4.4 and 9.5 and ion strengths between 0 and 700 mM. Less accurate but more widely used (74) for the calculation of the calcium carbonate saturation state is the

Langelier Index (42,43). The Langelier Index (LI) is the difference between the measured pH of a water and the hypothetical pH the water would have if it were in equilibrium with solid $CaCO_3$ at the existing concentrations of hydrogen carbonate (HCO_3^-) and calcium (Ca^{2+}):

$$LI = \Delta pH = pH\ measured - pH\ equilibrium$$

Provided that hydrogen carbonate makes up the majority of the total alkalinity, the hypothetical pH *equilibrium* can be calculated (48,66) using the following equation:

$$pH\ equilibrium = -\log \frac{K_{HCO_3^-}}{K_{CaCO_3}} - \log[Ca^{2+}] - \log[HCO_3^-]$$

Where $K_{HCO_3^-}$ is the second dissociation constant for carbonic acid, K_{CaCO_3} is the calcite solubility constant and [] denotes the activity of the free ion species.

The commonly used Saturation Index (SI) with respect to calcium carbonate has been derived from the Langelier Index (6):

$$SI = \log \frac{[Ca^{2+}][CO_3^{2-}]}{K_{CaCO_3}}$$

Negative SI-values indicate that carbon dioxide is dissolving calcium carbonate, whereas positive values are a sign of calcium carbonate over-saturation. A SI that equals zero indicates that the water is in equilibrium with respect to calcium carbonate. However, a positive SI value will not necessarily lead to the precipitation of calcium carbonate, as reports on sustained super-saturation at elevated temperatures during ATES have shown (22,90). Super-saturation with respect to calcite may be caused by a combination of factors, such as increase in solubility of calcite with increasing magnesium concentration (10,63), inhibition of precipitation by ortho phosphate (52,91) and inhibition by naturally occurring fulvic and humic acids (3). These organic compounds have been shown to either complex calcium and magnesium or to adsorb onto potential calcite seeds, thereby rendering the surfaces inactive as sites for crystal growth (31).

1.2.5 Iron precipitation

In anaerobic waterlogged soils that contain organic carbon the poorly soluble ferric iron is usually reduced to the more mobile ferrous form (80). During the extraction of groundwater from wet soils mobilized ferrous iron is readily oxidized near drainpipes and wells. This results in the formation of ochreous deposits, a mixture of ferric hydroxides and organic matter which may hamper or entirely clog the discharge system. The mobilization of manganese in anaerobic groundwater may cause analogous clogging phenomena. Because manganese clogging is less frequently observed than iron clogging (76) it will not be further considered here.

The mobilization and redox cycle of iron in aquifers is schematically represented in Figure 3. Iron reduction in anaerobic aquifers is a complex biogeochemical process involving both biotic and abiotic factors. Ferric iron can spontaneously be reduced to ferrous iron by metabolic end products such

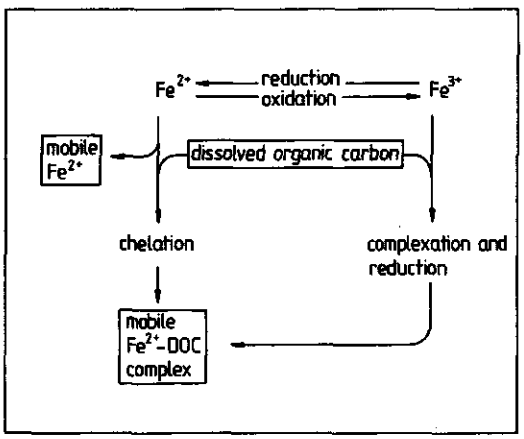

Fig. 3. Schematic representation of the iron redox cycle and the mobilization of ferrous iron in aquifers.

as sulphide and formate (53). Studies on ferric iron reducing enzyme systems indicate that bacterial respiratory pathways can be involved (4,15,16). Iron-respiration driven proton translocation has been reported (68) and coupling to oxidative phosphorylation is possible in some cases (7,46,47). Figure 3 shows that in the presence of dissolved organic carbon ferrous iron may occur in soluble complexes of humic substances (38,51). Some of these compounds either keep the chelated iron reduced or are able to reduce ferric iron

(70,85). Mobile non-chelated ferrous iron can also be relatively stable in aerobic aquifers provided that either the Ph or the oxygen concentration is low. Because of slow kinetics abiotic oxidation of ferrous iron does not occur below pH 3. However, at these pH's the acidophilic chemolithotroph *Thiobacillus ferrooxidans* can form ferric iron (2,41). At a pH between 6.0 and 7.6 and a pε between +200 and +320 mV spontaneous ferrous iron oxidation is very slow when oxygen concentrations are lower than 1 mgO_2/l. Under such circumstances iron oxidation is catalyzed by *Gallionella ferruginea* (26).

The possibility of well clogging as a result of iron precipitation (59) may require the need for treatment of iron rich groundwater. *In situ* treatment of groundwater with more than 5 mg Fe^{2+}/l can be accomplished with the Vyredox process (11,23). In this process groundwater is extracted from the aquifer, aerated and degassed. The aerated groundwater is injected back into the aquifer at a certain distance from the extraction well (Fig. 4). The Vyredox process is based firstly on the precipitation of ferric oxides around the injection wells and secondly on the adsorption of ferrous iron onto the ferric oxide complexes. When oxygenated groundwater is injected back into the aquifer the

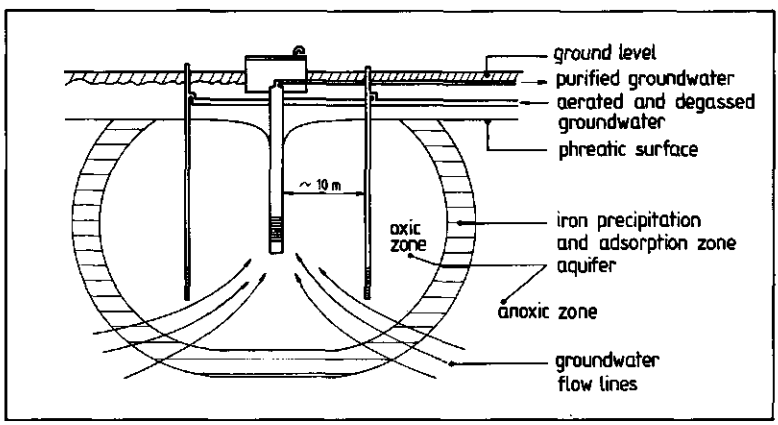

Fig. 4. With the Vyredox process an iron precipitation and adsorption zone is achieved in the aquifer. The groundwater is filtrated through this zone before it is pumped up to the ground level. After Hallberg (23).

adsorbed ferrous iron will be converted to ferric iron, and so the efficiency of the iron removal process is increased after each extraction and injection cycle. As a result the volume of the extracted, iron-free anaerobic water is

considerably larger than the volume of aerobic water injected. In addition, clogging of the aquifer is prevented, since the zone in which iron is precipitated is much increased, namely from just around the extraction well to an area exceeding 20 meters in diameter.

1.2.6 Anaerobic metal corrosion

Microbial corrosion. The most important anaerobic metal corroding microbes are the sulphate-reducing bacteria (88). Anaerobic corrosion of iron caused by this group of bacteria constitutes an economic problem of massive proportions (24). Therefore, it is important to understand the eco-physiology of sulphate-reducers if their corrosive impact should be minimized.

A mechanism for anaerobic metal corrosion by sulphate reducing bacteria was originally proposed by Von Wolzogen Kühr and Van der Vlugt in 1934 (83). These authors state that iron immersed in water releases ferrous ions. As a result the metal surface becomes negatively charged by the remaining electrons (Table 3, equation 1). The dissolving process will go on continuously as long as the electrons are removed by an oxidant. In the absence of oxygen, the electrons left on the metal surface reduce protons from the dissociation of water. The so formed hydrogen remains on the iron surface and protects the iron from further dissolving (equation 2 & 3). Thus, an equilibrium is established which keeps the iron polarized. The originally proposed mechanism of anaerobic metal

Table 3. An overview of the reactions during the depolarization of iron by sulphate-reducing bacteria (84).

[1] Anodic reaction	: $4Fe$	$\rightleftharpoons$	$4Fe^{2+} + 8e^-$
[2] Water dissociation	: $8H_2O$	$\rightleftharpoons$	$8H^+ + 8OH^-$
[3] Cathodic reaction	: $8H^+ + 8e^-$	$\rightleftharpoons$	$4H_2$
[4] Cathodic depolarization	: $SO_4^{2-} + 4H_2$	$\rightleftharpoons$	$H_2S + 2H_2O + 2OH^-$
[5] Sulphide precipitation	: $Fe^{2+} + H_2S$	$\rightleftharpoons$	$FeS + 2H^+$
[6] Hydroxide formation	: $3Fe^{2+} + 6OH^-$	$\rightleftharpoons$	$3Fe(OH)_2$
[7] Overall reaction	: $4Fe + SO_4^{2-} +$	$\rightleftharpoons$	$FeS + 3Fe(OH)_2 + 2OH^-$

corrosion (84) is a cathodic depolarization of the iron surface by hydrogen consuming sulphate reducing bacteria (equation 4). With hydrogenase-positive strains of *Desulfovibrio vulgaris* (12,58) it was shown that sulphate-reducers can provoke corrosion by oxidation of cathodically formed elemental hydrogen with sulphate as an electron acceptor (equation 3 & 4). The result is a net oxidation of the metal termed pitting corrosion (Fig. 5). Besides for sulphate reducers, growth at the expense of cathodic hydrogen has also been reported for several methanogenic genera (17) and homoacetogenic bacteria (62).
Several other models for bacterial mediated corrosion have been proposed which will be briefly outlined here. The corrosive activity of sulphate-reducers has also been ascribed to the metabolic production of highly corrosive reduced phosphorous compounds (33). Extensive corrosion of iron has been found in media from which sulphate reducing bacteria and sulphide have been removed. Iron phosphides have been identified among the corrosion products, but the corrosive metabolite itself has still not been characterized further than as a volatile phosphorous compound (86). A different type of severe iron corrosion may occur in intermittent anaerobic/aerobic environments where sulphide-oxygen gradients induce the formation of dissolved elemental sulphur (65). The corrosion mechanism was proposed to be the high local acidity generated on particles of solid sulphur reacting with water (25). A *Pseudomonas* spp. originally isolated from

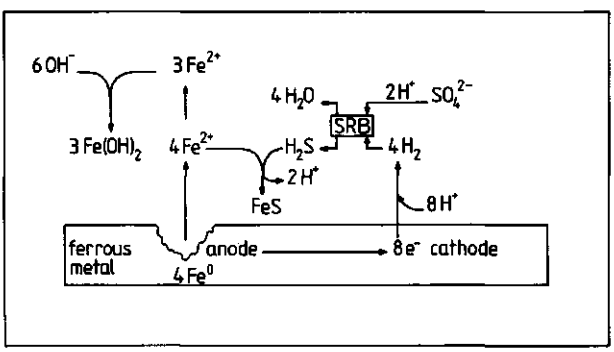

Fig. 5. Proposed cathodic depolarization reactions for pitting corrosion of ferrous metals by sulphate-reducing bacteria (SRB). Adapted from Widdel (88).

crude oil was described to be capable of generating a corrosive environment by reducing ferric iron (56). The mechanism of steel corrosion depended on the ability of *Pseudomonas* spp. to convert a protective, insoluble ferric oxide film into unprotective, soluble ferrous iron (56). Denitrifying and nitrate respiring bacteria may also cause steel corrosion (1). In such cases anaerobic

corrosion results from a chemical reaction between nitrite (from nitrate reduction) and elemental iron:

$$2Fe + NaNO_2 + 2H_2O \rightleftarrows \gamma\text{-}Fe_2O_3 + NaOH + NH_3$$

It was shown (5) with röntgen-analysis that a temporarily protective film of γ-Fe_2O_3 is formed according to this equation.

Control of sulphate-reducing bacteria. In absence of oxygen, growth of sulphate-reducing bacteria can be expected in groundwaters that contain organic carbon and sulphate. In aerobic aquatic environments biofilms attached to surfaces may become oxygen depleted through microbial respiration within a distance of 10 to 25 μm. Relatively thick biofilms can provide anaerobic microniches for sulphate-reducers (14). In analogy to the offshore oil and gas industry control of growth and activity of sulphate-reducing bacteria during aquifer thermal energy storage can be achieved by cathodic protection using a sacrificial magnesium anode (9,13). Another preventive measure could be the use of protective coating in pipes for instance with fibre glass, rubber derivatives and other inert materials. Biocides such as quaternary ammonium compounds (QAC's) with alkyl chain length C12-C18 and glutaraldehyde are widely used in the offshore industry to control sulphate-reducers (82). Obviously, the general toxicity of biocides used against desulphuricants militates against their use in aquifers. A treatment technique suitable for practical application against sulphate-reducers in aquifers might be the *in situ* injection of aerated groundwater as is done in the Vyredox process (11). Because biogenic sulphide production is not only inhibited by oxygen but also by nitrate (35), the Vyredox process would be even more effective against sulphate-reducers if the aerated groundwater contained nitrate.

1.3 OUTLINE OF THIS THESIS

Depending on the operational temperature, the performance of an ATES-facility can be considerably affected by bacterial growth and slime formation and biogeochemical precipitation reactions. Besides the influence of temperature on these processes, the effect of various concentrations of electron donors and electron acceptors on groundwater condition is of pivotal importance. When the present investigations were started, only limited knowledge was available

on the influence of thermal energy storage on groundwater quality. A more fundamental understanding of processes affecting groundwater quality was regarded necessary for the development of both groundwater treatment methods and hydrochemical modelling. Therefore, the objective of the present research was to study bacterial slime formation and the biogeochemical aspects of carbonate and iron precipitation during aquifer thermal energy storage.

Chapter 2 describes the contribution of bacterial growth and slime formation to well clogging in an ATES-process that was simulated in continuous column experiments. Studies with columns were carried out under aerobic and anaerobic conditions at 10 and 30°C with synthetic groundwater and aquifer material from an ATES test site. In order to quantify slime formation the various column effluents were passed through a hollow fiber membrane that was periodically backflushed. The recovered organic material was analyzed for proteins and polysaccharides. Chapter 3 deals with the influence of dissolved organic matter and carbon dioxide production on the precipitation of calcium and magnesium carbonates at temperatures between 4 and 95°C in samples from two different aquifers used as ATES test sites. The kinetics of carbon dioxide production have been measured. An attempt has been made to distinguish between biologically mediated processes and purely chemical reactions. In Chapter 4 it is reported that ferric iron precipitation resulting from anaerobic ferrous iron oxidation in denitrifying mixed cultures is caused by nitric oxide formation. This subsequently caused a total inhibition of sulphate reduction in these cultures. Nitric oxide is spontaneously formed from the reaction of nitrite (from nitrate reduction) with ferrous iron. In Chapter 5 nitric oxide production was studied in detail using *Escherichia coli* E4 as a model bacterium. The influence of nitrate on the L-lactate-driven ferric iron reduction in *E.coli* E4 is described. Considerable amounts of nitric oxide were formed during the concurrent reduction of nitrate and ferric iron. Nitric oxide was found to be inhibitory for L-lactate oxidation and concomitant nitrate reduction in *E.coli* E4. Chapter 6 reports aerobic reduction of nitrate to ammonium, a hitherto unknown feature of *E.coli* which is of general biogeochemical interest. The influence of molecular oxygen on nitrate and nitrite reduction was studied. It was shown that both enzymes, nitrate and nitrite reductase, are active in the presence of oxygen when *E.coli* E4 was grown in a chemostat. In addition it was found that *E.coli* E4 is able to grow aerobically on nitrate as sole source of nitrogen. Finally, concluding remarks and the summary of this thesis are presented in Chapter 7.

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CHAPTER 2

MICROBIOLOGICAL ASPECTS OF WELL CLOGGING DURING AQUIFER THERMAL ENERGY STORAGE

ABSTRACT

In the present investigation column experiments were conducted to simulate well clogging during the process of aquifer thermal energy storage (ATES). Using aquifer material from a heat storage site and synthetic groundwater, the well clogging potential of column effluents was studied at 10° and 30°C under both oxic and anoxic conditions. The clogging potential of the various column effluents was determined with a hollow fiber membrane from which slime depositions were recovered. A temperature increase from 10° to 30°C caused a slight increase in slime deposition only under oxic conditions. No significant difference was observed in the bacterial plate counts of oxic and anoxic column effluents, despite the increase in dissolved organic material concentrations at elevated temperatures. This material was mobilized from the soil organic carbon. The available organic carbon concentration was less than 1% of the dissolved organic carbon concentration, which was not enough to either allow excessive growth of bacteria or slime formation.

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2.1 INTRODUCTION

The reduction of hydraulic conductivity or clogging of saturated porous media during periods of prolonged liquid flow has been observed by many investigators (12). Reductions in conductivity may hamper or entirely block the functioning of wells that are used for the withdrawal or artificial recharge of groundwater. Since withdrawal and injection of groundwater is used in aquifer thermal energy storage (ATES), clogging might also hamper this process.

Clogging in the strictly physical sense is defined as the process of settling of particles that are hydrologically transported into the original pores of a porous medium, thus reducing hydraulic conductivity (18). Field observations have indicated that besides particle transport processes the hydrological performance of a well can decrease through a combination of physicochemical and microbiological phenomena (25). Therefore, various types of clogging processes can be distinguished around water wells. For instance for aquifers containing a sulphide-oxygen gradient near a well, the transport of soluble iron will result in the precipitation of ferrous sulphides and ferric hydroxides (31). For soils containing clay, reductions in conductivity may be caused by the flocculation of clay particles at high electrolyte concentrations of the permeating water (11). Gas accumulation in the porous medium may also reduce the conductivity, either from air entrapped during initial wetting of the medium or from gas released by the permeating water (7). The work of Allison (1) has established that microbiological processes near groundwater wells can account for marked conductivity reductions. Microbiological clogging is caused by polysaccharides excreted by bacteria when the available organic carbon to nitrogen ratio in the permeating water is higher than 10 (3). For the prolonged operation of wells used for thermal energy storage in oligotrophic aquifers, a groundwater dissolved organic carbon concentration lower than 10 mgC/l and a suspended solids concentration below 2 mg/l are to be recommended (23). To significantly limit bacterial growth the available organic carbon concentration in groundwater should remain below 10 μg/l (15,20).

In the present study a test system was developed to investigate and quantify clogging by bacteria and microbial slime formation under oxic and sulphate reducing conditions. Continuous column experiments were carried out at 10° and

30°C with synthetic groundwater and sediments obtained from an ATES testing site. Slime was collected in a hollow fiber membrane and its polysaccharide content was quantified.

2.2 MATERIALS AND METHODS

Organism. The organism used in this study was *Pseudomonas fluorescens* P8. It was obtained from the culture collection of the Department of Microbiology of the Wageningen Agricultural University. The organism was maintained on yeast extract glucose agar slants, containing (g/l): yeast extract (2,5), glucose (5) and agar (12). The agar slants were stored at 4°C and subcultured every 2 months. *Rhizobium meliloti* TA-1 was kindly provided by M.W. Breedveld, Department of Microbiology, Wageningen Agricultural University.

Growth conditions, media and determination of bacteria. *Pseudomonas fluorescens* P8 was cultivated in demineralized water (pH = 7.2) containing (g/l): sodium-acetate.$3H_2O$ (1), $NHCl_4$ (0.82), K_2HPO_4 (0.25), $MgSO_4.7H_2O$ (0.1), $NaHCO_3$ (0.5) and 1 ml/l of a trace element solution. This trace element solution was composed as follows (gram per liter of distilled water): $CaCl_2.2H_2O$ (10), NaCl (10), $FeCl_3.6H_2O$ (1), HBO_3 (0.05), $CuSO_4.5H_2O$ (0.01), $CoCl_2.6H_2O$ (0.02), $NiCl_2.6H_2O$ (0.02), KI (0.01), $MnCl_2.4H2O$ (0.04), $NaMoO_4$ (0.02), $ZnSO_4.7H_2O$ (0.04). *P.fluorescens* P8 was grown at 30°C on an orbital shaker and was used for the determination of available organic carbon in column effluents.
Rhizobium meliloti TA-1 was cultivated in demineralized water (pH = 7.2) containing (g/l): mannitol (2.5), sodium-acetate.$3H_2O$ (5.2), K_2HPO_4 (3), KH_2PO_4 (2), $MgSO_4.7H_2O$ (0.1) and 1 ml/l of the trace element solution described above. Nitrogen was added as NH_4Cl in concentrations varying from 0.15 g/l to 5.9 g/l. *R.meliloti* TA-1 was grown for 6 days at 30°C on an orbital shaker. The organism was used as a reference for the production of extracellular polysaccharides (34).
The number of colony forming units (C.F.U.) per milliliter in oxic and anoxic column effluents was determined after two and four days in a series of dilutions on agar plates. The agar plates were incubated at 30°C and consisted of (g/l): agar (10), nutrient broth (4), and $NaHCO_3$ (0.5).
The number of sulphate reducing bacteria per milliliter of the anoxic column effluent was determined 1 and 3 weeks after diluting and plating in an anoxic chamber (Coy Laboratory Products, Ann Arbor, MI, USA). The anoxic counting

plates were incubated at 30°C and were composed as follows (g/l): agar (10), sodium-acetate.$3H_2O$ (6.6), Na_2SO_4 (5), NH_4Cl (0.82), K_2HPO_4 (3), KH_2PO_4 (2), $MgSO_4.7H_2O$ (0.10), cysteine (0.12), $Na_2S.7\text{-}9H_2O$ (0.025), $NaHCO_3$ (0.5) and 1 ml/l of the trace element solution described above.
Gram-negative aerobic rods present in the 30°C oxic column effluent were characterized as follows: Gram-staining, pigmentation on nutrient broth agar plates, motility, oxidase-reaction (19), oxic and anoxic degradation of glucose (16), anoxic degradation of arginine (29) and the capability of denitrification (27).

Sediment and groundwater. The sediment that was used in this study was obtained from an ATES test site at Bunnik, The Netherlands. The sediment comprised medium fine sand with an organic matter content of 0.48% volatile solids, a porosity of 40% and a cation exchange capacity of 0.4 meq/100g (14). The sediment was obtained from the Betuwe Formation (Holocene) at a depth of 5.6 to 6.3 meter below surface. The *in situ* temperature of the groundwater at the test site was 10°C, the pH 7.1 and the electric conductivity 74 mS/m . The chemical oxygen demand (COD) of the groundwater was 15 mgO_2/l, the total organic carbon (TOC) 41 mgC/l and the dissolved organic carbon (DOC) 5 mgC/l. The composition in mg/l of the inorganic ions was: 48 Na^+, 15 K^+, 15 Mg^{2+}, 131 Ca^{2+}, 2Mn^{2+}, 10 Fe^{2+}, 0.9 NH_4^+, 25 Cl^-, 484 HCO_3^-, 95 SO_4^{2-}, 0.3 NO_3^- and 1.8 PO_4^{3-} (14). Sediment and groundwater were stored at 4°C for not longer then 3 days before further use.

Column experiments. Oxic and anoxic column experiments were carried out at 10° and 30°C with 1600 g wet weigth of sand (7.4 g volatile solids) in perspex columns (length: 45 cm; internal diameter: 5 cm). The experiments were done with sterilized synthetic groundwater which was pumped through the column in an up-flow mode at a rate of 40 ml/h. The synthetic groundwater had a COD identical to the *in situ* groundwater and was composed as follows (mg/l): Na-acetate.$3H_2O$ (30), NH_4Cl (2.7), K_2HPO_4 (3.5), $MgSO_4.7H_2O$ (120), $NaHCO_3$ (500) and 1 ml/l of the trace element solution. After autoclaving in 10 liter carboys the synthetic groundwater was continuously flushed at a rate of 2 l/h with filter sterilized air/CO_2 (99.5%/0.5%) or with nitrogen/CO_2 (99.5%/0.5%). 0.1 mg/l Resazurin as a redox indicator (17) and 25 mg/l $Na_2S.7\text{-}9H_2O$ (24) were added to the anoxic carboy. A bacterial filter was placed between carboy and sand column to prevent back growth from the column. This bacterial filter was

a 5 ml glass tube with a P-oo type bottom plate containing 2 grams of bactericidal iodine resin (10). The column effluents were led through a hollow fiber membrane (volume: 10 ml;internal surface: 0.03 m^2) with an average pore diameter of 0.1 μm (Amicon Nederland B.V., Rotterdam, The Netherlands).

Quantification of the available organic carbon. *Pseudomonas fluorescens* P8 was used for the determination of available organic carbon in the oxic and anoxic column effluents. Strain P8 was precultured in the carbon limited acetate containing medium described above. At the stationary growth phase of strain P8, 0.1 ml of the culture medium was used as an inoculum for 100 ml of sterilized column effluent. To avoid growth limitation by a lack of minerals the following salts were added before sterilization (g/l): NH_4Cl (0.82), K_2HPO_4 (0.25) and $MgSO_4.7H_2O$ (0.1). The number of *Pseudomonas* P8 (CFU/ml) in the inoculated medium was determined with plate counts. A calibration curve made with acetate as a standard. It showed that the yield of strain P8 was $3.7x10^6$ CFU per μg acetate-carbon added.

Chemical determinations. For the determination of dissolved organic carbon the samples were filtered through a 0.22 μm filter (Millipore B.V., Etten-Leur, The Netherlands) that was previously washed with 15 ml of 10 mM HCl. Total organic carbon and dissolved organic carbon (DOC) were determined with a Beckman 915A type organic carbon analyzer (Beckman Instruments Nederland B.V., Mijdrecht, The Netherlands) using K,H-phthalate in 0.1 M H_2SO_4 as standard. The oven temperature was 1000°C, carrier gas was CO_2-free air at a rate of 200 ml/min.

Polysaccharides with hexose units were determined with anthrone-sulphuric acid reagent using glucose as a standard (9).

Polysaccharides with hexuronic acid residues were determined with M-OH-diphenyl-sulphuric acid using glucuronic acid as a standard (6).

Protein was determined after boiling for 5 min. in 0.5 M NaOH according to a modified Lowry method (8) with bovine serum albumin as a standard.

Volatile fatty acids were analyzed on a Varian type GC (Varian Benelux B.V., Amsterdam, The Netherlands) equipped with a Chromosorb 101 glass column (200 cm x 1/8 inch internal diameter). Flow-rate was 30 ml/min of formic acid saturated nitrogen gas. Oven 190°C, FID-detector 240°C and injection port was 170°C.

Sulphate was measured with a LKB type 2142 Differential Refractometer

(Pharmacia LKB Biotechnology, Woerden, The Netherlands). Column length was 100 mm x 3 mm internal diameter, packed with Ionosphere-tm-A (Chrompack B.V., Middelburg, The Netherlands). Injection temperature was 30°C, injection volume was 20 μl, flow-rate was 0.4 ml/min 27 mM K,H-phthalate.

Dissolved oxygen in carboy and column samples was determined in a thermostatically controlled reaction vessel of a YSI model 53 biological oxygen monitor equipped with a polarographic oxygen probe (Yellow Springs Instruments, Tamson B.V., Zoetermeer, The Netherlands).

The hydraulic conductivity in the sand columns was measured as centimeter watercolumn using a glass tubing (3 mm internal diameter) connected to a column sample port.

The dry matter content of aquifer material was determined after overnight heating at 100°C; ash percentage was determined after reheating at 600°C for 2 hours.

Determination of chemical oxygen demand (chromic acid method), nitrate (alkaline salicylate method), ammonium (nesslerization), and organic nitrogen (Kjeldahl method) were done following standard methods (2).

2.3 RESULTS

Chemical groundwater composition. In Table 1 the observed changes in synthetic groundwater composition are given for the oxic and anoxic column effluents at 10°C and 30°C. Nitrogen was partially nitrified in the oxic columns. The effluents contained 0.17 mg/l nitrate-N and 0.35 mg/l ammonium-N at 10°C and 0.16 mg/l nitrate-N and 0.34 mg/l ammonium-N at 30°C. The effluent COD varies little between the oxic and the anoxic incubations at 10° and 30°C. The COD/DOC ratios are inversely proportional to the operational temperature, suggesting that at 30°C relatively oxidized organic compounds are mobilized from the organic content of the sediment. Material that accumulated in the hollow fiber membrane was backwashed after volume throughputs of 5, 10, 15 and 20 liters and analyzed for total organic carbon (Fig. 1). The results show that in contrast to oxic conditions, the organic carbon accumulation under anoxic conditions is both smaller and independent of the operational temperature. The accumulated organic carbon in the membrane device was analyzed for carbohydrates and protein after an effluent throughput of 20 liters (Table 2). Under oxic conditions at 30°C hexoses and hexuronic acids were both found in the 'slime fraction' of the membrane retentate. Under anoxic conditions much less

slime was formed and no hexoses and hexuronic acids were found. The effluent temperature and the presence or absence of oxygen are more important parameters

Table 1. The composition of influent and effluent from oxic and anoxic sulphate-reducing sand columns incubated at 10° and 30°C. All effluent values are the average of at least three independent measurements.

	Influent	Oxic effluent		Anoxic effluent	
		10°C	30°C	10	30°C
Acetate (mg/l)	13	1.9	-[a]	8.4	-
Nitrogen compounds[b] (mgN/l)	0.70	0.52	0.50	0.48	0.56
Sulphate (mg/l)	49	45	46	42	34
Oxygen (%)[c]	100/-	40	35	-	-
pH	7.8	7.8	7.8	7.8	7.8
COD (mgO_2/l)	15	19	21	18	19
DOC (mgC/l)	7.8	4.4	13	6.3	10
AOC (mgC/l)	5.3	0.63	0.05	2.8	0.02
Plate counts (CFU[d]/ml)	n.d.[e]	$3.2x10^4$	$5.3x10^4$	$2.0x10^3$	$1.5x10^3$
SRB[f](CFU/ml)	n.d.	n.d.	n.d.	$1.8x10^2$	$6.2x10^2$

[a]: Not detected. [b]: Nitrogen was added as NH_4Cl. [c]: Oxygen is given as percentage air saturation; influent values refer to aerobic and anaerobic columns respectively. [d]: CFU means Colony Forming Units. [e]: SRB means Sulphate Reducing Bacteria.

for slime formation than the C/N ratios presented in Table 3. The dissolved organic carbon concentrations increased with temperature and as a consequence also the C/N ratio because in all effluents inorganic nitrogen compounds varied only little (Table 1). Organic nitrogen was never detected in the effluent. The temperature dependent mobilization of organic carbon results an increase of the DOC/N ratio at 30°C. From the concomitant decrease of the AOC/N ratio at 30°C, it can be calculated that less than 0.5 % of the organic carbon present in the effluent can be metabolized. This is in good agreement with

results presented by others (19,29) who reported that the available organic carbon fraction in groundwater from similar oligotrophic aquifers is usually less than 1% of the dissolved organic carbon fraction.

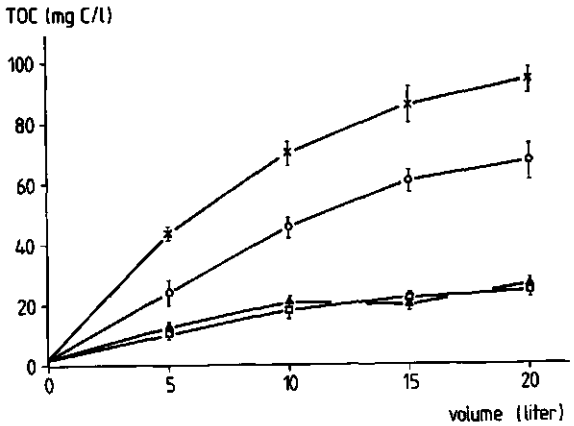

Fig.1. Accumulation of organic carbon (mg C/l) in a hollow fiber membrane from column effluent. The volume indicates the amount of effluent which passed through the hollow fiber membrane. TOC in the hollow fiber membrane was recovered by backwashing with 30 ml demineralized water. Each point respresents the average of two independent determinations. Bars indicate standard deviation. (o): oxic 10°C, (□): anoxic 10°C; (x): oxic 30°C; (▲): anoxic 30°C.

Table 2. Total organic carbon, carbohydrate and protein content of the hollow fiber membrane retentate recovered in 30 ml of demineralized water after an effluent throughput of 20 liters. The columns were operated at 10° and 30°C under both oxic and anoxic conditions. The data given are the average of two independent experiments.

	Oxic effluent		Anoxic effluent	
	10°C	30°C	10°C	30°C
TOC (mgC/l)	67	94	24	27
Glucose-units (mg/l)	10	20	-	-
Glucuronic acid units (mg/l)	-	5	-	-
Protein (mg/l)	40	40	15	1

(-): not detected

Table 3. The dissolved- and available organic carbon to nitrogen ratios of column influent and effluent. The sand columns were operated at 10° and 30°C under oxic and anoxic conditions. The data given are the average of three independent measurements.

	Influent	Oxic effluent		Anoxic effluent	
		10°C	30°C	10°C	30°C
DOC/N	11	8.4	27	13	18
AOC/N	7.6	1.2	0.09	5.8	0.04

Microbial groundwater composition. The majority (90%) of the bacteria in the 'slime fraction' from the 30°C oxic effluent collected in the hollow fiber membrane were Gram-negative rods (Fig. 2). Up to 50% of the Gram-negative rods

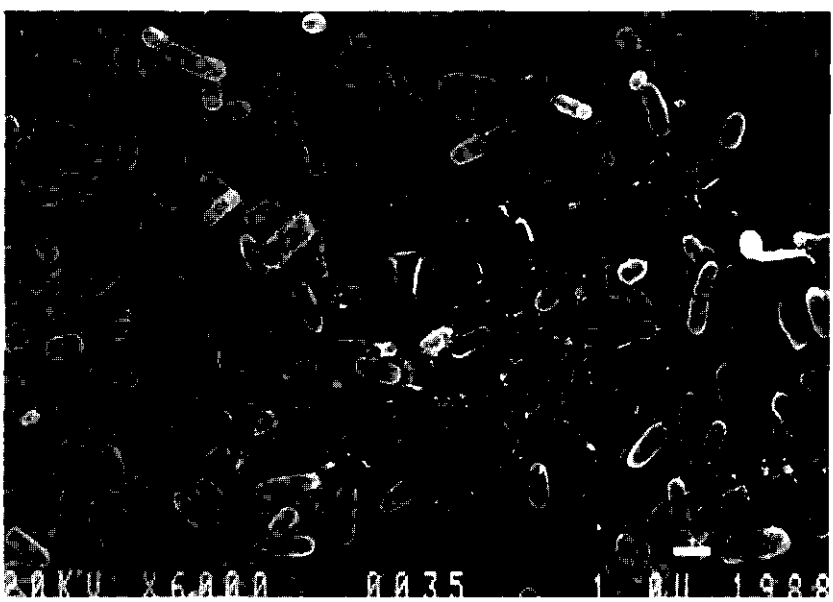

Fig. 2. Scanning electron micrograph of small amounts of slime that enclosed a microbial population present in the hollow fiber membrane after filtration of 20 liters effluent from an oxic column. Rod shaped Gram-negative bacteria were predominant. Bar represents 1 micrometer.

belonged to the genus *Acinetobacter* and *Moraxella*. About 30% of the bacterial population was relativly equally composed of *Pseudomonas*, *Flavobacterium*, *Aeromonas* and *Corynebacterium* species. The high-nutrient medium used for total cell counts in this study could have inhibited growth of oligotrophic bacteria and reduced the number of CFU/ml by a factor 2-3 as compared with a low nutrient medium (28). The predominant genera of Gram-negative heterotrophic bacteria were nevertheless similar to those reported in the literature (28) for well-water isolates. The small amounts of polysaccharides found in the membrane retentate (Table 3 and Fig. 2) seems not only to be due to the low AOC/N ratio in the aerobic 30°C column effluent but also to the disability of the endogenous column bacteria to form slime in large amounts. Slime formation was tested at different C/N ratios using the bacterial population found in the membrane retentate of the oxic 30°C effluent after throughput of 20 liter and

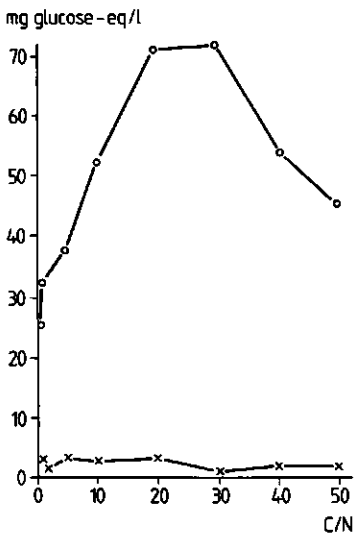

Fig. 3. Production of extracellular polysaccharides by *Rhizobium meliloti* TA-1 and a bacterial mixed culture versus the C/N ratio in the growth medium. The bacterial mixed culture originated from the hollow fiber membrane after a throughput of 20 liters oxic effluent at 30°C and was recovered by backwashing in 30 ml demineralized water. Hexoses present in extracellular polysaccharides were determined with anthrone-H_2SO_4 and are expressed as glucose-equivalents. Each point represents the average of three independent determinations. Symbols refer to inocula used in the growth medium. (o): *Rhizobium meliloti* TA-1, (x): bacterial mixed culture.

Rhizobium meliloti TA-1, as inoculants. The medium was composed of mannitol and acetate. While *R.meliloti* TA-1 produced considerable amounts of polysaccharide, the membrane retentate population hardly formed any reducing sugars containing polymers (Fig. 3).

2.4 DISCUSSION

In the hydrological literature (18) the reduction in hydraulic conductivity in a physical clogging process is expressed by the time dependent water velocity V(t) being a function of the initial water velocity (Vo),time (t), the porosity (ϵ) and the suspended solids concentration (S). Besides physical particle deposition biological processes are also important in clogging of groundwater wells (1,15). Biologically mediated clogging processes will also be influenced by the operational temperature. Therefore, the time dependent water velocity V(t) is determined not only by the initial water velocity (Vo), time (t), porosity (ϵ) and suspended solids concentration including bacteria (S) but also by available organic carbon (AOC) and temperature (T). This leads to a function: $V(t)=f(Vo,t,\epsilon,S,AOC,T)$ which can only be solved empirically, provided the soil composition is well known and both the temperature and the available organic carbon concentration do not fluctuate significantly (26). Consequently, the prediction of well clogging rates during ATES is only possible with site specific models that have no general value. Modelling of clogging processes is even more difficult because field studies (25,26,33) have shown that the hydrological performance of groundwater wells is actually hampered by the poorly understood growth of bacteria in organic deposits located at or near the aquifer-gravel interface. This also applies to laboratory studies (12,15) in which a decrease of hydraulic conductivity of sand filters in relation to the AOC-*charge* ($mgC/m^2.h$) is reported. The AOC-*charge* results from the product of AOC concentration ($mgC/m^2.h$) and the flux of groundwater through a sand filter ($m^3/m^2.h$). The two columns investigated here had an AOC-*charge* of 96 $mgC/m^2.h$. This AOC-*charge* was calculated from the acetate-carbon concentration of 5.3 mgC/l, a flow rate of 40 ml/h and an internal column diameter of 5 cm. Laboratory studies with clogging by bacterial cells have shown that an AOC-*charge* of 120 $mgC/m^2.h$ resulted in a decrease in hydraulic conductivity of 10 kPascal (100 cm watercolumn) in 4 weeks (15). However, the quantification of bacterial biomass can be a problem in laboratory studies with sand columns (12,15,25). Despite various washing procedures, plate counts

techniques and ATP measurements are not as accurate as for instance direct counts with a transmission electron microscope (4). In these laboratory studies (12,15,25) the total volume of bacteria represented by the CFU number per gram of sand was relatively small. As a consequence this low number of bacteria could not entirely explain clogging phenomena in the pores of the sand columns. Field observations around groundwater wells have shown that microbial slime formation must be taken into consideration for the prediction of the loss of hydraulic conductivity (23,26). In our study clogging as a result of microbial activities was expected to occur near the inlet of the column. Only under oxic conditions and a throughput of 20 liters in three weeks a decrease in hydraulic conductivity of 5 cm watercolumn at 10°C and 10 cm watercolumn at 30°C was measured near the inlet of the sand column (data not shown). It was not intended to specifically investigate where and by what mechanism a loss in hydraulic conductivity near the inlet of the oxic sand column occurred. In the present study the oxic sand column is a black box that actually functions independently from the hollow fiber membrane. The data given in Tables 1 and 2 indicate that such a system may yield reproducible column effluents and membrane retentates. However, with this experimental set-up it is not possible to draw specific conclusions about the material collected from the hollow fiber membrane and *in situ* clogging in the oxic sand column.

The results listed in Table 2 show that the excretion of bacterial polysaccharides is markedly lower under anoxic conditions as was previously reported by others (21). Anoxic conditions, the presence of sulphate and the AOC-*charge* around a groundwater well favour the growth of sulphate-reducers. The accumulation of iron sulphides seems to be the primary cause for anoxic well clogging and not slime formation by sulphate reducing bacteria (31). Under oxic conditions bacteria can actively cause clogging as was reported for *Flavobacterium* spp. in a laboratory sand filter which was fed with infiltration water supplemented with 2% casein (21). Such a high amount of organic carbon in infiltration water is incompatible with common groundwater. The amounts of polysaccharides that were found to be produced with *Flavobacterium* spp. can be compared with those excreted by *R.meliloti* TA-1 but not with the bacteria enriched in the hollow fiber membrane (Fig. 3). Besides *Flavobacterium*, also *Pseudomonas* and *Corynebacterium* have been described in the literature as bacteria capable of extracellular polysaccharide production (13). Yet, our results show that the presence of these species in the 30°C oxic column

effluent does not necessarily lead to excessive slime production, even under ideal carbon to nitrogen ratios (Fig. 3).

The predominant Gram-negative heterotrophs isolated from the hollow fiber membrane are common to aquifers with a dissolved organic carbon concentration lower than 10 mgC/l (28). Nutritionally versatile aquifer bacteria such as coryneform bacteria and pseudomonads can subsist on low concentrations of humic acids without forming specialized resting cells (5). Even bacterial growth was possible on humic acids from lake water (30). In our study humic acids may be present in the available organic carbon fraction of column effluent. The contribution of humic acids to microbial growth has not been investigated.

In conclusion it can be stated that in our study the observed mobilization of dissolved organic carbon compounds has only limited significance for prediction of microbiological slime formation during aquifer thermal energy storage on the large scale. These dissolved organic carbon compounds may determine the mobility and the precipitation iron, calcium and magnesium in groundwater, however.

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CHAPTER 3

BIOGEOCHEMICAL REACTIONS IN AQUIFER MATERIAL FROM A THERMAL ENERGY STORAGE SITE

ABSTRACT

The mobilization of organic compounds and the release of CO_2 was studied in aquifer material from a site chosen for thermal energy storage. These processes have been measured aerobically and anaerobically within a temperature range of 4° to 95°C in sediment samples consisting of either quartz-rich coarse sand or peaty clay. At temperatures above 45°C organic carbon compounds, including fulvic acids, were mobilized from both sediments resulting in an increased chemical oxygen demand of the water phase. Complexation of calcium and magnesium by fulvic acids resulted in the super-saturation of the water phase with regard to calcite and dolomite and thus prevented the precipitation of these carbonates. The highest rates of CO_2 release were observed during the first four days. Aerobically, the maximum velocity for CO_2 formation varied between 35 and 800 (sand) or 15 and 150 (peaty clay) μmol CO_2 per gram volatile solids per day. Anaerobically, similar rates were observed, namely 25 to 500 (sand) and 10 to 110 (peaty clay) μmol CO_2 per gram volatile solids. At temperatures above 55°C, CO_2 was produced purely chemically.

Brons H.J., Griffioen J. Appelo C.A.J. and Zehnder A.J.B. (1991) (Bio)geochemical reactions in aquifer material from a thermal energy storage. Water Research 25: 729 - 736.

3.1 INTRODUCTION

Aquifer thermal energy storage (ATES) is used to balance seasonal differences in energy surplus and demand. In ATES systems, groundwater is extracted and heated by the summer's surplus heat. The warm water is injected into the aquifer at a certain depth in another place. During winter, the heated water is pumped up, its heat recovered and the cool water reinjected where it was originally extracted. This technique results in considerable fluctuations of *in situ* groundwater temperatures. As a consequence, both the biological and chemical composition of the groundwater will change markedly. These changes can lead to the precipitation of ferric hydroxides, ferrous sulphides, calcium carbonates, *etc.* in the aquifers, wells, pipes and heat exchangers. These precipitates may hamper the operation of ATES (Iihola *et al.*, 1988).

The solubility of carbonates is dependent on pH, CO_2 partial pressure, presence or absence of specific cations and complexing agents (Inskeep and Bloom, 1986A; Stumm and Morgan, 1981). In a given constant environment, CO_2 partial pressure may increase as a result of organic matter mineralization either by chemical or biological means. In aquifers humic substances are generally the major natural source of organic matter (Mathess, 1982). The biological mineralization of organic compounds depends, besides on the degradability of these molecules, on the presence of adequate electron acceptors such as oxygen, nitrate and sulphate. These acceptors are used according to their decreasing level of redox potentials (Zehnder and Stumm, 1988). Dutch aquifers used for ATES contain only little nitrate and no oxygen. Therefore, sulphate reduction and methanogenesis are expected to contribute significantly to the CO_2 production and hence affect carbonate solubility during ATES-processes. The kinetics of these redox reactions depend on (i) the biodegradability of organic compounds present, (ii) the presence and concentration of inorganic electron acceptors and (iii) the operational temperature of the thermal energy storage system.

A study has been initiated to measure the influence of dissolved organic material and carbon dioxide production on the precipitation of calcium and magnesium carbonates, at temperatures between 4 and 95°C in samples from two different aquifers used as ATES test sites. The kinetics of carbon dioxide production have also been measured. An attempt has been made to distinguish between biologically mediated processes and pure chemical reactions.

3.2 MATERIALS AND METHODS

Sediments and groundwater. The two sediments that were used in this study were obtained from an ATES test facility at Delft, NL, by an anaerobic sampling procedure (Appelo *et al.*, 1989). The sediment rich in organic matter (27.9% volatile solids) was a peaty clay with 1.2% $CaCO_3$; it originated from the Westland Formation and was sampled at 2.7 to 3.5 m below the surface. The sediment low in organic matter (0.38% volatile solids) was obtained from the Kreftenheye Formation at a depth of 14.8 to 15.5 m below surface. The Kreftenheye Formation comprises the aquifer in which the thermal energy is actually stored. This sediment is a quartz-rich coarse sand with 1.5% $CaCO_3$. These two sediments will be further referred to as peat and sand. The groundwater used in this study was sampled at the same site with a submersible pump from a depth of 16 m and was collected into an argon flushed 10-l carboy. The *in situ* groundwater temperature was 10.8°C, the pH 6.6 and the electric conductivity 420 mS/m. The chemical oxygen demand (COD) of the groundwater was 72 mg O_2/l, the total organic carbon (TOC) and the dissolved organic carbon (DOC) were both 63 mg C/l. The composition of the inorganic ions (in mg per liter) in the sampled groundwater was 640 Na^+, 23 K^+, 72 Mg^{2+}, 240 Ca^{2+}, 16 NH_4^+, 1040 Cl^-, 1220 HCO_3^-, and 10 PO_4^{3-}. Manganese (Mn^{2+}) and iron (Fe^{2+}) were both below 1 mg/l; sulphate and nitrate were not detected. Both sediments and groundwater were stored at 4°C for 3-5 days before further use.

Batch experiments. Aerobic and anaerobic batch experiments were carried out at 4, 10, 20, 30, 37, 45, 55, 65, 75, 85 and 95°C with either 50 g wet weight of peat (4.9 g volatile solids) or 100 g wet weight of sand (0.34 g volatile solids) together with 250 ml groundwater in 500 ml serum flasks sealed with a butyl rubber stopper (Rubber B.V., Hilversum, NL). Serum flasks (headspace 280 ml) were made anaerobic both by flushing with argon during 15 min and by adding 25 mg/l $Na_2S.9H_2O$, (Postgate, 1979); 0.1 mg/l resazurin was used as a redox indicator (Hungate, 1969). To allow sulphate reduction to occur, 1.74 g/l K_2SO_4 was added to a series of anaerobic incubations. All aerobic incubations were supplemented with 80 mg/l K_2SO_4 as sulphur source. To avoid inhibition of microbial activities due to nutrient depletion, 820 mg/l NH_4Cl, 250 mg/l K_2HPO_4 and 1 ml/l of a trace element solution were added to all incubations. The trace element solution consisted of (per liter of distilled water): 1000 mg $FeCl_3.6H_2O$, 50 mg H_3BO_3, 10 mg $CuSO_4.5H_2O$, 20 mg $CoCl_2.6H_2O$, 20

mg $NiCl_2.6H_2O$, 10 mg KI, 40 mg $MnCl_2.4H_2O$, 2 mg $NaMoO_4$ and 40 mg $ZnSO_4.7H_2O$. Controls with groundwater only were used for the correction of CO_2 produced by the groundwater. Prior to all gaschromatographic analyses, the incubated serum flasks were equilibrated at room temperature (20 ± 2°C) for 3 to 4 hours. Oxygen, carbon dioxide and methane were sampled from the headspace and analyzed for by gaschromatography.

Dialysis experiments. Dialysis membranes with molecular weight cut off 1.000 and 10.000 (Hicol B.V., Oud Beyerland, NL) were conditioned for 30 minutes in 5 mM $NaHCO_3$ and were then filled with 25 ml groundwater. The sample was dialysed at 4°C against 50 ml of demineralized water for 16 hours. Subsequently calcium and magnesium concentrations inside and outside the membrane were measured.

Analytical methods. The headspace samples were injected into a gaschromatograph using a 250 µl syringe equipped with a mininert type syringe valve (Supelco Inc., Applikon B.V., Schiedam, NL). Henry's law constant (H) for the partitioning of CO_2, O_2 and CH_4 between groundwater and headspace of serum flasks was determined experimentally at room temperature (Stumm and Morgan, 1981). O_2 was measured with a Packard Becker 417 type gaschromatograph (Chrompack B.V., Middelburg, NL) equipped with a 180 cm x 0.6 cm internal diameter column, packed with a 13 x molecular sieve (60-80 mesh). Argon was the carrier gas at a flow rate of 30 ml/min. Thermal conductivity detector (TCD) was set at 60°C, the oven at 100°C. CO_2 and CH_4 were quantified with the same GC but a Poropak Q (60-80 mesh) column (600 cm x 0.3 cm internal diameter) was used. Carrier gas was argon at a flow rate of 30 ml/min. TCD and oven were both set at 100°C.

Bicarbonate and sulphate were measured by ionchromatography with a LKB type 2142 Differential Refractometer (Pharmacia LKB Biotechnology, Woerden, NL). The column (100 mm x 3 mm internal diameter) was packed with Ionosphere-tm-A (Chrompack B.V., Middelburg, NL). Injection temperature was 30°C, injection volume 20 µl. The mobile phase was 30 mM potassium hydrogen phthalate at a flow rate of 0.6 ml/min. Sulphide was determined spectrophotometrically at 665 nm by a ferric iron mediated oxidation of leucomethylene blue (Pachmayr, 1961). The sulphide ($Na_2S.9H_2O$) for the calibration curve was standardized iodometrically. Chloride was measured potentiometrically by titration with silver nitrate using a Microchlorocounter (Marius Instruments, Utrecht, NL).

Sodium, potassium and calcium were determined with a Perkin Elmer model 51 flame-photometer (Perkin Elmer Nederland B.V., Gouda, NL). Magnesium was quantified with an Instrumentation Laboratory model 357 atomic absorption spectrophotometer (Van Hengel Instruments B.V., Breda, NL).

Lactate and acetate were measured with a LKB model 2158 Uvicord-SD HPLC (Pharmacia LKB Biotechnology, Woerden, NL) equipped with a 30 cm x 3/8 inch internal diameter organic acids column (Chrompack B.V., Middelburg, NL) which was operated at 65°C and 80 bar and protected by a guard column (anion exchanger). The wavelength of the UV-detector was set to 206 nm. The mobile phase was 10 mM H_2SO_4 at a flow rate of 0.8 ml/min.

For the determination of dissolved organic carbon (DOC), samples were filtered through a 0.22 μm filter (Millipore B.V., Etten-Leur, NL) that was pre-washed with 15 ml of 10 mM HCl. Total organic carbon (TOC) and DOC were determined with a Beckman model 915A organic carbon analyzer (Beckman Instruments Nederland B.V., Mijdrecht, NL) using potassium hydrogen phthalate in 0.1 M H_2SO_4 as a standard. The oven temperature was 1000°C and the carrier gas was CO_2-free air at a flow-rate of 200 ml/min.

The pH of groundwater samples was measured with a Knick model Porta Mess pH meter (Hilcomy B.V., Den Haag, NL).

The electric conductivity of the groundwater sampled at the ATES testing-site was measured with a LF530 type of E.C. meter (Retsch B.V., Ochten, NL) using KCl as standard.

Determinations of the chemical oxygen demand (chromic acid method), calcium carbonate (Scheibler's barometric method using hydrochloric acid), phosphate (molybdenum-ascorbic acid method), nitrate (alkaline salicylate method) and ammonium (nesslerization) were all done following standard methods (American Public Health Association, 1976). Iron and manganese were measured spectrophotometrically with Spectroquant reagent kits (E. Merck Nederland B.V., Amsterdam, NL).

Sediment dry solids were measured after heating for 24 hours at 100°C; volatile solids (VS) were determined after reheating the samples at 600°C for 2 hours. A röntgen diffractometer model PW 1050 (Philips B.V., Eindhoven, NL) with a cobalt tube (lambda = 1.7889 Å) was used for mineralogical analysis of random powder samples. The generator was set at 40 kV and 32 mA. The scanning speed was 0.03° (2 θ) per second, scanning range was 5-70° (2 θ). The powder diffraction file of the International Centre for Diffraction Data (1987) was used for identification.

The hydrogeochemical data were analyzed with the computer program PHREEQE (Parkhurst *et al.*, 1980).

Chemicals. Sodium L-lactate was from J.T. Baker B.V., Deventer, NL. Sodium acetate and mineral salts which were added to the incubations were obtained from E. Merck Nederland B.V., Amsterdam, NL. All chemicals were of analytical grade. Gases were purchased from Hoek Loos B.V., Schiedam, NL.

3.3 RESULTS

Temperature dependent change of groundwater composition. After 23 days of incubation at 10 or 75°C, the chemical composition of the aqueous phase in the peat and sand aquifer material has been quantified for anaerobic (Table 1) and aerobic (Table 2) conditions. Sodium (640 mg/l), potassium (135 mg/l), ammonium (830 mg/l) and chloride (1560 mg/l) remained constant at all conditions tested. Therefore, they were not included in these Tables. The increase in DOC/COD ratios in peat incubations indicates that the organic substances that are thermally mobilized from the solid phase are relatively oxidized. Bicarbonate remains fairly constant in all incubations. The decrease in pH is thus the result of carbon dioxide or organic acid production. Calcium and magnesium concentrations decreased with increasing temperature. For the sake of electroneutrality, some anions have to diminish also. The data in Tables 1 and 2 show only a reduction of phosphate concentrations. Besides phosphate, carbonate (formed from carbon dioxide) could also act as counter ion for calcium and magnesium. Since an exact carbon and redox balance is not possible in this system (the precise composition of the organic carbon pool is not known), a loss of carbonate can only be quantified indirectly. To obtain information on possible carbonates of calcium and magnesium, a röntgen diffraction analysis was made. Precipitates found anaerobically at 95°C showed the highest crystal plane distance (d-value) at 2.92 Å. Hence, the precipitate is best characterized as $CaMg(CO_3)_2$, a dolomite (d = 2.89 Å) type of material that is akin to calcite (d = 3.03 Å). Column experiments at 90°C, in which an ATES-cycle was simulated with the same groundwater and sediment from the Kreftenheye Formation, also indicated that precipitation of Ca^{2+} and Mg^{2+} with CO_3^{2-} and PO_4^{3-} occurred upon heating (Griffioen *et al.*, 1989).
With the data sets from Table 1, the amount of carbonate precipitation was calculated from the decrease in Ca^{2+}, Mg^{2+} and ortho-PO_4^{3-} concentrations.

Table 1. Chemical oxygen demand (COD), dissolved organic carbon (DOC), bicarbonate, carbon dioxide produced, pH, calcium, magnesium, phosphate and sulphate determined after 23 days of anaerobic incubation at 10 and 75°C. All values are the mean of three batch replicates.

	Native groundwater	Groundwater + sand		Groundwater + peat		Groundwater control	
		10°C	75°C	10°C	75°C	10°C	75°C
COD (mgO_2/l)	72	85	260	120	1100	74	74
DOC (mgC/l)	63	72	370	140	1980	69	69
DOC/COD	0.88	0.85	1.4	1.2	1.8	0.93	0.95
HCO_3^- (mM)	20	22	23	22	22	22	21
CO_2 ($mmol.g^{-1}VS$)	-	0.48	1.6	0.12	0.38	-	-
pH	6.6	6.9	6.8	6.9	6.5	6.9	6.9
Ca^{2+} (mM)	6.0	6.0	4.4	6.0	5.4	5.9	4.1
Mg^{2+} (mM)	3.0	3.0	2.5	3.0	2.8	3.0	2.2
PO_4^{3-} (mM)	0.1	1.4	0.84	1.5	0.82	1.5	0.90

(-): not detected

Table 2. Chemical oxygen demand (COD), dissolved organic carbon (DOC), bicarbonate, carbon dioxide produced, pH, calcium, magnesium, phosphate and sulphate determined after 23 days of aerobic incubation at 10 and 75°C. All values are the mean of three batch replicates.

	Native groundwater	Groundwater + sand		Groundwater + peat		Groundwater control	
		10°C	75°C	10°C	75°C	10°C	75°C
COD (mgO_2/l)	72	81	230	85	930	74	76
DOC (mgC/l)	63	78	310	92	1470	68	70
DOC/COD	0.88	0.96	1.4	1.1	1.6	0.92	0.92
HCO_3^- (mM)	20	22	23	22	22	22	22
CO_2 ($mmol.g^{-1}VS$)	-	0.63	2.6	0.14	0.43	-	-
pH	6.6	6.9	6.6	6.8	6.3	6.9	6.9
Ca^{2+} (mM)	6.0	5.9	4.4	6.0	5.3	5.9	4.2
Mg^{2+} (mM)	3.0	3.0	2.5	3.0	2.8	3.0	2.2
PO_4^{3-} (mM)	0.1	1.5	0.72	1.5	0.95	1.5	0.90
SO_4^{2-} (mM)	-	0.50	0.45	0.48	0.52	0.50	0.41

(-): not detected

Since no crystalline phosphate was found in the röntgen diffraction analysis, phosphate must have precipitated in an amorphous HPO_4^{2-} or PO_4^{3-} form. Therefore, the amount of carbonate precipitation has to be calculated for a calcium-phosphate ratio of 1 to 1 and 3 to 2. During incubation at 10°C, the concentration of Ca^{2+}, Mg^{2+} and ortho-PO_4^{3-} remained constant in time for the groundwater-, sand- and peat vials. The saturation indices with respect to calcite and dolomite are 2.8 and 3.2, respectively, with a calculated pH (10°C) of 6.97 (Table 3). The calculated pH's are all lower than the measured values which can most likely be ascribed to carbon dioxide degassing during the sampling procedure. Increasing the temperature to 75°C, without any CO_2 production, would result in saturation indices of 12.8 and 142 for calcite and dolomite, respectively. These values were used as reference points for the calculations of the 75°C experiments.

Besides the effect of CO_2 production on the calculated saturation indices, also the calcium and magnesium complexing ability of organic matter is also important at 75°C. At higher temperatures considerable amounts of organic matter are mobi-

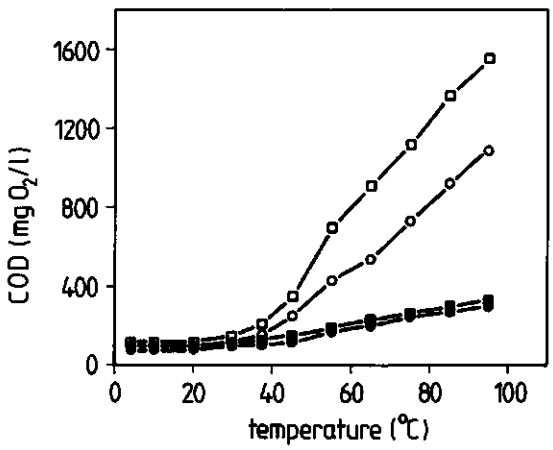

Fig. 1. Chemical oxygen demand (COD) of groundwater as a function of incubation temperature after 23 days with two sediments. These experiments were done in serum flasks to which 50 g fresh weight peat (4.9 g volatile solids), 100 g fresh weight sand (0.34 g volatile solids) and groundwater (250 ml) had been added. Sulphate was absent in anaerobic incubations; data are the mean of two replicates. Symbols used are: (□) anaerobic peat, (o) anaerobic peat, (■) anaerobic sand, (●) aerobic sand.

lized (Fig. 1) which strongly affect the solution chemistry of calcium and magnesium (Fig. 2). The calcium and magnesium chelating properties of the thermally

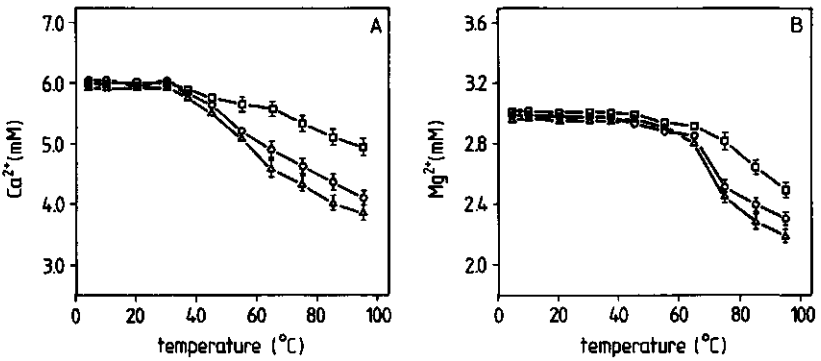

Fig. 2. Calcium (**A**) and magnesium (**B**) concentrations in the aqueous phase of aquifer material incubated anaerobically at different temperatures as described for Fig. 1. No sulphate was added in this series of experiments. Calcium and magnesium were determined after 23 days of incubation at the temperatures indicated. Error bars denote the standard deviations of four replicate experiments. Symbols are: (□) groundwater + peat, (o) groundwater + sand, (▲) groundwater only.

mobilized material could be assessed using two kinds of dialysis membranes. One with a molecular weight cut off (M_γ) of 1.000 and another with a cut off of 10.000. Only the M_γ 1.000 membrane acted as a barrier for part of the magnesium and calcium (Fig. 3). This was an indication that a fraction of the thermally mobilized DOC consisted of small molecules which were able to complex the two cations. The solubility of this DOC over the entire pH-range and the ratio of absorbances at 465 and 665 nm of all water samples collected after 23 days from 95°C incubation indicated that some fulvic acids were mobilized from the solid phase and probably responsible for complexing calcium and magnesium (Schnitzer, 1971). Fulvic acids typically have molecular weights in the range 500-2.000 (Stevenson, 1982). Therefore, some of the chelated calcium and magnesium might pass the membrane with an average molecular weight cut off of 1.000. Free calcium and magnesium ions which could also pass the M_γ 1.000 membrane, are not likely to be present, since the solutions were already super-saturated with respect to calcite and dolomite (Table 3).

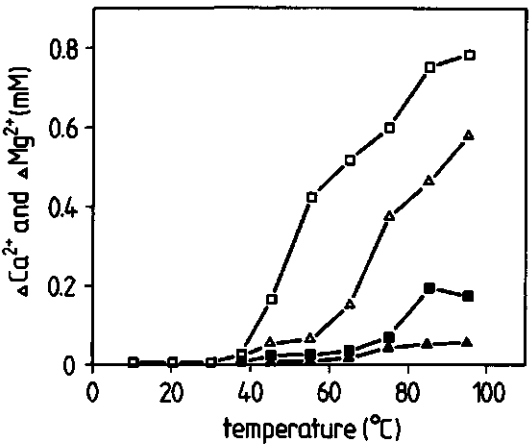

Fig. 3. Calcium and magnesium differential concentrations in dialysis experiments with groundwater that was anaerobically incubated with two sediments for 23 days in the temperature range 10 to 95°C. The incubations were done as described in Fig. 1. Dialysis was carried out with 25 ml groundwater sample in a membrane with a molecular weight cut off of 1.000. After 16 hours at 4°C dialysis equilibrium was established and calcium and magnesium concentrations inside and outside the membrane were determined. The differences are given in this Figure. Symbols: (□) calcium-peat, (△) magnesium-peat, (■) calcium-sand, (▲) magnesium-sand.

Table 3. Calculated pH, calcite- and dolomite saturation indices (SI) for anaerobic groundwater-, sand-, and peat incubations at 10 and 75°C based on data in Table 1. A: precipitation of calcium and phosphate in a 1 to 1 ratio. B: precipitation of calcium and phosphate in a 3 to 2 ratio.

	10°C	75°C-groundwater		75°C-sand		75°C-peat	
		A	B	A	B	A	B
pH (exp.temp)	6.97	6.75	6.71	6.69	6.65	6.58	6.57
pH (20°C)	6.93	6.75	6.71	6.69	5.4	6.58	6.57
SI-calcite	2.8	5.8	5.3	5.8	5.4	6.3	6.2
SI-dolomite	3.2	31	26	34	29	35	34

Mineralization of organic matter. To ensure the potential degradability of the organic matter in peat and sand, the respective aquifer material was first incubated aerobically. In general, natural organic material is less recalcitrant

in presence of oxygen than in its absence (Eijsackers and Zehnder, 1990). A temperature range between 4 and 95°C was chosen, since these two temperatures represent the limits reached during ATES. It was assumed that biological processes will be the most important contributors to mineralization, showing two activity maxima: one between 25 and 40°C and the other between 55 and 65°C. In material from areas without geothermal activities (volcanoes, hotsprings, *etc.*) no biological contribution was expected above 65°C (Zeikus and Winfrey, 1976). Surprisingly, oxygen was consumed with increasing rates between the two temperature limits in peat, as well as in sand (Figs. 4 and 5).

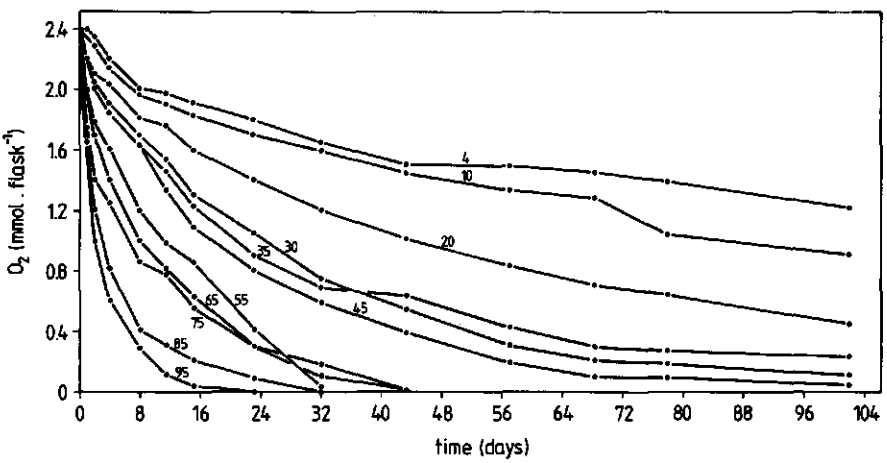

Fig. 4. Time course of oxygen consumption in the headspace (280 ml) of aerobic peat and groundwater incubations. This series of experiments was done as described for Fig. 1 in the temperature range from 4 to 95°C. The presented data are the average of two replicates.

The maximum rates of CO_2 production and O_2 consumption were for both aquifer materials equal over the entire temperature range but differed among the aquifer materials (Fig. 6A). Though the maximum rates of CO_2 release were lower anaerobically, these rates also increased steadily with increasing temperature (Fig. 6B). In Figs. 6A and 6B it is shown that the maximum mineralization rates in groundwater incubations with sand is higher per gram of organic material added than with peat. The ratio between aerobic and anaerobic CO_2 production was for both aquifer materials over the whole temperature range about 1.7.

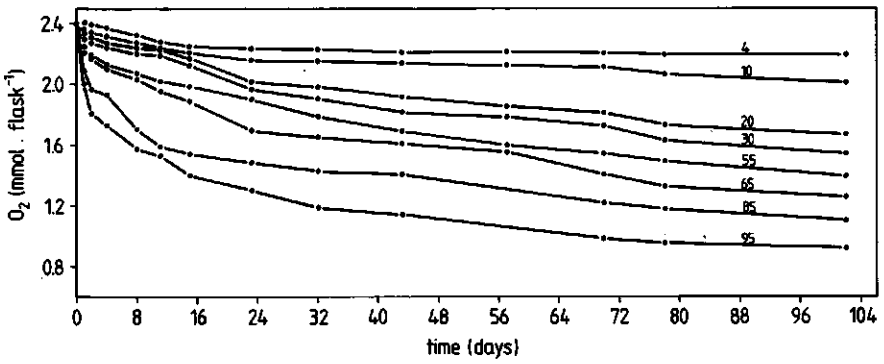

Fig. 5. Time course of oxygen consumption in the headspace (280 ml) of aerobic sand and groundwater incubations. This series of experiments was done as described for Fig. 1 in the temperature range from 4 to 95°C. The presented data are the average of two replicates.

In all peat incubations, aerobic as well as anaerobic, CO_2 production and O_2 consumption followed first order kinetics ($r^2 > 95\%$). For sand, this was only true between 20 and 45°C. Outside this range, the correlation coefficient was statistically not significant ($r^2 < 95\%$), therefore first order kinetics was not applicable. The activation energy calculated from the linearized Arrhenius equa-

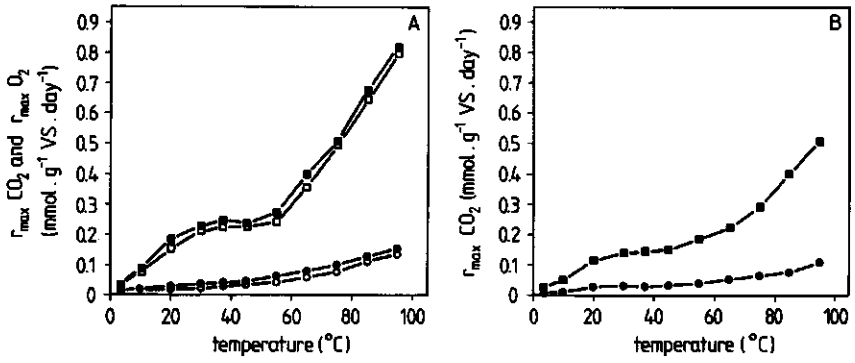

Fig. 6. Temperature dependent maximum rates of oxygen consumption and carbon dioxide production in the headspace (280 ml) of aerobic (**A**) and anaerobic (**B**) peat, sand and groundwater incubations. The series of experiments was done as described for Fig. 1. r_{max} Values were determined on basis of the highest rates observed in the first four days of two replicate experiments. Symbols: (□): O_2 sand, (■): CO_2 sand, (o): O_2 peat, (●): CO_2 peat.

tion varied between 360 and 860 $J.mol^{-1}$, which is about ten times lower than would be expected for relative stable organic material (Characklis and Gujer, 1979).

Table 4. Carbon dioxide production in sterilized aerobic and anaerobic groundwater plus sand incubations determined after 23 days of 30, 55, 75 and 95°C. All values are in mmol CO_2 per gram volatile solids and are the mean of two batch replicates. Sterilization was done by heating at 120°C for 20 minutes. During sterilization 0.12 and 0.08 mM CO_2 were released under aerobic and anaerobic conditions, respectively.

	Aerobic incubation (°C)				Anaerobic incubation (°C)			
	30	55	75	95	30	55	75	95
Control	1.3	1.7	2.4	3.2	0.89	1.0	1.4	1.9
Sterile	0.16	1.7	2.5	3.7	0.11	1.2	1.5	2.3

The mineralization of organic matter above 55°C began without a lag period (Figs. 4 and 5), which suggested that chemical reactions prevailed in these incubations. A series of experiments with previously sterilized aerobic and anaerobic sand incubations demonstrated that at temperatures above 55°C the mineralization processes are purely chemically (Table 4). This shows that some chemical mineralization also occurs at 30°C, although most carbon dioxide produced originates from biological activities at this temperature.

Influence of alternative electron acceptors. In anaerobic sand and peat incubations, sulphate reduction and methanogenesis was not observed. The potential ability of peat and sand aquifer material to utilize alternative electron acceptors was tested in a series of anaerobic experiments at 30 and 55°C. Denitrification could be measured at both temperatures only after addition of nitrate. Within 32 days 55% and 40% of 1 mM nitrate was converted at 30° and 55°C, respectively. Sulphate reduction took place at both temperatures exclusively after sulphate and lactate was added. Methane was produced stoichiometrically from 10 mM acetate. In the absence of added acetate or with only 1 mM acetate, no methane was released from the incubation mixture. These findings indicate that sulphate reduction and methanogenesis should not

cause problems at this ATES site. However, because samples have been taken from one depth and one place only, general conclusions on possible impacts from sulphate reduction and methanogenesis cannot be drawn.

3.4 DISCUSSION

The data on saturation indices and acidity given in Table 3 are in accordance with carbonate chemistry for closed systems (Stumm and Morgan, 1981). Firstly, the increase in saturation indices with respect to calcite and dolomite results from a temperature increase from 10 to 75°C. The saturation indices then increase slightly more because of the increased carbon dioxide pressure resulting from the mineralization of organic matter. Acidity can be produced from organic matter in two ways, namely by thermally mobilized fulvic acids and carbon dioxide production. Although fulvic acids behave like weak-acid polyelectrolytes that contain carboxilic acid groups (Ephraim *et al.*, 1989), the acidity produced by these compounds is negligible as compared to carbon dioxide production.
In $CaCO_3$ super-saturated solutions containing sediment, phosphate precipitation occurs *via* amorphous Ca-HPO_4-hydrate which is then transformed to crystalline apatite (Stumm and Leckie, 1971; Griffin and Jurrinak, 1973). Since super-saturated solutions with respect to apatite may remain stable under natural conditions for a long time (Fixen *et al.*, 1983), the amount of carbonate precipitation given in Table 3 was calculated for a calcium-phosphate ratio of 1 to 1 and 3 to 2. Precipitation of magnesium phosphate is unlikely, because Mg-phosphates are more soluble than Ca-phosphates (Lindsay, 1979).

Super-saturation with respect to calcite at 10°C (Table 3) might be caused by a combination of factors, such as increase in solubility of calcite with increasing magnesium concentration (Berner *et al.*, 1979; Reddy and Wang, 1980), inhibition of precipitation by ortho phosphate (Yadav *et al.*, 1984; Mucci, 1986) and inhibition by organic acids such as fulvic and humic acids (Amrhein and Suarez, 1987). These organic compounds have been shown to adsorb onto potential calcite seeds, thereby rendering the surfaces inactive as sites for crystal growth (Inskeep and Silvertooth, 1988). The effectiveness of surface coverage by fulvic acids of crystal growth sites would explain why groundwater with a low dissolved organic carbon concentration (5 mM carbon) is super-saturated with respect to calcite at 10°C (Inskeep and Bloom, 1986A; Inskeep

and Bloom, 1986B). The presence of magnesium and phosphate and the action of fulvic acids as inhibitor of crystal growth can also explain super-saturation of calcite and dolomite after 23 days of incubation at 75°C. Moreover, above approximately 45°C the excess of fulvic acids may also complex calcium and magnesium (Fig. 2). It should be noted that the computer model that was used for the hydrogeochemical calculations does not account for the presence of any organic acids and complexing agents. Therefore, the computed activities of free calcium and magnesium will exceed those in our incubations. This results in an overestimation of the saturation indices for calcite and dolomite in the experiments presented here. Since the temperature dependency of the complexation process has not been determined no attempts were made to calculate saturation indices for the two minerals in presence of fulvic acids at 75°C.

Mineralization of organic matter to carbon dioxide occurred over a wide temperature range under both aerobic and anaerobic conditions (Fig. 6). At temperatures around 30°C oxygen consumption and carbon dioxide release was predominantly microbially mediated. At temperatures above 55°C chemical reactions prevailed. In the absence of oxygen, denitrification was possible but actually not occurring and no methanogenesis or sulphate reduction was observed, apparently because adequate electron donors lacked. Hence, anaerobic carbon dioxide production resulted from disproportioning of the solid organic material (Tables 1 and 2). Such a disproportioning (redox-reaction) could be facilitated by quinone-like structures present in humic substances. Electron transfer reactions can be catalyzed by these structures because of the tautomerization between keto- and enol-forms (Stevenson, 1982). Since carbon dioxide is not reduced and no other external electron acceptor is present in our anaerobic incubations, the disproportioning of the organic material should, after release of carbon dioxide, result in an increased COD as compared to aerobic experiments. An increase of COD has actually been measured (Fig. 1). The increase in groundwater DOC could result from the dissipation of hydrogen bonds (4-40 kJ/mol) that interlink constitutive units in humic substances (Ziechman, 1988). The relatively low activation energy for CO_2 production is conceivably also associated with the thermal disintegration of humic substances into smaller units because (i) the specific surface of the humic substances increases and (ii) easily degradable non-humic substances e.g. carbohydrates may become available. This hypothesis does not explain the mechanisms of CO_2 production, however. The reactivity of organic matter in terms of carbon

dioxide production at temperatures up to 95°C is as yet not clear and deserves further consideration. It also remains to future research to clearly differentiate between microbial and chemical mineralization reactions in ATES-processes below 55°C.

3.5 CONCLUSIONS

The current study shows that a complex interplay between carbon dioxide production from mineralization of organic material and mobilization of fulvic acids controls the precipitation of calcium and magnesium minerals during ATES-processes. The rate of carbon dioxide production augments with increasing temperature. This mineralization process can, depending on the temperature, either be purely chemically or biologically mediated. Without contamination from outside, interference of ATES by sulphide, iron and manganese precipitates is not very likely at this site. The process of carbon dioxide formation and the release of organic molecules has to be understood in more details before the response of an aquifer to ATES can be predicted merely based on the chemical analysis of the subsoil and its groundwater.

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CHAPTER 4

FERROUS IRON DEPENDENT NITRIC OXIDE PRODUCTION AND SUBSEQUENT INHIBITION OF SULPHATE REDUCTION

ABSTRACT

Seawater was anaerobically incubated, buffered with 50 mM Tris-HCl and supplemented with 40 mM sodium L-lactate, 15 mM NH_4Cl and mineral salts. In these cultures, lactate was rapidly oxidized to acetate by sulphate reducing bacteria after a lag period of three days. Acetate was mineralized only slowly. In presence of added nitrate or ferric iron (both at 1 mM) or a combination of both, the initial lactate consumption rate was slowed down for the first four days and sulphate reduction started only after 4 days, though with a similar rate as the incubations without added nitrate and ferric iron. Nitrate in combination with ferrous iron totally inhibited sulphate reduction. Some L-lactate was initially oxidized, but its concentration did not change after day 6 of incubation. In these incubations ferrous iron was oxidized chemically to ferric iron with a concomitant reduction of nitrite to nitric oxide (chemodenitrification). Nitrite was formed biologically from nitrate. The production of nitric oxide and its inhibition of sulphate reduction might, besides thermodynamic reasons, be responsible for the spatial separation of denitrification,iron reduction and sulphate reduction in anaerobic environments.

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4.1 INTRODUCTION

Iron reduction in soils was explained more than half a century ago (28) as an indirect microbial process in which microorganisms lowered the pH and oxygen concentration to an extent that favored the chemical reduction of ferric hydroxides. To date the direct reduction of iron by bacteria belonging to different genera has been documented (8), although the underlying biochemical mechanisms are not yet completely understood. In some bacteria iron reduction may be linked to the respiratory chain but until recently coupling to growth and energy production could not be shown unequivocally (8). With a novel bacterium designated GS-15 it was demonstrated for the first time that the oxidation of organic compounds can be effectively coupled to the reduction of ferric iron (18).

Thermodynamic predictions imply that in microbially mediated reactions inorganic electron acceptors are used according to their decreasing level of redox intensities (29). Accordingly, ferrous iron can be an appropriate electron donor for nitrate respiration and denitrification. It has been reported that ferrous iron is oxidized in nitrate respiring cultures of *Escherichia coli* E4 (3), during denitrification in anaerobic waterlogged soils (16) and in anaerobic lake sediments to which nitrate was added (7,11). To date no pure cultures of bacteria capable of nitrate reduction or denitrification using ferrous iron as an electron donor have been documented (8,19).
In anaerobic habitats denitrifying and nitrate reducing bacteria may also be involved in enzymatic iron reduction, indicating that both,iron and nitrate may be reduced simultaneously (14). Under equilibrium conditions in anaerobic aquifers, lake- and marine sediments (9,14,26) microbial iron reduction occurs as predicted by thermodynamics between denitrification and sulphate reduction zones, and ferrous iron is not found in the presence of nitrate (8,9).

In the current investigation the kinetics of iron and nitrate conversion in the presence of sulphate were studied. Interestingly, the spatial separation between nitrate, iron, and sulphate reduction observed in natural habitats could with our results also be explained by inhibition of sulphate reduction by nitric oxide.

4.2 MATERIALS AND METHODS

Media and growth conditions. The medium used was North Sea water, sampled from a shallow-water site at Westerschouwen, The Netherlands. The *in situ* pH of the seawater was maintained at pH 7.4 by the addition of 6.0 g/l Tris-HCl buffer. The following chemicals were added to Tris-HCl buffered seawater (in g/l): Na-L-lactate (4.4); NH_4Cl (0.82); K_2HPO_4 (0.25); $MgCl_2.6H_2O$ (0.10); 1 ml of a trace elements solution and 0.1 ml of a vitamin solution. The composition of the trace elements solution was (g/l): $CaCl_2.2H_2O$ (10), NaCl (10), $FeCl_3.6H_2O$ (1), HBO_3 (0.05), $CuSO_4.5H_2O$ (0.01), $CoCl_2.6H_2O$ (0.02), KI (0.01), $MnCl_2.4H_2O$ (0.04), $NaMoO_4$ (0.02), $ZnSO_4.7H_2O$ (0.04). The vitamin solution composition was (g/l): thiamine-HCl (0.02), riboflavin (0.01), nicotinic acid (0.50), 4-amino-benzoic acid (0.03) pyridoxal-HCl (0.10), Ca-pantothenate (0.20), biotin (0.01). Resazurin was added as a redox indicator to a final concentration of 0.1 mg/l (10).
The nutrient amended seawater samples (50 ml) were incubated on an orbital shaker at 30°C in anaerobic argon flushed 122 ml (total volume) vials sealed with a rubber stopper. Nitrate, ferrous iron and ferric iron were added in various combinations, each to a final concentration of 1 mM. For the study of nitric oxide toxicity 0.5 μmol nitric oxide, nitrous oxide or nitrite were added to each vial as well. Precipitation of iron did not occur because the buffer Tris-HCl (2-amino-2-hydroxymethylpropane-1,3-diol) kept it in solution.

Chemical and biochemical determinations. Sulphate was determined with liquid chromatography using a refraction index detector (Pharmacia LKB Biotechnology B.V., Woerden, NL) coupled to a 100 mm x 3 mm internal diameter Ionosphere-tm-A column (Chrompack B.V., Middelburg, NL). Injection volume was 20 μl, injection temperature 30°C, flow rate 0.6 ml/min 30 mM K,H-phthalate.
Sulphide was measured spectrophotometrically at 665 nm by a ferric iron mediated oxidation of leucomethyleneblue (25). The sulphide calibration curve was determined iodometrically with $Na_2S.7-9H_2O$. Sulphide containing samples were fixed in 30 mM zinc acetate and stored at -18°C until further analysis.
Samples for iron determinations were directly acidified to pH 1 with HCl to prevent autoxidation and to dissolve iron sulphides. Ferrous iron was

measured as a magenta complex. For this purpose [3-(4-Phenyl-2-pyridyl)-5-(phenyl sulfonic acid)-1,2,4-triazine sodium salt] was used as a chelating agent and the complex analyzed spectrophotometrically at 565 nm. This chelating agent is commercially available as Ferrospectral II. For ferric iron determinations, 5 mM ammonium thioglycollate had been added to the 1 mM Ferrospectral II solution in order to convert all iron into the ferrous form.

Headspace samples were injected into a gaschromatograph using a 250 μl syringe equipped with a mininert type syringe valve (Supelco Inc., Applikon B.V., Schiedam, NL) Henry's law constant (H) for the partitioning of nitric oxide, nitrous oxide, carbon dioxide, hydrogen and methane between seawater and headspace of vials was determined experimentally at room temperature (29). Nitric oxide, nitrous oxide, carbon dioxide, hydrogen and methane were measured gaschromatographically using a Packard Becker GC (Chrompack B.V., Middelburg, NL) equipped with a Poropak Q (60-80 mesh) column (600 cm x 0.3 cm internal diameter). Carrier gas was argon at a flow rate of 30 ml/min (60 psi). Thermal conductivity detector and oven temperature were both kept at 100°C.

Lactate and acetate were quantified by HPLC with a LKB model 2158 Uvicord SD (Pharmacia LKB Biotechnology B.V., Woerden, NL) equipped with a 30 cm x 3/8 inch internal diameter organic acids column (Chrompack B.V., Middelburg, NL) which was operated at 65°C and 80 bar and protected by a guard column (anion exchanger). The detection wavelength was 206 nm, the elution rate 0.8 ml/min of 10 mM H_2SO_4 and the injection volume 20 μl. Samples for organic acid analyses were fixed in 30 mM zinc sulphate and stored at -18°C.

Nitrate was quantified with alkaline salicylate and nitrite with acid sulfanilamide (1). The biochemical oxygen demand (BOD) of the seawater as it was sampled on site was measured at 20°C in Voith Sapromat type B12 automated respirometer (Proton Wilten Instruments B.V., Etten-Leur, NL). Thioureum (1 mM final concentration) was used to inhibit nitrification.

Chemicals. Sodium L-lactate was from J.T. Baker B.V., Deventer, NL. Growth media constituents, Ferrospectral II, ammonium thioglycollate, resazurin and reagents for sulphide determinations were from E. Merck Nederland B.V., Amsterdam, NL. Nitric oxide (chemically pure), nitrous oxide, carbon dioxide, hydrogen and methane were from Hoek Loos B.V., Schiedam, NL.

4.3 RESULTS

Lactate, sulphate, nitrate and iron conversion. The biochemical oxygen demand of the sampled seawater was 55 mgO_2/l. Nitrate could not be detected and sulphate was present at a concentration of 25 mM. Therefore, sulphate was anaerobically the major electron acceptor. Lactate added to the nutrient amended seawater was anaerobically converted to a large extent into acetate (Fig. 1). Lactate oxidation was paralleled by a decrease in sul-

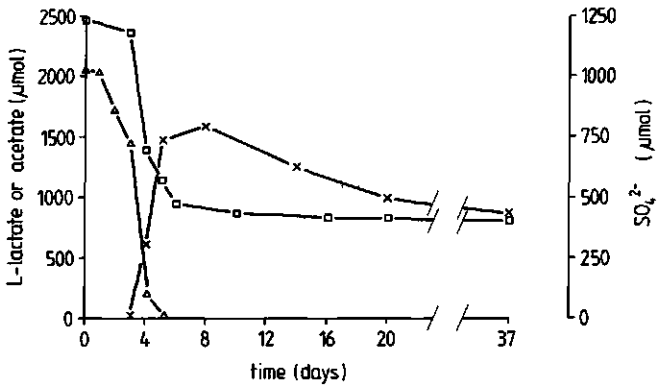

Fig. 1. Time course of the L-lactate(Δ), acetate (x) and sulphate (□) in anaerobic nutrient amended seawater samples. Absolute amounts are given for 50 ml medium. All data are the average of two independent experiments.

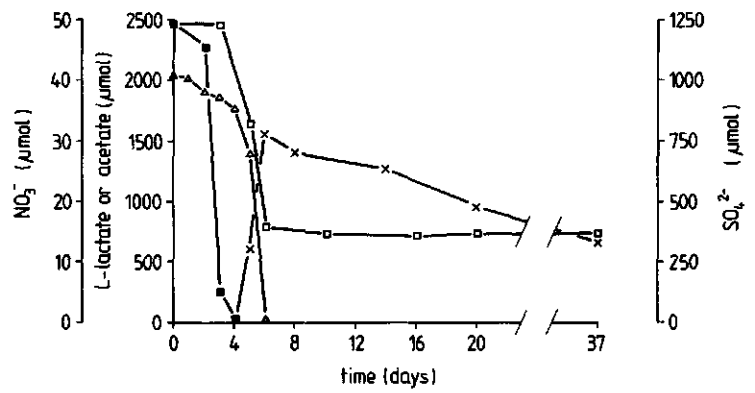

Fig. 2. Time course of L-lactate (Δ), acetate (x) and sulphate (□) in the presence of nitrate (■) in anaerobic nutrient amended seawater samples. Concentrations and statistics are as in Fig.1.

phate concentration. Phase-contrast microscopy showed a relatively large number of curved motile non-sporulating cells indicating the enrichment of *Desulfovibrio* spp. (34). After the depletion of lactate, sulphate reduction at the expense of acetate proceeded rather slowly. Methane formation (25 μmol) was detected only after 10 weeks. Incubations amended with 1 mM nitrate or 1 mM ferric iron resulted in a delay of sulphate reduction, sulphate was only reduced after reduction of the more potent electron acceptors nitrate and ferric iron (Figs. 2 and 3A). Except for conspicuous ferrous sulphide formation lactate oxidation and sulphate reduction in the cultures supplemented with 1 mM Fe^{2+} (Fig. 3B) were identical to the control experiment (Fig. 1).

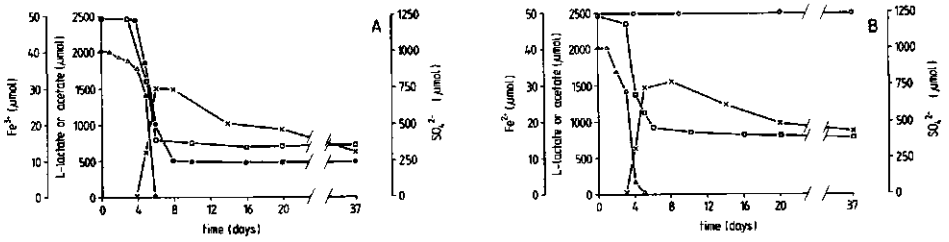

Fig. 3. Time course of L-lactate(Δ), acetate(x) and sulphate(□) in the presence of (**A**) ferric iron (●)or (**B**) ferrous iron (o) in anaerobic nutrient amended seawater samples. Concentrations and statistics are as in Fig.1.

Iron conversion in the presence of nitrate. The presence of both 1 mM nitrate and ferric iron did not significantly alter lactate oxidation, acetate production and decrease in sulphate concentration (Fig. 4). Such in contrast to 1 mM ferrous iron plus 1 mM nitrate (Fig. 5). Only small amounts of lactate were oxidized and no sulphate reduction could be observed. Nitrate reduction was slowed down and ferrous iron was oxidized to ferric iron. No reduction products of nitrate could be detected with the analytical methods used.

Nitric oxide. To obtain more information on the reduced nitrogen compounds, a series of experiments was conducted in which increasing amounts of nitrate and five times more ferrous iron were added to anoxic nutrient amended seawater (Table 1). Nitric oxide and nitrous oxide formation increased with nitrate. No sulphate reduction could be measured in the pre-

sence of nitrate even at its lowest concentration (1 mM). In autoclaved nutrient amended seawater samples nitrate and ferrous iron concentrations did not change. Despite the fact that these two compounds constitute a redox couple with a $\Delta G^{\circ'}$ of -109 kJ/mol (30) this spontaneous reaction is ap-

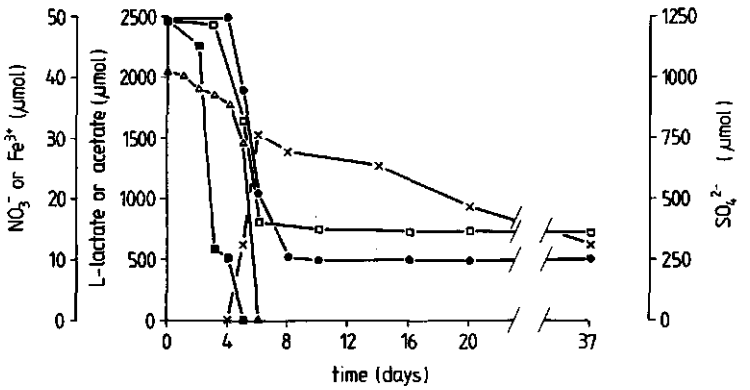

Fig. 4. Time course of L-lactate, acetate and sulphate in the presence of both ferric iron and nitrate in anaerobic nutrient amended seawater samples. Concentrations and statistics are as in Fig. 1. Symbols: (Δ) L-lactate, (□) sulphate, (x) acetate, (■) nitrate, (●) ferric iron.

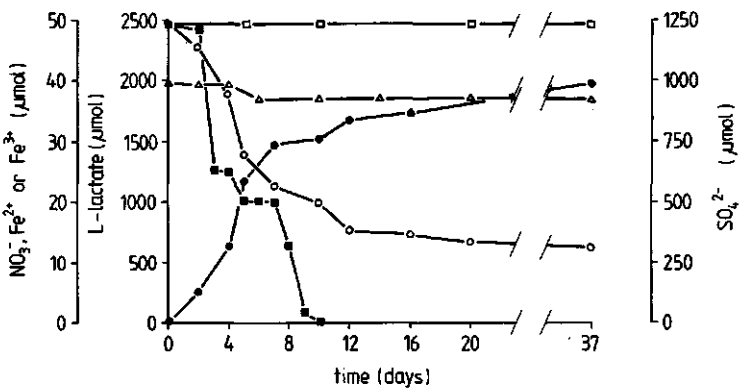

Fig. 5. Time course of L-lactate and sulphate in the presence of both ferrous iron and nitrate in nutrient amended seawater samples. Concentrations and statistics as in Fig. 1. Symbols: (Δ) L-lactate, (□) sulphate, (o) ferrous iron, (●) ferric iron formed from ferrous iron, (■) nitrate. Acetate was not formed in these incubations.

parently prevented by slow kinetics. It was observed that ferric iron was formed spontaneously during autoclaving 5 mM ferrous iron and 40 mM nitrate in the presence of small amounts of copper (5 mgCu^{2+}/l) which probably acted as a catalyst (not shown).

Table 1. Nitrate dependent nitric oxide, nitrous oxide, ferrous-, ferric iron and L-lactate concentrations after 6 days in anoxic nutrient amended seawater samples. L-lactate was 40 mM and ferrous iron was 5 mM. All values are given in μmol added or produced in 50 ml reaction mixture and are the means of duplicates.

NO_3^--added	NO[a)]	N_2O[a)]	Fe^{2+}	Fe^{3+}	L-lactate
0	-[b)]	-	250	-	-
50	-	-	240	6	1700
100	2	-	200	41	1750
200	7	2	175	70	1700
400	32	2	125	130	1550
800	60	3	47	205	1400
1600	75	4	21	220	1500

[a)] The minimum amounts of NO and N_2O detectable in a 100 μl sample with the methods used were 10 nmoles and 18 nmoles, respectively. [b)] not detected.

The presence of L-lactate was necessary for the anaerobic ferrous iron oxidation and nitric oxide formation (Table 2). Nitrite was never found in the experiments presented in Tables 1 and 2. Nitrite as intermediate could not be excluded, however, since the concentration of the various compounds were measured after six days only. Nitrite is reduced spontaneously in a two step process with ferrous iron as a reductant, first to nitric oxide and subsequently to nitrous oxide in a process called chemodenitrification (31). To check whether in our incubation such a reaction occurred 40 mM nitrite was incubated anoxically with and without 5 mM ferrous iron in sterile buffers at different pH. Both nitric oxide and nitrous oxide were found to be produced between pH 4 and 9 (Figs. 6A and 6B). The maximum rates of nitric oxide and nitrous oxide formation were found below pH 5.

Table 2. Iron and L-lactate dependent nitric oxide, nitrous oxide, ferrous iron and ferric iron concentrations measured after six days in anaerobic nutrient amended seawater samples. L-lactate was 4 mM and 40 mM, nitrate was 40 mM, both ferrous iron and ferric iron were 5 mM. All values are given in μmol added or produced in 50 ml reaction mixture and are the means of duplicates.

	NO_3^-		NO_3^- and Fe^{2+}		NO_3^- and Fe^{3+}	
	L-lactate		L-lactate		L-lactate	
	200	2000	200	2000	200	2000
Nitrate	1250	-	1500	1100	1300	-
Nitric oxide	-	-	4	75	-	-
Nitrous oxide	-	-	-	8	-	-
Ferrous iron	-	-	200	15	-	190
Ferric iron	-	-	4	225	240	30

(-): not detected

Without ferrous iron self-decomposition of nitrite resulting in nitric oxide formation was only found at pH 4 (33; not shown). Under such conditions self-decomposition of nitrite did not result in nitrous oxide formation. Nitric

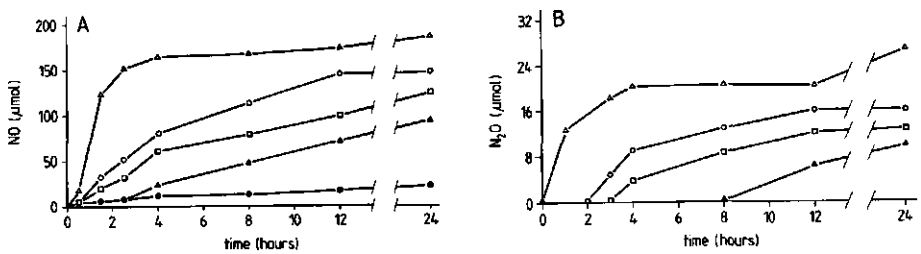

Fig. 6. Time course of nitric oxide (**A**) and nitrous oxide (**B**) production at different pH-values. Nitric- and nitrous oxide were measured in the headspace (72 ml argon) of anoxic vials containing 50 ml buffer to which 2000 μmol NO_2^- and 250 μmol Fe^{2+} had been added. Sodium citrate-HCl (pH 4 and 5), sodium acetate-HCl (pH 6) and Tris-HCl (pH 7,8 and 9) were used as buffers at a concentration of 50 mM. Symbols are: (Δ) pH 4, (o) pH 5, (□) pH 6, (▲) pH 7, (●) pH 8. All data are the mean of duplicates.

oxide and nitrous oxide were not found after 24 hours at pH 9 and 8, respectively. The addition of 0.5 μmol nitric oxide to freshly prepared nutrient amended seawater completely inhibited sulphate reduction for at least two months. The same amount of nitrite or nitrous oxide did not affect the start of sulphate reduction.

4.4 DISCUSSION

Sulphate reduction was readily observed after the addition of lactate, indicating that viable sulphate reducers were still present in originally oxic seawater made anoxic. The survival of oxic conditions by sulphate reducing bacteria was reported in detritus particles suspended in oxic seawater (15) and in sulphate free systems containing oxygen-sulphide gradients (5). As could be predicted thermodynamically (29), only a transient delay in the onset of sulphate reduction was observed in nutrient amended seawater samples where 1 mM nitrate was added solely or combined with 1 mM ferric iron. Such in contrast to the combination of 1 mM nitrate plus 1 mM ferrous iron. Lactate dissimilation stopped after 6 days and sulphate reduction was totally inhibited. Interestingly, ferrous iron was slowly oxidized in this incubation (Fig. 5), but evidence for nitric oxide formation could not be obtained because of limitations of the analytical methods used in this study. A buildup of nitric oxide could be readily detected in incubations with nitrate and ferrous iron concentrations higher than 1mM (Tables 1 and 2). In these incubations nitrite had first to be formed biologically from nitrate with electrons from lactate. Subsequently, in a chemodenitrification process ferrous iron was oxidized chemically to ferric iron with a concomitant reduction of nitrite to nitric oxide and nitrous oxide. Chemodenitrification is generally referred to as a process in which nitrate and nitrite are reduced to gaseous nitrogen oxides with an abiotic agent (31). The ferrous iron mediated reduction of nitrate is thermodynamically feasible (30) but was not observed to occur in this study during the experimental time frame. The chemodenitrification rate of nitrate is probably determined by slow kinetics at low temperatures (-30°C).

In chemodenitrification processes occurring in acidic soils (pH < 5) nitrite and ferrous iron can be regarded as key compounds (33). Likewise, our results shown in Fig. 6 demonstrate that under acidic conditions the

redox reaction between ferrous iron and nitrite is considerably accelerated. Such an acceleration is in line with thermodynamic considerations predicting an increasingly negative ΔG with decreasing pH (below pH 7) for the reduction of nitrite to nitric oxide with ferrous iron. Nitric oxide is the predominant gaseous nitrogen oxide found during chemodenitrification, although minute amounts of nitrous oxide have also been reported (22). In fact, soil borne nitric oxide can be considered as a preliminary indication of chemodenitrification (31).

The formation of nitric oxide and nitrous oxide in soils has been attributed to nitrifiers (20), denitrifiers (6), nitrate respirers (2) as well as chemodenitrification (13). The precise role of ferrous iron in the chemistry of these gaseous nitrogen compounds is often not accounted for. In the literature (8) direct evidence for chemodenitrification in soils and sediments, including information on both iron and nitrate transformations is not conclusive. For instance, anaerobic oxidation of ferrous iron during denitrification in waterlogged soils has been reported (16), but oxidation of ferrous iron has not been quantified. Evidence for chemodenitrification was provided only on the basis of a nitric oxide yielding *in vitro* experiment with nitrite and ferrous iron (16). In another study, inhibition of ferric iron reduction by nitrate with a denitrifying *Pseudomonas* species was reported (24). This inhibition was attributed to a chemical reaction between nitrite and ferrous iron although only iron conversions were measured.

In addition to chemodenitrification the influence of nitrate and nitrite on iron reduction and sulphate reduction in estuarine enrichment cultures has also been attributed to high redox potentials (32). For instance, an accumulation of nitric oxide in denitrifying coastal marine sediment formed a zone with an increased redox potential between the oxidized surface layers and sulphate reduction in the deeper sediment layers (27). Analogously, an inhibitory effect of nitrate on sulphate reduction was ascribed to an increased redox potential resulting from a buildup of nitrous oxide in a sewage sludge digestor (12). In the present study it was found that contrary to the addition of 0.5 μmol nitric oxide the addition of either 0,5 μmol nitrite or nitrous oxide did not prevent the onset of sulphate reduction. This finding strongly indicates that the inhibitory effect of nitric oxide on the onset of sulphate reduction is not caused by a high redox potential. Therefore, another mechanism must be reponsible for the

nitric oxide mediated inhibition of sulphate reduction. Nitric oxide is known to be a bacteriostatic agent and its toxicity is caused by direct binding to heme iron (4,17,21,23). In cultures of *E.coli* E4 which simultaneously reduce ferric iron and nitrate ferric iron reduction is not energetically coupled to growth and resulted in a nitric oxide mediated inhibition of L-lactate oxidation (3). In the presence of nitrate and ferric iron, the cultures of *E.coli* E4 reacted different from what was observed in the present mixed culture study. L-lactate-driven nitrate respiration was inhibited by 50% in the presence of ferric iron but only by 20% in the presence of ferrous iron (3). With ferrous iron most nitric oxide was bound in [Fe^{2+}-NO] complex and as a consequence could not bind to other for *E.coli* E4 essential compounds. In the current investigation ferro-nitrosyl complexes were not determined and their role in the system studied could therefore not be quantified. It remains to future investigations to establish whether a specific hydrogenase, bisulphite reductase, or other enzymes typical for sulphate reducing bacteria bind nitric oxide stronger than free ferrous iron. A stronger binding could explain the differences observed between the sulphate reducing mixed culture and cultures of *E.coli* E4.

In conclusion it can be stated that thermodynamic considerations alone are not necessarily sufficient to explain the sequence of microbially mediated redox processes in anaerobic habitats. Therefore, besides the thermodynamic mechanism the inhibitory effect of nitric oxide may also be responsible for the spatial separation of denitrification, iron reduction and sulphate reduction in anaerobic aquifers and sediments.

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CHAPTER 5

FERROUS IRON DEPENDENT NITRIC OXIDE PRODUCTION IN NITRATE REDUCING CULTURES OF *ESCHERICHIA COLI*

ABSTRACT

L-lactate-driven ferric iron and nitrate reduction was studied in *Escherichia coli* E4. Ferric iron reduction activity in *E.coli* E4 was found to be constitutive. Contrary to nitrate, ferric iron could not be used as electron acceptor for growth. "Ferric iron reductase" activity of 9 nmol $Fe^{2+}.mg^{-1}protein.min^{-1}$ could not be inhibited by inhibitors for the respiratory chain, like rotenone, quinacrine, antimycin A, or potassium cyanide. Active cells and L-lactate were required for ferric iron reduction. The L-lactate-driven nitrate respiration in *E. coli* E4 leading to the production of nitrite, was reduced to about 20% of its maximum activity with 5 mM ferric iron, or to about 50% in presence of 5 mM ferrous iron. The inhibition was caused by nitric oxide formed by a purely chemical reduction of nitrite by ferrous iron. Nitric oxide was further chemically reduced by ferrous iron to nitrous oxide. With electron paramagnetic resonance spectroscopy, the presence of a free [Fe^{2+}-NO] complex was shown. In presence of ferrous or ferric iron and L-lactate, nitrate was anaerobically converted to nitric oxide and nitrous oxide by the combined action of *E. coli* E4 and chemical reduction reactions (chemodenitrification).

Brons H.J., Hagen W.R. and Zehnder A.J.B. (1991) Ferrous iron dependent nitric oxide production in nitrate reducing cultures of *Escherichia coli*. Archives of Microbiology 155: 341-347.

5.1 INTRODUCTION

Many bacteria have the capacity to reduce iron under conditions in which it would not be spontaneously reduced. Several enzymatic mechanisms can account for bacterial ferric iron reduction. Enzymatic iron reduction may serve either as an electron sink for excess reductant (Jones *et al.*, 1984) or it may act as a terminal acceptor in an electron transfer chain (Short and Blakemore, 1986). The iron reductase systems in *Spirillum itersonii* (Daily and Lascelles, 1977), *Staphylococcus aureus* (Lascelles and Burke, 1978) and *Pseudomonas aeruginosa* (Cox, 1980) were studied in cell free extracts and the effects of respiratory inhibitors suggested that reduced components of the electron transfer chain that precede cytochrome b or c serve as a reductant for ferric iron. An induction of two ferri reductase systems was observed in *Pseudomonas* spp. strain 200 (Arnold *et al.*, 1986). At high oxygen concentrations iron reduction occurred *via* an abbreviated electron transfer chain. Low oxygen tension (< 0.01 atm) gave rise to the induction of alternative respiratory pathways, and iron reduction was accelerated six- to eight-fold, although the increased activity apparently was uncoupled from oxidative phosphorylation.

Iron reduction can be inhibited by oxygen and nitrate. This inhibition can either be predicted based on thermodynamic relationship of the chemical reactions involved (Zehnder and Stumm, 1988), or be the result of a direct action of nitrate on the process of ferric iron reduction. Ottow (29) proposed that in facultative anaerobic bacteria nitrate reductase can transfer electrons to either ferric iron or nitrate. Lascelles and Burke (21) found ferric iron to be reduced independently of nitrate at a level in the electron transfer chain preceding cytochrome b and nitrite inhibited iron reduction. Based on studies with *Pseudomonas* spp. Obuekwe *et al.* (28) suggested that the inhibitory effect of nitrate was due to ferrous iron oxidation by nitrite. Direct evidence for the oxidation of ferrous iron was obtained by Williams and Poole (34) with the addition of nitrite to ferric iron reducing cultures of *Escherichia coli* K12. The chemical oxidation of ferrous iron to ferric iron with nitrite results in nitric oxide formation. Such a reaction has actually been reported for an *in vitro* system by Komatsu *et al.* (19). For *in vivo* systems this reaction is poorly understood and is not yet well quantified (Ghiorse, 1988).

In the following the influence of nitrate on the L-lactate-driven ferric iron reduction in *E.coli* E4 was investigated. Considerable amounts of nitric oxide were formed during the concurrent reduction of nitrate and ferric iron. Nitric oxide was found to be inhibitory for lactate oxidation and concomitant nitrate reduction in *E.coli* E4.

5.2 MATERIALS AND METHODS

Organism. *Escherichia coli* E4 (NCTC 9002) used in this study was obtained from the culture collection of the Department of Microbiology, Wageningen Agricultural University, Wageningen, The Netherlands. The organism was maintained on yeast extract-glucose agar slants, containing (g/l): yeast extract (2.5), glucose (5) and agar (12). The agar slants were stored at 4°C and subcultured every 2 months.

Cultivation. *Escherichia coli* E4 was cultivated in demineralized water (pH = 7.2) containing (g/l): Na-L-lactate (5.6), NH_4Cl (0.82), $MgSO_4.7H_2O$ (0.1), K_2HPO_4 (3) and KH_2PO_4 (2). To 1 liter of this culture medium 1 ml of a trace element solution was added as was described previously (Brons and Zehnder, 1990). The pH was set at 7.2 with HCl.
Escherichia coli E4 was grown at 30°C in continuous culture using a 1 liter working volume chemostat (Applikon B.V., Schiedam, NL) which was sterilized and recultivated every 30-35 volume changes to prevent the selection of spontaneous mutants. The growth rate of *E.coli* E4 was fixed at 0.10 h^{-1}. The pH was monitored with a steam sterilizable glass electrode (Ingold, Van Oortmessen B.V., Den Haag, NL) connected to a pH meter and titrator (Radiometer, Instrumenthandel Zuid-Holland B.V., Den Haag, NL). The pH was maintained at 7.2 by the automatic addition of sterile 3M KOH. Sterile air was passed through the medium at a rate of 1 liter per liter working volume per minute. The impeller speed was set at 600 r.p.m.

Batch experiments. Steady state cultures of *E.coli* E4 were harvested and centrifuged in portions of 50 ml at 10.000 x *g* for 10 minutes. Pellets were washed in a 50 mM Tris-HCl buffer (pH = 7.2) centrifuged again, resuspended in 50 ml of the same buffer and supplemented with 1 ml/l each of a trace element solution. Batch experiments with 50 ml resuspended whole cells of *E.coli* E4 (12 mg protein) were carried out in anaerobic argon flushed vials

(headspace 72 ml) sealed with a rubber stopper, on an orbital incubator in a temperature controlled room at 30 ± 1°C.
Experiments for chemodenitrification were done anaerobically under argon in the following buffers (50 ml, 50 mM): sodium citrate-HCl (pH 4 and 5), sodium acetate-HCl (pH 6) and Tris-HCl (pH 7 and 8). If necessary, KNO_3, KNO_2, $FeCl_3$, $FeCl_2$ or Na-L-lactate were added as well.

Preparation of cell free extracts. Steady state cultures of *E.coli* E4 were harvested and centrifuged for 10 minutes at 10.000 x *g*. Pellets were washed in a 50 mM Tris-HCl buffer (pH = 7.2; 4°C), centrifuged again and resuspended in 2 ml of the same buffer. Ultrasonic disruption of the cells was achieved by treatment with a Branson model B12 sonifier (Marius Instruments B.V., Utrecht, NL) applying 36 W for 3 minutes at intervals of 1 minute while cooling one ice. The crude extracts were centrifuged at 10.000 x *g* for 15 minutes at 4°C to remove the cell debris. The supernatant was used as a cell free extract and was stored on ice until further use.

Oxygen respiration. Oxygen respiration was monitored at 30°C in the thermostatically controlled reaction vessel of a Yellow Springs Instruments model 53 biological oxygen monitor equipped with a polarographic oxygen probe (Tamson B.V., Zoetermeer, NL). The oxygen uptake rate of 0.3 ml (0.24-0.36 mg protein) cell free extract was determined in 3 ml final volume of an air saturated Tris-HCl buffer (pH = 7.2; 50 mM). The endogenous oxygen uptake rate was subtracted from the respiration rate observed in the presence of 10 mM L-lactate.

Electron paramagnetic resonance spectroscopy. Electron paramagnetic resonance spectroscopy of whole cell incubations was done with a Bruker model ER 200 D EPR spectrometer (Bruker Spectrospin N.V., Wormer, NL). The microwave frequency was measured with a Systron Donner model 1292 A frequency counter (Intechmij B.V., Diemen, NL). The magnetic field was modulated with a frequency of 100 kHz and an amplitude 8 Gauss. The microwave power was 5 mW. Cooling was done with a helium-flow cryostat (Lundin and Aasa, 1972). The detection temperature was 20 K.
High-spin (S=5/2) ferric iron was quantified by comparing the amplitude at g = 4.3 with that of a standard solution of 5 mM Fe^{3+} in 50 mM Tris-HCl, pH 7.2. Signals from ferrous-NO complex (S=3/2) were quantified by comparing

amplitudes with that from a standard solution of 5 mM Fe^{2+} in 50 mM Tris-HCl, pH 7.2, reacted with NO(g). The spin concentration of the iron nitrosyl in the latter standard solution was determined by double integration with respect to the spectrum from 5 mM Fe^{3+}, with correction for different Boltzmann distributions and transition probabilities (Aasa and Vänngård, 1975).

Chemical and biochemical determinations. Whole cells were removed by centrifugation at 10.000 x *g* for 5 min. Nitrate was measured with alkaline salicilate, nitrite with acid sulfanilamide and ammonium after direct Nesslerization according to Deutsche Einheitsverfahren (11). Autoxidation of iron was prevented by acidification to pH 2 with HCl prior to centrifugation. Ferrous iron was measured as a magenta Fe^{2+}-complex. For this purpose [3-(4-Phenyl-2-pyridyl)-5-(phenyl sulfonic acid)-1,2,4-triazine sodium salt] was used a chelating agent and the complex analyzed spectrophotometrically at 565 nm. This compound is commercially available as Ferrospectral II. For ferric iron determinations, 5 mM ammonium thioglycollate had been added to the 1 mM Ferrospectral II solution in order to convert all iron into the ferrous form.

Ferric iron reduction activity was assayed anaerobically at 30°C in a Beckman model 25 recording spectrophotometer (Beckman Instruments Nederland B.V., Mijdrecht, NL). The assay was carried out in a 1 cm argon flushed cuvette sealed with a rubber stopper. The reaction mixture, final volume 1 ml in Tris-HCl (50 mM; pH = 7.2), comprised 10 mM Na-L-lactate, 1 mM $FeCl_3.H_2O$, and 0,1 mM Ferrospectral II. The reaction was started by the addition of 100 μl (80-120 μg protein) cell free extract.

Nitric oxide, nitrous oxide, and carbon dioxide were measured gaschromatographically using a Packard Becker model 417 GC (Chrompack B.V., Middelburg, NL) equipped with a Poropak Q (60-80 mesh) column (600 cm x 0.3 cm internal diameter). Carrier gas was argon at a flow rate of 30 ml/min (60 psi). Thermal conductivity detector and oven were kept at 100°C.

Organic acids were analyzed by HPLC with a LKB model 2158 Uvicord SD detector (Pharmacia LKB Biotechnology B.V., Woerden, NL) equipped with a 30 cm x 3/8 inch internal diameter organic acids column (Chrompack B.V., Middelburg, NL) which was operated at 65°C and 80 bar and protected by a guard column (anion exchanger). The detection wavelength was 206 nm, elution rate of 10 mM H_2SO_4 0.8 ml/min, and injection volume 20 μl. Pyruva-

te was quantified as described by Katsuki *et al.* (17).
Volatile alcohols were determined on a Packard Becker model 417 GC (Chrompack B.V., Middelburg, NL) using a Sil5CB column (10 m x 0.53 mm internal diameter). Flowrate 10 ml N_2/min, oven 60°C, flame ionization detector (FID) 150°C, injection port 140°C.
Volatile fatty acids were analyzed on a Varian model 2400 GC (Varian Benelux B.V., Amsterdam, NL) equipped with a Chromosorb 101 glass column (200 cm x 1/8 inch internal diameter). Flowrate of formic acid saturated N_2 was 30 ml/min. Oven 190°C, FID-detector 240°C, injection port 170°C.
For protein determinations of cell free extracts and whole cells a modified Lowry method (DeMoss and Bard, 1957) with bovine serum albumin as a standard was used. Whole cells were boiled 10 min in 0.5 M NaOH previous to protein determination.
Glycogen was quantified after extraction and acid hydrolysis as glucose by the anthrone reaction (Hanson and Phillips, 1981).

Chemicals. Sodium L-lactate was from J.T. Baker B.V., Deventer, NL. Growth media constituents, ammonium thioglycollate and Ferrospectral II were from E. Merck Nederland B.V., Amsterdam, NL. Yeast extract and agar used in slants were from Difco (Brunschwig Chemie B.V., Amsterdam, NL). Rotenone, antimycin A, KCN and bovine serum albumin were obtained from Sigma (Brunschwig Chemie B.V., Amsterdam, NL). Quinacrine was purchased from Aldrich Chemie B.V., Brussels, Belgium. Nitric oxide (chemically pure) was from Matheson Gas Products (Hoek Loos B.V., Schiedam, NL).

5.3 RESULTS

Nitrate reduction. *Escherichia coli* cells grown aerobically at a dilution rate of 0.1 h^{-1} show nitrate and nitrite reductase activity (Brons and Zehnder, 1990). Cells washed in Tris-buffer started to reduce nitrate to nitrite by oxidizing L-lactate after a lag of 90 min (Fig. 1). Only minor amounts of nitrite were further reduced to ammonium (50 μmol within 24 h, not shown), despite the fact that nitrite reductase was present in cell free extracts at an activity of 80 μmol NH_4^+.mg^{-1}protein.min^{-1}. Most of the lactate was converted to acetate (1150 μmol) and intracellular glycogen (215 μmol as glucose). In the absence of nitrate or nitrite some lactate (150 μmol) was converted to pyruvate (60 μmol), acetate (30 μmol), carbon

dioxide (30 μmol), and intracellular glycogen (20 μmol) after 24 h. The protein content increased from 12 mg to 15 mg in presence of nitrate and from 12 mg to 12.5 mg in its absence within 24 hours. This slight increase is due to the lack of essential nutrients such as sulphur and phosphorous in the first case and in addition nitrogen in the second case.

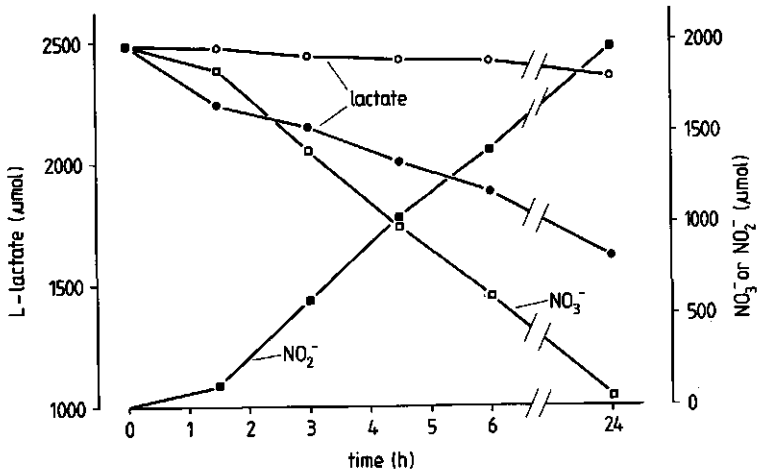

Fig. 1. L-lactate utilization by anaerobic non-growing batch cultures of *Escherichia coli* E4 in presence (•) and absence (○) of nitrate. Nitrate consumption (□) and concomitant nitrite (▪) production are also given. Absolute amounts are reported for 50 ml medium. All data are means of two independent experiments.

Iron reduction. In presence of 50 mM Tris-buffer both 5mM Fe^{3+} and Fe^{2+} remained in solution at pH 7.2. L-lactate could not be used by *E.coli* E4 to a significant extent as electron donor for the reduction of Fe^{3+}. Within 24 h only 40% of Fe^{3+} was reduced to Fe^{2+} (Fig. 2) and the decrease in L-lactate was the same whether Fe^{3+}, Fe^{2+} or no iron at all was added to the incubation mixture (Figs. 1 and 2). Higher Fe^{3+} concentrations (10 and 20 mM) did not increase CO_2 or acetate production from lactate (not shown). After 24 h of incubation, the HCl extractable iron of the biomass (15 mg protein) increased from 14.4 μmol to 62.2 μmol. This cellular iron accumulation may explain the drop of Fe^{2+} from 250 μmol to 200 μmol in the experiments shown in Figure 2.

To obtain indications where a possible ferri reductase is located in the respiratory chain, experiments were done with some specific inhibitors. These inhibitors acted on NADH-dehydrogenase (1 mM rotenone; Boogerd *et al.*, 1980), flavinedehydrogenase (1 mM quinacrine; Wolfe, 1975), cytochrome b (10 μg/ml antimycin A; Boogerd *et al.*, 1980), and terminal cytochrome oxidase (1 mM potassium cyanide). None of these inhibitors had any effect on the rate of Fe^{3+} reduction in cell free extracts. This rate was actually linearly proportional to the amount of extract present. The constant specific rate was 9 nmol $Fe^{2+}.mg^{-1}$protein.min^{-1}. However, oxygen respiration was clearly reduced to various degrees by these chemicals in cell free extracts. Rotenone inhibited oxygen respiration by 13%, quinacrine by 40%, antimycin A by 52%, and potassium cyanide by 84%.

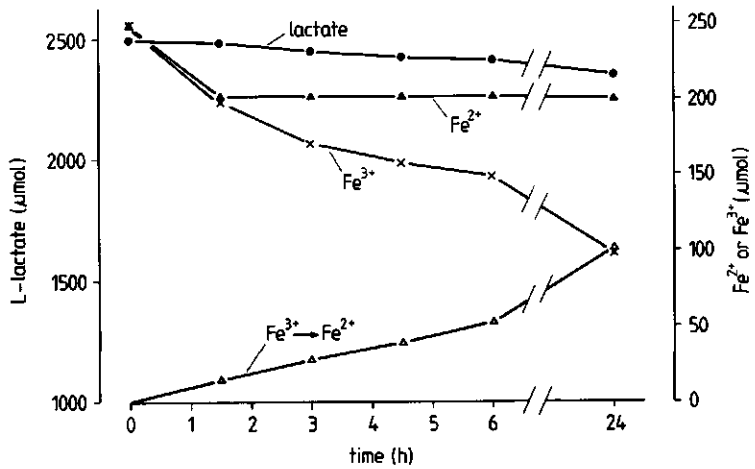

Fig. 2. L-lactate consumption (•) by anaerobic non-growing batch cultures of *Escherichia coli* E4 after addition of either $FeCl_2$. "Uptake" of Fe^{2+} (▲) and conversion of Fe^{3+} (X) to Fe^{2+} (Δ) are also given. Concentrations and statistics as in Fig. 1.

Iron reduction in presence of nitrate. Fe^{3+} seems to inhibit L-lactate oxidation with nitrate as electron acceptor. But also the rate of Fe^{3+} reduction is diminished in presence of nitrate (Fig. 3A). Fe^{2+} inhibits nitrate reduction with L-lactate but to a lesser extent than Fe^{3+} (Fig. 3B). Interestingly, in both cases with Fe^{3+} and Fe^{2+} more nitrate is reduced than nitrite formed, namely 420 μmol versus 350 μmol with Fe^{3+} and 850 μmol versus 700 μmol with Fe^{2+} after 6 hours of incubation (Figs. 3A and 3B).

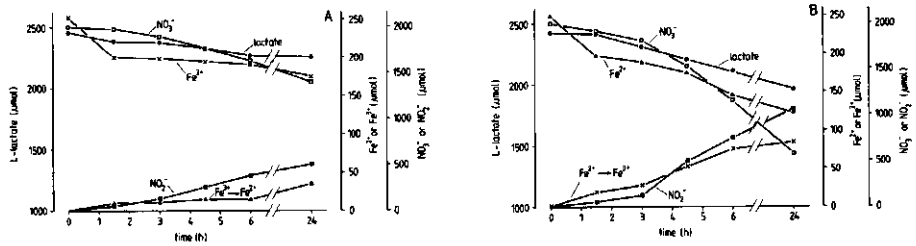

Fig. 3. (A) L-lactate consumption (•), nitrate conversion (□) to nitrite (▪) and Fe^{3+} reduction (X) to Fe^{2+} (Δ) by anaerobic non-growing batch cultures of *Escherichia coli* E4 after addition of $FeCl_2$. (B) Same as in (A) but after addition of $FeCl_3$. Concentrations and statistics as in Fig. 1.

This loss of nitrogen could be explained in three ways: (i) nitrate was assimilated for growth, (ii) nitrite was converted to ammonium, or (iii) nitrite was transformed to something else than ammonium. Almost no growth has been observed in batches with either iron ions. Ammonium was only present in very small amounts (40 μmol) and could not account for the whole difference. The only other possible nitrogen compounds were N_2O and NO. In fact both were found (Fig. 4). Only NO when Fe^{3+} was present, and NO and N_2O with Fe^{2+}. NO could account for the lacking 60 μmols of nitrogen in incubations with Fe^{3+}. However, NO and N_2O could only make up for one fourth of the missing nitrogen in the batch cultures with Fe^{2+}. Electron paramagnetic resonance (EPR) showed the presence of an [Fe^{2+}-NO] complex at concentrations of about 135 μmol (Fig. 5). This is enough to explain the missing nitrogen. Data in Figure 5 confirm also the observation that in absence of nitrate a considerable amount of Fe^{3+} is converted to the EPR invisible Fe^{2+} (Fig. 2) and that this conversion is strongly inhibited when nitrate was added (Fig. 3A). [Fe^{3+}-NO] complexes could not be detected with the methods used. Interestingly, the rate of Fe^{3+} reduction was not affected by the presence of nitrate in cell free extract (not shown).

Nitric oxide. In the range between 0 and 5 mM Fe^{3+} added, the production of NO increased as the amount of added Fe^{3+} increased (Fig. 6). At higher Fe^{3+} concentration, the rate of NO increased only slightly. Parallel to the production of free NO, nitrate formation and lactate consumption is reduced (Fig. 6) indicating an inhibitory effect of NO. The addition of NO to cell suspensions reduced in fact the lactate-driven nitrate respiration rates by

50% already at a NO concentration of 85 μM (Fig. 7). The reduction of NO_2^- to NO and further to N_2O (Moraghan and Buresh, 1977) by the simultaneous oxidation of Fe^{2+} is probably a purely chemical process (Table 1) and is referred to in

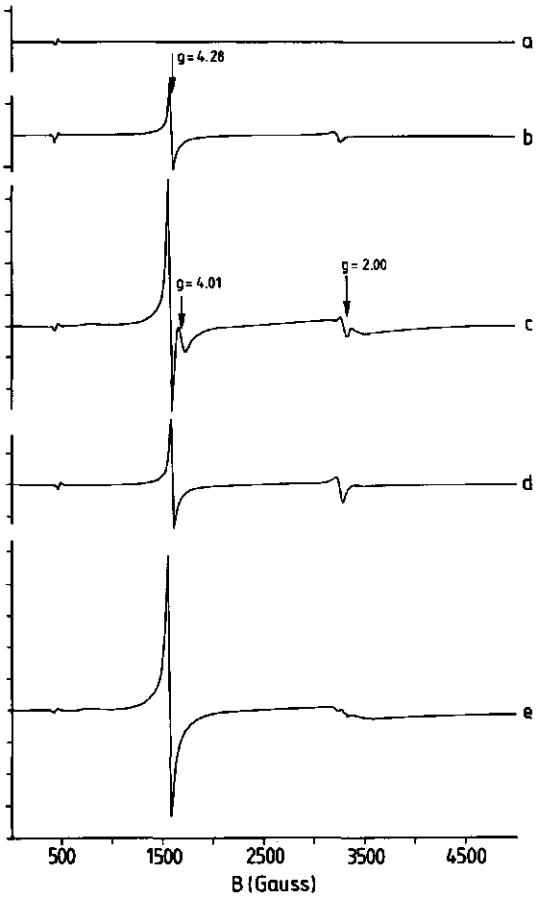

Fig. 5. EPR spectra detected after 6 hours in anaerobic non-growing batch cultures of *Escherichia coli* E4 (12 mg protein; 50 mM L-lactate as a source of energy). (a): control experiment (*E.coli* E4, L-lactate and 2 mmol NO_3^-). (b): 250 μmol Fe^{2+} were added, signal at g = 4.28 represent 0.57 mM Fe^{3+}. (c): 250 μmol Fe^{2+} and 2 mmol NO_3^- were added, signal at g = 4.28 is 1.5 mM Fe^{3+}, signals at g $\approx$ 4.01 and g = 2.00 were quantified 2.7 mM [Fe^{2+}-NO]. (d): 250 μmol Fe^{3+} was added, g = 4.28 represents 0.71 mM Fe^{3+}. (e): attenuation 0.5: 250 μmol Fe^{3+} and 2 mmol NO_3^- were added, signal g = 4.28 is 4.8 mM Fe^{3+}. The microwave frequency was 9329 $\pm$ 3 MHz.

the literature as chemodenitrification (Tiedje, 1988). In the system investigated here the role of *E.coli* E4 is to provide continuously NO_2^- and Fe^{2+} by reducing NO_3^- and Fe^{3+} with lactate.

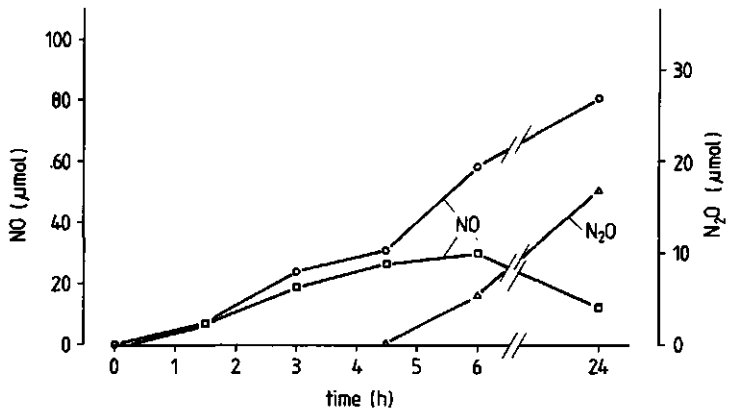

Fig. 4. Time course of nitric oxide and nitrous oxide production in anaerobic non-growing batch cultures of *Escherichia coli* E4. Symbols referring to nitric oxide, (○) when 250 µmol $FeCl_3$ was added or (□) when 250 µmol $FeCl_2$ were added. (△): N_2O production in response to the addition of 250 µmol $FeCl_2$. Concentrations and statistics as in Fig. 1.

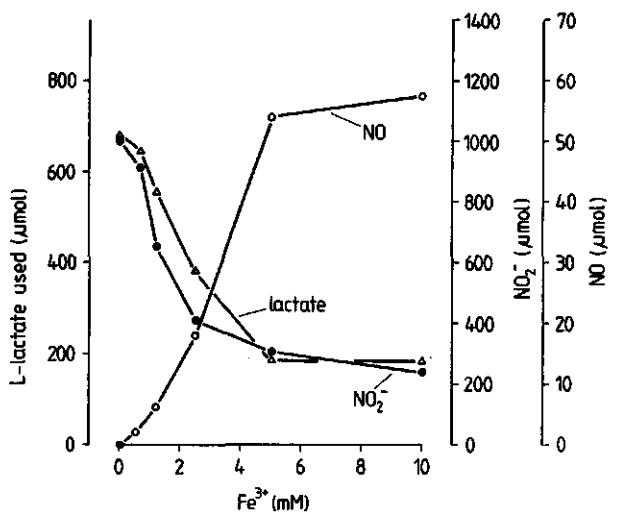

Fig. 6. Nitrite and nitric oxide formation and L-lactate uptake determined after 6 hours in anaerobic non-growing batch cultures of *Escherichia coli* E4 as a function of initial ferric iron concentration. Concentrations of L-lactate, NO_2^-, and NO and statistics as in Fig. 1.

Table 1. Formation of nitric oxide, nitrous oxide, [Fe^{2+}–NO], and ferric iron after 6 hours of incubation. Experiments were done at pH 7.2, in 50 mM Tris–HCl buffer containing 40 mM KNO_3 or KNO_2, 5 mM $FeCl_2$ or $FeCl_3$, and 50 mM Na–L–lactate and were indicated *Escherichia coli* E4 cells at a concentration of 12 mg protein per 50 ml. Nitric oxide, nitrous oxide, [Fe^{2+}–NO] and ferric iron are given in μmol produced in 50 ml reaction mixture.

Reaction mixture	NO[a]	N_2O	[Fe^{2+}- NO]	Fe^{3+}
Fe^{3+}	-[b]	-	-	250[c]
Fe^{3+} + L-lactate	-	-	-	250
Fe^{2+}	-	-	-	-
NO_3^- + Fe^{2+}	-	-	-	-
NO_3^- + Fe^{2+} + *E.coli* E4 (without L-lactate)	-	-	-	-
NO_3^- + Fe^{2+} + *E.coli* E4 (autoclaved)[d]	-	-	-	-
NO_3^- + Fe^{2+} + *E.coli* E4 (plus L-lactate)	16	10	135	75
NO[e] + Fe^{2+} + *E.coli* E4 (plus L-lactate)	4	85	72	90
NO_3^- + Fe^{3+} + *E.coli* E4 (plus L-lactate)	59	-	-	241[f]
NO_2^- + Fe^{2+}	170	23	< 40[g]	200
NO[h] + Fe^{2+}	2	10	25	10

a) NO and N_2O were quantified gaschromatographically, [Fe^{2+}–NO] with EPR, and Fe^{3+} either spectrophotometrically or with EPR.

b) Means not detected.

c) All data are means of at least two independent experiments.

d) *E.coli* E4 cells were autoclaved at 120°C for 20 min in the Tris–HCl buffer before sterile addition of KNO_3, $FeCl_2$, and L–lactate.

e) 150 μmol NO added.

f) Total amount of Fe^{3+} including the portion adsorbed to the cells.

g) [Fe^{2+}–NO] can only be detected with this method if the concentration of this complex is at least 20% of the Fe^{3+} concentration.

h) 35 μmol NO added.

The chemodenitrification rates in our system are pH dependent (Knowles, 1981). The apparent rate constants for the initial nitric oxide formation were 2.0 h^{-1} at pH 4 and 0.12 h^{-1} at pH 5, 0.05 h^{-1} at pH 6 and 0.01 h^{-1} at pH 7 and << 0.01 h^{-1} at pH 8.

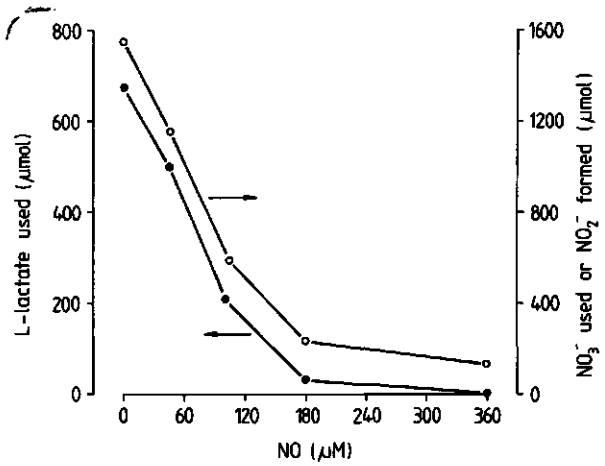

Fig. 7. Inhibition of L-lactate-driven nitrate respiration by nitric oxide in anaerobic non-growing batch cultures of *Escherichia coli* E4. Concentrations and statistics as in Fig. 1.

5.4 DISCUSSION

Ferric iron is a suitable electron acceptor under anaerobic conditions (Zehnder and Stumm, 1988). Experiments in this chapter with *Escherichia coli* E4 have shown that this organism can reduce ferric iron with electrons from lactate but is not able to specifically couple iron reduction to the respiratory chain. This is in accordance with the findings of Williams and Poole (34) that in *E.coli* K12 the respiratory chain is not involved in the reduction of ferric iron. Yet Short and Blakemore (30) report a translocation of protons with ferric iron as terminal electron acceptor in *E.coli* CSII27, but no evidence was presented for energy conservation *via* this reaction. As a result of the presence of ferric iron some minor increase of cell yield was found by Jones *et al.* (16) for a malate fermenting *Vibrio* spp., although ferric iron reduction was primarily associated with the diversion of the metabolism to energetically more favorable end products. Positive evidence for the direct coupling of dissimilatory iron

reduction to growth was obtained with a hydrogen metabolizing *Pseudomonas* spp. (Balashova and Zavarzin, 1979). Growth at the expense of acetate oxidation coupled to ferric iron reduction was recently reported for a strictly anaerobic Gram-negative rod, isolated from fresh water sediments (Lovley and Phillips, 1988).

Ferric iron inhibited nitrate reduction by *E.coli* E4 (Fig. 3A). This inhibition was not due to the competition for electrons by both electron acceptors but to the formation of a considerable amount of toxic nitric oxide. In these cultures nitric oxide is formed by a process which can schematically be represented by Fig. 8. Ferric iron is reduced to ferrous iron by compounds which are directly or indirectly reduced by lactate. Lactate cannot reduce ferric iron without an electron carrier in our cultures (Table 1). Ferrous iron then reduces nitrite by chemodenitrification (Tiedje, 1988) to nitric oxide without the mediation of electron carriers or enzymes (Table 1). Nitric oxide can further react with ferrous iron by the same process to nitrous oxide. In case ferrous iron is added together with nitrate, in total more nitric oxide is formed than with ferric iron. Most nitric oxide is subsequently bound to excess ferrous iron by the formation of a $[Fe^{2+}\text{-NO}]$ complex. Ferric iron can be converted back to

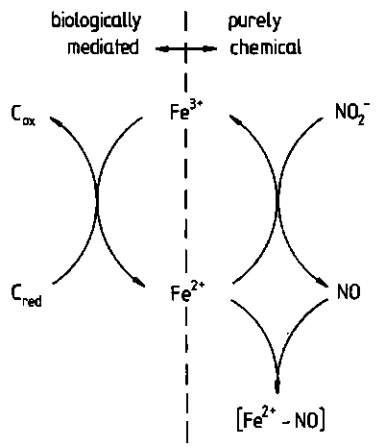

Fig. 8. Schematic representation of the coupling of biologically mediated and purely chemical processes leading to the formation of free nitric oxide and the $[Fe^{2+}\text{-NO}]$ complex from nitrite and ferrous iron. The biologically mediated nitrate reduction and the further purely chemical reduction of nitric to nitrous oxide are not included in this figure. C_{red} and C_{ox} means reduced and oxidized "coenzyme(s)" which can reduce ferric to ferrous iron.

ferrous iron as long as the biological system is functioning.

Nitrate reduction is much stronger inhibited by ferric iron than by ferrous iron (Fig. 3). This is probably due to the higher free NO concentration in cultures supplemented with ferric iron (Fig. 4). When ferrous iron was added most nitric oxide was bound in the [Fe^{2+}-NO] complex. The data in Fig. 3 and Table 1 together with the scheme presented in Fig. 8 suggest that the purely chemical oxidation of ferrous iron by nitrite is much faster than the subsequent biologically mediated reduction of ferric iron by lactate. This difference keeps ferrous iron concentration low. As a consequence the [Fe^{2+}-NO] complex is not formed and nitric oxide is not removed. This mechanism suggest that the binding constant between free Fe^{2+} and NO is relatively low, since [Fe^{2+}-NO] does not act as an ultimate sink of either Fe^{2+} and NO. In addition, the iron in the [Fe^{2+}-NO] complex seems still to be able to become oxidized, thereby releasing NO or perhaps even N_2O. The production of NO is probably also responsible for the inhibition of ferric iron reduction by nitrate in *Staphylococcus aureus* (Lascelles and Burke, 1978), *Pseudomonas* spp. (Obuekwe *et al.*, 1981) and *E.coli* K12 (Williams and Poole, 1987).

According to Muhoberac and Wharton (27) nitric oxide toxicity is due to direct binding to heme iron. Stouthamer (32) hypothesizes that the specific binding of nitric oxide to ferrous iron is a possible reason that during denitrification and dissimilatory nitrate reduction to ammonium free nitric oxide is not found or only measured in trace amounts. In fact some nitric oxide reductases are clearly heme proteins (Heiss *et al.*, 1989; Carr and Ferguson, 1990). Nitrite reduction by purified nitrite reductases from *Thiobacillus denitrificans* (Le Gall *et al.*, 1979) and *Desulfovibrio desulfuricans* (Liu *et al.*, 1980) occurs also *via* a heme-nitric oxide complex as intermediate. In both cases evidence for an enzyme-NO complex was obtained with the characteristic ^{14}N hyperfine pattern in the EPR signal in the g = 2.0 region. The spectrum for *E.coli* E4 does not exhibit this pattern in the g = 2.0 region (Fig. 5C). The observed spectrum is characteristic for a non-heme iron nitric oxide complex, suggesting that in our experiments nitric oxide is directly bound to the free ferrous iron. This could be confirmed with ferrous iron and nitric oxide in the absence of *E.coli* cells (Table 1). The binding of nitric oxide to heme iron has been used recently to prove that nitric oxide can indeed be a free intermediate in denitrification (Goretski and Hollocher, 1988; Kučera, 1989).

The data presented in this chapter can explain N_2O production by enterobacteria and are an indication for the mechanism leading to the change of N_2O in human breath before and after a meal containing nitrate and nitrite (Bleakley and Tiedje, 1982; Smith, 1982). The results also show that organisms, other than nitrifiers and denitrifiers, able to produce nitrate and to reduce ferric iron, can contribute to the loss of nitrogen to the atmosphere. It remains to further investigations to quantify the contribution of the reactions described in this chapter for the nitric and nitrous oxide content of the atmosphere.

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CHAPTER 6

AEROBIC NITRATE AND NITRITE REDUCTION IN CONTINUOUS CULTURES OF *ESCHERICHIA COLI*

ABSTRACT

Nitrate and nitrite was reduced by *Escherichia coli* E4 in a L-lactate (5 mM) limited culture in a chemostat operated at dissolved oxygen concentrations corresponding to 90-100% air saturation. Nitrate reductase and nitrite reductase activity was regulated by the growth rate, and oxygen and nitrate concentrations. At a low growth rate (0.11 h^{-1}) nitrate and nitrite reductase activities of 200 $nmol.mg^{-1}protein.min^{-1}$ and 250 $nmol.mg^{-1}protein.min^{-1}$ were measured, respectively. At a high growth rate (0.55 h^{-1}) both enzyme activities were considerably lower (25 and 12 $nmol.mg^{-1}protein.min^{-1}$). The steady state nitrite concentration in the chemostat was controlled by the combined action of the nitrate and nitrite reductase. Both nitrate and nitrite reductase activity were inversely proportional to the growth rate. The nitrite reductase activity decreased faster with growth rate than the nitrate reductase. The chemostat biomass concentration of *E.coli* E4, with ammonium either solely or combined with nitrate as a source of nitrogen, remained constant throughout all growth rates and was not affected by nitrite concentrations. Contrary to batch, *E.coli* E4 was able to grow in continuous cultures on nitrate as the sole source of nitrogen. When cultivated with nitrate as the sole source of nitrogen the chemostat biomass concentration is related to the activity of nitrate and nitrite reductase and hence, inversely proportional to growth rate.

Brons H.J. and Zehnder A.J.B. (1990) Aerobic nitrate and nitrite reduction in continuous cultures of *Escherichia coli* E4. Archives of Microbiology 153:531-536.

6.1 INTRODUCTION

Anaerobically, *Escherichia coli* can reduce nitrate to nitrite and nitrite to ammonium. These reactions serve the regeneration of reduced co-enzymes (Cole and Brown, 1980; Ingledew and Poole, 1984; Zehnder and Svensson, 1986). Oxygen may affect the activity and synthesis of dissimilatory nitrate reductase in *E.coli* in different ways. Four control mechanisms have been proposed, namely: (i) The synthesis of nitrate reductase is repressed by the presence of dioxygen gas (Kobayashi and Ishimoto, 1973; Showe and DeMoss, 1968). (ii) Oxygen competes with nitrate for electrons from the respiratory chain and as a consequence of its higher electron affinity will diminish the *in vivo* reduction rate of nitrate (John, 1977; Stouthamer, 1988; Zehnder and Svensson, 1986). (iii) Nitrate uptake is inhibited by oxygen (Noji and Taniguchi, 1987), and (iv) aerobically nitrate reductase is not incorporated into the cytoplasmic membrane (Hackett and MacGregor, 1981).

Oxygen regulation of nitrate reductase synthesis was interpreted by Showe and DeMoss (29) as evidence for the existence of a redox sensitive repressor. Their interpretation was based on the observation that nitrate reductase in *E.coli* required not only nitrate but also anaerobiosis for full induction. The presence of such a simple redox sensitive control mechanism was doubted (Pecher *et al.*, 1983). It was proposed instead that a hierarchy of regulatory proteins could either interact directly with the promoter DNA, or modify the fnr (fumarate/nitrate reductase regulation) product (fnr protein) itself (Griffiths and Cole, 1987). According to Kaprálek *et al.* (14) oxygen may also inhibit the synthesis of nitrate reductase at the transcription level.

A competition which is merely based on thermodynamic factors would not exclude the concomitant use of nitrate and oxygen under certain environmental conditions. In fact, in recent years increasing evidence appears in the literature that the use of nitrate as terminal electron acceptor in presence of oxygen is possible (Dunn *et al.*, 1979; Krul and Veeningen, 1977; Meiberg *et al.*, 1980; Robertson and Kuenen, 1984; Strand *et al.*, 1988). *Alcaligenes* spp. (Krul and Veeningen, 1977) uses nitrate and oxygen at the same time when grown in batch even at air saturation above 200%. *Klebsiella* (Dunn *et al.*, 1979) and *Hyphomicrobium* (Meiberg *et al.*, 1980)

still contain considerable nitrate reductase activity when grown in a chemostat at 10 and 35% air saturation, respectively. *Zoogloea ramigera* reduces nitrate at considerable rates at 8 mg O_2 per liter (Strand *et al.*, 1988). *Thiosphaera pantotropha*, a denitrifying mixotroph, could at 90% air saturation use oxygen and nitrate concomitantly as terminal electron acceptors at 90% air saturation. The presence of excess nitrate together with oxygen even increased the growth rate (Robertson and Kuenen, 1984). Most of the data on oxygen tolerance of nitrate reductase were obtained with denitrifying organisms. Because of the similarity of the dissimilatory nitrate reductases in a variety of different organisms (Stouthamer, 1988) it can be assumed that these enzymes may show similar behaviours toward oxygen. Therefore the influence of molecular oxygen on nitrate and nitrite reduction was studied in *E.coli*. It was shown that both enzymes are active in presence of oxygen when the organism was grown in a chemostat. In addition it was found that *E.coli* is able to grow aerobically on nitrate as sole source of nitrogen.

6.2 MATERIALS AND METHODS

Organism. The organism used in this study was *Escherichia coli* E4 (NCTC 9002) and was obtained from the culture collection of our laboratory. The organism was maintained on yeast extract-glucose agar slants, containing per liter: 2.5 g yeast extract, 5 g glucose and 12 g agar. The agar slants were stored at 4°C and subcultured every 2 months.

Cultivation. *Escherichia coli* E4 was cultivated in a medium (pH ≈ 7.2) containing per liter demineralized water: 0.56 g Na-L-lactate, 0.82 g NH_4Cl, 0.1 g $MgSO_4.7H_2O$, 3 g K_2HPO_4, and 2 g KH_2PO_4. To 1 liter of this culture medium 1 ml of a trace element solution was added. This trace element solution contained the following elements (g/l): $CaCl_2.2H_2O$ (10), NaCl (10), $FeCl_3.6H_2O$ (1.0), H_3BO_3 (0.05), $CuSO_4.5H_2O$ (0.01), $CoCl_2.6H_2O$ (0.02), KI (0.01), $MnCl_2.4H_2O$ (0.04), $NaMoO_4$ (0.02), $ZnSO_4.7H_2O$ (0.04). Continuous cultures were performed at 30°C in a 1 liter working volume chemostat (Applikon B.V., Schiedam, NL). To avoid the selection of specific spontaneous mutants every 30-35 volume changes a new continuous culture was started up. To assure constant conditions, at least six volume changes have been allowed for each steady state before measurements have been made.

The maximal growth rate (μ_{max}) of *E.coli* E4 was determined by a step wise increase of the chemostat's dilution rate in the range 0.10-0.65 h^{-1} until total cell wash out was achieved. The pH in the continuous culture was monitored with a steam sterilizable glass electrode (Ingold, Van Oortmessen B.V., Den Haag, NL) connected to a pH meter (Radiometer, Instrumenthandel Zuid-Holland B.V., Den Haag, NL). As the pH could be maintained at 7.2 with the 30 mM phosphate buffer further pH control was not necessary. Dissolved oxygen concentration in the chemostat was monitored with a steam sterilizable oxygen electrode (Biolafitte, Marius Instruments B.V., Utrecht, NL) connected to a potentiometric recorder (Goerz, Brown Boveri Nederland B.V. Rotterdam, NL). Dissolved oxygen concentration in the chemostat was maintained at 90-100% air saturation by both passing sterile air through a submerged nozzle and setting the impeller speed at 800 rpm.

Induction experiments. Steady state cultures (D = 0.11 h^{-1}) were harvested and centrifuged in portions of 50 ml at 10.000 *g* for 10 min. Pellets were washed in a 50 mM Tris-HCl buffer (pH = 7.2) centrifuged again, resuspended in 50 ml of the same buffer and supplemented with (1 ml/l) of the trace element solution. Nitrate- and nitrite reductase induction in the presence of chloramphenicol were carried out with 50 ml resuspended whole cells in anaerobic, argon flushed vials (headspace 72 ml) on an orbital incubator (120 rpm) at 30 ± 1°C. Cyclic AMP to a final concentration of 1 mM was added directly to aerobic steady state cultures (pO_2 > 90%; D = 0.11 h^{-1}) grown on L-lactate (5 mM), ammonium (15 mM) and nitrate (40 mM). After 3 h nitrite was quantified in the chemostat and nitrate reductase was measured in cell free extracts.

Preparation of cell free extracts. Cells from different steady state cultures were harvested and washed as above. Cells were disrupted at 0°C with a sonifier (Branson, Marius Instruments B.V., Utrecht, NL) by applying 36 W three times for one minute. The crude extracts were centrifuged at 4°C for 15 min at 15.000 *g* to remove the cell debris. The supernatant was used as cell free extract.

Enzyme assays. Nitrate reductase and nitrite reductase were assayed at 30°C in anaerobic argon flushed Hungate tubes (16.6 ml) that were fixed on an orbital shaker (120 rpm). Unless otherwise stated the electron donor was

Na-L-lactate (50 mM) in both assays and the final reaction volume was 4 ml Tris-HCl buffer (50 mM; pH = 7.2). Occasionally formate (50 mM) and NADH (2.5 mM) were also used as electron donors. The assay was started by the addition of cell free extract (0.8 - 1.2 mg of protein per assay) and the reaction products nitrite and ammonium were determined after 5 min.
Nitrate reductase activity was assayed with 40 mM KNO_3 as an electron acceptor and is expressed in nmol nitrite formed per mg protein in the cell free extract per minute. A correction for the formation of ammonium from nitrite was made because nitrate reductase provides the substrate for nitrite reductase.
Nitrite reductase activity was assayed with 0.5 mM $NaNO_2$ as an electron acceptor and is expressed in nmol ammonia formed per mg protein in the cell free extract per minute.

Analytical methods. L-lactate, ammonium, nitrite and nitrate were determined after cells had been removed by filtration through a 0.22 μm Millipore filter (Millipore Nederland B.V., Etten-Leur, NL). Ammonium, nitrite and nitrate were measured according to Deutsche Einheitsverfahren (5).
L-lactate was quantified with HPLC equipped with a Chrompack organic acid column (Chrompack, Middelburg, NL) connected to a 2142 refractive index detector (LKB, Woerden, NL). Analysis were run at 60°C with a liquid phase of 5 mM H_2SO_4 at a flow rate of 0.6 $ml.min^{-1}$.
Biomass concentration in the chemostat was quantified as particulate organic carbon (POC) in a Beckman model 915A Organic Carbon Analyzer (Beckman Instruments Nederland B.V., Mijdrecht, NL). K,H-phthalate in 0.1 M HCl served as a standard.
The protein concentration in cell free extracts was determined according to a modified Lowry method (DeMoss and Bard, 1957) using bovine serum albumin as a standard.
The oxygen respiration of sampled chemostat cultures (D = 0.11 h^{-1}) was monitored at 30°C in the thermostatically controlled reaction vessel of a YSI model 53 biological oxygen monitor (Yellow Springs Instruments, Tamson B.V., Zoetermeer, NL) equipped with a polarographic oxygen probe. The oxygen uptake rate of washed whole cells was determined in 3 ml final volume of an air saturated 50 mM Tris-HCl buffer (pH = 7.2). The endogenous oxygen uptake rate was subtracted from the respiration rate measured in the presence of 10 mM L-lactate.

Chemicals. Sodium L-lactate and formate were obtained from J.T. Baker B.V., Deventer, NL. All other growth media constituents were from E. Merck B.V., Amsterdam, NL. Yeast extract and agar used in slants were obtained from Difco (Brunschwig Chemie B.V., Amsterdam, NL). α-Naftylamino, sulfanilamide, and chloramphenicol were purchased from Sigma (Brunschwig Chemie B.V., Amsterdam, NL). NADH, and cAMP were from Boehringer Mannheim B.V., Almere, NL.

6.3 RESULTS

In a chemostat at dilution rates between 0.11 to 0.45 h^{-1} *E.coli* E4 required 1.7 mM ammonium for cell synthesis when grown aerobically under L-lactate (5 mM) limiting conditions. With ammonium as only nitrogen source no oxidized nitrogen compounds were found in the medium. In a strictly aerobic chemostat nitrite was formed when nitrate (40 mM) was used besides ammonium as nitrogen sources (Fig. 1). The steady state concentration of ammonium was higher than was expected from the experiments with ammonium alone. The excess ammonium is also shown in Fig. 1. Both nitrite and excess ammonium concentration decreased with increasing growth rate between 0.11 and 0.45 h^{-1}. Interestingly, ammonium production ceased and more nitrite was formed above 0.45 h^{-1}. This rate is close to μ_{max} (0.48 h^{-1}) determined

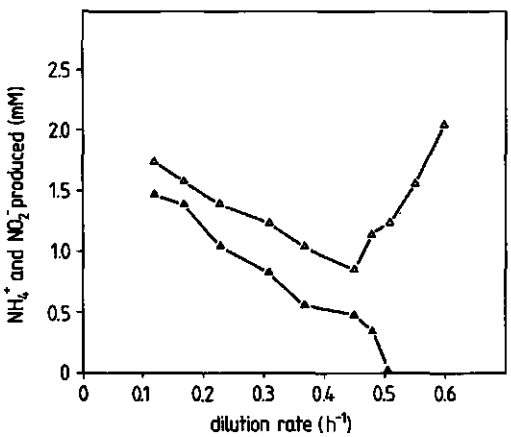

Fig. 1. Nitrite (△) and ammonium (▲) concentrations additionally produced in aerobic (pO_2 > 90%) chemostat cultures of *Escherichia coli* E4 as a function of growth rate. *E.coli* E4 was cultivated under L-lactate (5 mM) limitation in the presence of both NH_4Cl (15 mM) and KNO_3 (40 mM). Each point represents the average of three independent measurements.

for growth on nitrate as the sole nitrogen source. Biomass production did not differ when ammonium alone or in combination with nitrate was used as nitrogen source (Fig. 2). However, nitrate alone was not able to maintain the same biomass concentration and biomass decreased slowly with increasing growth rate (Fig. 3). The high steady state L-lactate concentration in the

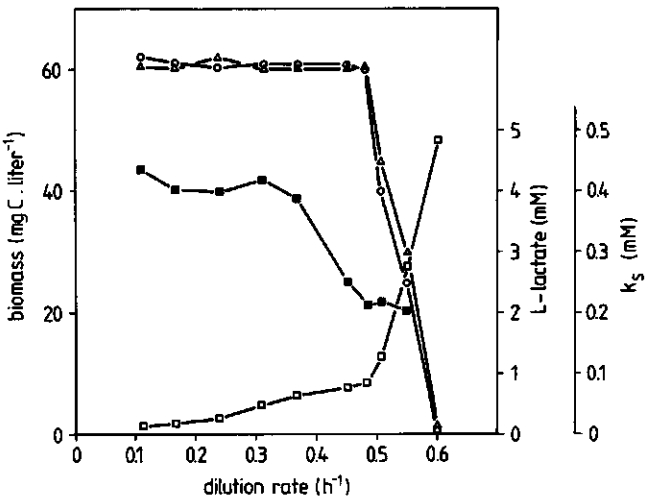

Fig. 2. Biomass, L-lactate concentration and substrate affinity constant (K_s) as a function of growth rate of *Escherichia coli* E4 in aerobic (pO_2 > 90%) chemostat cultures. *E.coli* E4 was grown under (5mM) L-lactate limiting conditions either solely with ammonium (15 mM) or combined with nitrate (40 mM). (o) biomass with NH_4^+; (Δ) biomass with NH_4^+ and NO_3^-; (□) steady state L-lactate concentrations and K_s (■) with NH_4^+ as the sole nitrogen source. Each point represents the mean of three replicates.

nitrate fed chemostat indicates that the culture is not lactate limited. From the low concentrations of ammonium found in this culture it can be concluded that *E.coli* E4 had first to convert nitrate in a dissimilatory way to ammonium before it could use it as a nitrogen source. Thus, despite of the high nitrate concentration this culture was nitrogen limited at all growth rates. In fact, by calculating the possible biomass production from the surplus ammonium in Fig. 1 (60 mg C in biomass per 1.7 mM ammonium) theoretical and measured biomass concentration where almost identical over the whole range of growth rates when nitrate was the sole nitrogen source (Fig. 3). In batch cultures *E.coli* E4 was not able to grow on nitrate as sole nitrogen source.

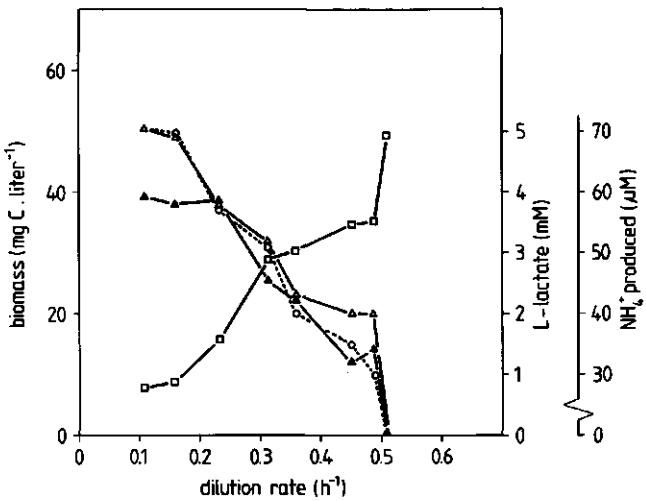

Fig. 3. Growth rates of *Escherichia coli* E4 on nitrate (40 mM) and L-lactate (5 mM) in aerobic (pO_2 > 90%) chemostat cultures. (▲) biomass observed; (o) biomass calculated; (▲) ammonium produced from nitrate; (□) L-lactate steady state concentration. Data represent the mean of three replicates.

Based on the data given in Fig. 2 a substantial energetic contribution of aerobic nitrate reduction as was shown for *Thiosphaera pantotropha* (28) is not likely. 40 mM nitrate even inhibited oxygen respiration rate by 10% in cells harvested at a growth rate of 0.11 h^{-1}. Such a minor inhibition is difficult to measure as reduction of biomass formation.

By calculating K_s for lactate with the Monod equation (Monod, 1950) from the data given in Fig. 2 two apparent constant K_s can be distinguished. One in the range between 0.11 and 0.37 h^{-1} and the other for growth rates above 0.48 h^{-1}. Between 0.37 h^{-1} and 0.48 h^{-1} the K_s gradually decreases (Fig. 2). Fig. 2 also demonstrates that *E.coli* is able to increase its μ_{max}, however with growth rates exceeding 0.48 h^{-1} less biomass is formed and lactate concentrations increased accordingly. Similar results have been reported for glucose-limited chemostat cultures of *Klebsiella pneumoniae* (Rutgers *et al.*, 1987). At all dilution rates with ammonium, either solely or combined with nitrate as nitrogen source, the growth yield of *E.coli* E4 was 1.06 ± 0.10 mmol cellular carbon per mmol of L-lactate used. No increase in μ_{max} was observed for growth of *E.coli* E4 on nitrate as the sole source of nitrogen. The steady state concentrations of ammonium formed from nitrate

appeared to be inversely related to growth rate. Since here ammonium was limiting instead of lactate a K_s for lactate could not be calculated.

In cells grown with ammonium as only nitrogen source, nitrate reductase activity was very low even after a three hours anaerobic induction period. When nitrate was present in addition, a distinct nitrate reductase activity was present in the aerobic cells at low growth rates. This activity decreased with increasing growth rates. An anaerobic induction period enhanced the nitrate reductase activity markedly (Fig. 4). Nitrite reductase showed a very similar pattern but its activity decreased faster at growth rates above 0.45 h^{-1} (Fig. 5). This fact explains the sudden increase of the steady state nitrite concentration above this dilution rate in the chemostat (Fig. 1). Induction of nitrate reductase was clearly inhibited by chloramphenicol in short term batch cultures. Nitrite reductase could not be induced under the same circumstances and its activity was not affected by chloramphenicol (Table 1). Contrary to nitrate reductase, nitrite reductase was independent of NADH (Table 2). The results given in the

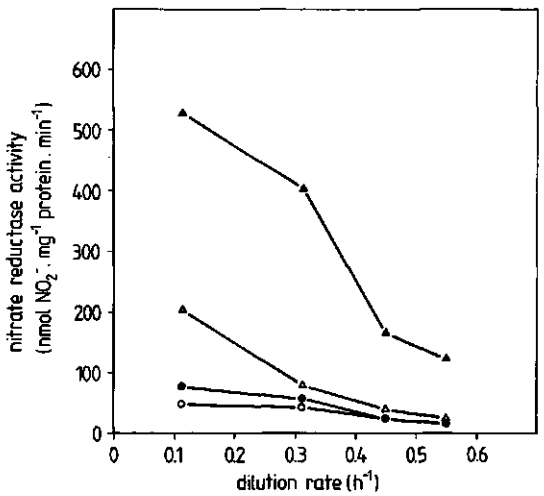

Fig. 4. Nitrate reductase activity in chemostat cultures of *Escherichia coli* E4 was grown under aerobic (pO_2 > 90%) L-lactate limiting (5 mM) conditions in the presence of NH_4^+ (15 mM) solely or combined with NO_3^- (40 mM). Induction of nitrate reductase under anaerobic conditions was achieved by replacing air in the chemostat culture by dinitrogen gas for 3 to 4 h. (o): aerobic + NH_4^+; (●): anaerobic + NH_4^+; (△): aerobic, NH_4^+ + NO_3^-; (▲): anaerobic, NH_4^+ + NO_3^-. All points are the average of duplicates.

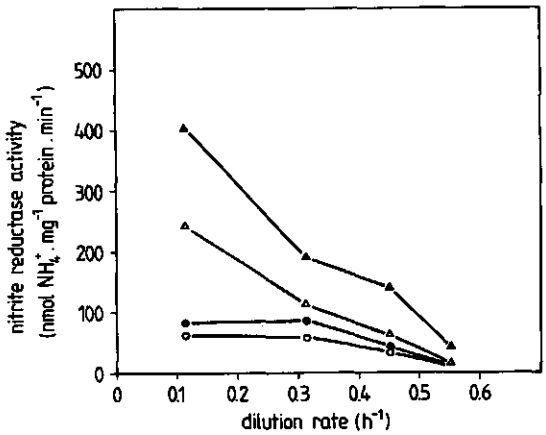

Fig. 5. Nitrite reductase activity in aerobic (pO_2 > 90%) L-lactate limited (5 mM) continuous cultures of *Escherichia coli* E4. Source of nitrogen was NH_4^+ (15 mM) solely or combined with NO_3^- (40 mM). Induction of nitrite reductase was achieved by replacing air in the chemostat culture by dinitrogen gas for 3 to 4 h. (o): aerobic + NH_4^+; (●): anaerobic + NH_4^+; (△): aerobic, NH_4^+ + NO_3^-; (▲): anaerobic, NH_4^+ + NO_3^-. All points are the average of duplicates.

Table 1. Nitrate reductase and nitrite reductase activities (nmol.mg^{-1} protein.min^{-1}) in aerobic and anaerobic batch experiments with *Escherichia coli* E4. This series of experiments was done with cells grown aerobically in the absence of nitrate at a dilution rate of 0.11 h^{-1}. Cell free extracts were made three hours after 50 mM L-lactate, 40 mM KNO_3 and 0.5 mM chloramphenicol (C) had been added to the Tris-HCl buffer (pH 7.2) containing trace elements.

Incubation	Nitrate reductase		Nitrite reductase	
	-C	+C	-C	+C
Aerobic - NO_3^-	137	133	70	70
Aerobic + NO_3^-	165	137	87	78
Anaerobic - NO_3^-	153	137	80	72
Anaerobic + NO_3^-	472	130	78	78

Table 2. The effect of different electron donors on the nitrate and nitrite reductase activities in *Escherichia coli* E4. *E.coli* E4 was grown in aerobic (pO_2 > 90%) L-lactate (5 mM) limited chemostat cultures at D = 0.11 h^{-1}. Source of nitrogen was ammonium (15 mM) combined with nitrate (40 mM). Induction of nitrate and nitrite reductase activities was achieved by replacing air with dinitrogen gas for 3-4 h in the chemostat culture. Enzyme activities were measured in cell free extracts, are expressed as nmol.mg^{-1}protein.min^{-1}, and represent the mean of two replicates.

Electron donor (mM)	Nitrate reductase	Nitrite reductase
L-lactate (50)	500	420
Formate (50)	510	410
NADH (2.5)	470	0

Figs. 4 and 5 indicate that repression of nitrate reductase and nitrite reductase in *E.coli* E4 was relieved in aerobic L-lactate limited chemostats at relatively low growth rates. After the addition of 1 mM cAMP at D = 0.11h^{-1} the steady state nitrite concentration and the nitrate reductase activity increased by a factor 2.0 and 1.8, respectively (data not shown). Interestingly nitrite reductase was not depressed in batch cultures under anaerobic conditions (Table 1) albeit 40 mM nitrate was almost entirely converted to nitrite and only 1% of the nitrate nitrogen was converted to ammonium. Similar results on the repression of nitrite reductase in batch as opposed to continuous culture were previously reported for a nitrate-ammonifying marine *Vibrio* species (Rehr and Klemme, 1986).

6.4 DISCUSSION

It is generally accepted in the literature that assimilatory nitrate reduction is inhibited by oxygen (Stewart, 1988). In *E.coli* E4 grown in continuous culture nitrate was reduced to nitrite in presence of 15 mM ammonium and at oxygen concentrations of about 7 mg per liter (100% air saturation at 30°C). At the same time nitrite was reduced to ammonium (Fig. 1). To our knowledge this is the first time that strictly aerobic nitrate reduction *via* nitrite to ammonium has been shown for *E.coli*. The aerobic reduction of nitrate to ammonium by *E.coli* allows this organism to grow on

nitrate as sole nitrogen source under oxic conditions. This seems to be in variance with observations made by others (Kapràlek *et al.*, 1982; Lin and Kuritzkes, 1987; Stewart, 1988) who reported that *E.coli* failed to grow aerobically with nitrate in batch. In batch cultures microorganisms grow initially at μ_{max} or very close to it. At dilution rates which allow growth around μ_{max} only small amounts of ammonium is formed from nitrate and growth rates decrease drastically (Fig. 3). For the specific case of μ_{max} our observation and the measurements in batch by Kobayashi and Ishimoto (15) agree quite well. However, at lower growth rates in the chemostat *E.coli* behaves differently from batch cultures.

The ability of *E.coli* E4 to grow aerobically on nitrate at low growth rates was paralleled by substantial nitrate and nitrite reductase activities (Figs. 4 and 5). Nitrate and oxygen had a regulatory influence on the enzymes. As expected nitrate and probably also nitrite as a product of nitrate reduction induces the respective enzyme activities whereas oxygen partially represses them. At lower growth rates (> 0.45 h^{-1}) oxygen could not entirely inhibit nitrate and nitrite reductase or their expression in *E.coli* E4. From these data no clearcut conclusions can be drawn with respect to the mechanism of oxygen repression of these two enzymes. In batch experiments nitrate and nitrite reductase activity are not augmented in the presence of nitrate and oxygen (Table 1), just as has been reported by many others (Cole, 1968; Kobayashi and Ishimoto, 1973; Showe and DeMoss, 1968). Only with a mutant of *E.coli* K12, a lack of repression of nitrate reductase in aerobic batch cultures has been measured (Giordano *et al.*, 1977). But as was noted by these authors the presence of nitrate did not significantly induce nitrate reductase above the constitutive level.

In the present study it was observed that L-lactate, formate and NADH were electron donors for nitrate reductase in cell free extracts (Table 2). Formate and NADH driven nitrate reduction has extensively been studied in *E.coli* K12 (Ingledew and Poole, 1984), however, little is known about L-lactate as electron donor. For *E.coli* K12 Nishimura *et al.* (22) showed that L-lactate dehydrogenase is membrane bound and closely linked to nitrate reductase.

Two enzyme systems have been described in *E.coli* for the reduction of nitrite to ammonium. One utilizes NADH as electron donor (Jackson *et al.*, 1981), the other formate and lactate (Stewart, 1988). According to Cole and Browne (4), the NADH dependant nitrite reduction serves as electron sink

during anaerobic growth and the reductase is considered to be a cytoplasmatic enzyme (Pope and Cole, 1982). Electrons are transferred to nitrite *via* NADH-oxidase and a periplasmatic cytochrome c_{552} (Liu *et al.*, 1981). In our experiments no nitrate reduction occurred with NADH (Table 2), either because the enzyme was absent or because one or the other component was lost when cells were opened. Although, NADH may act as electron donor for nitrite reduction in whole cells of *E.coli* E4, it is unlikely that under aerobic L-lactate limited growth electrons are spilled *via* a NADH-oxidase linked nitrite reduction.

The formate and lactate dependant nitrite reductase is membrane bound and is considered to be used by *E.coli* to generate a proton motive force (Abou-Jaoudé *et al.*, 1979; Pope and Cole, 1982). This enzyme was present in our strain (Table 2). However, from our growth data it cannot be decided whether *E.coli* E4 disposes reducing equivalents or conserves energy *via* this enzyme system, since the difference would be less than 10%.

According to the data presented in this chapter the measured nitrate and nitrite reductase activities in *E.coli* E4 are those of dissimilatory enzymes. Both are influenced by the presence of oxygen but apparently not by ammonium (Fig. 1, 4 and 5) which is the contrary of the expected response of an assimilatory enzyme (Stewart ,1988). Interestingly we have here a case where a dissimilatory enzyme system has first to convert the nutrient (nitrate or nitrite to ammonium) before it can be assimilated.

Activities of nitrate and nitrite reductase in cell free extracts were inversely proportional to growth rate of *E.coli* E4 (Fig. 4 and 5). Relaxation at the level of transcriptional control might be involved here (Buettner *et al.*, 1973; Unden and Duchene, 1987) as suggested by the experiments with cyclic AMP. Such changing enzyme activities are often reported, but their regulation is generally poorly understood (Matin, 1981). Increased enzyme activities with decreased growth rate has been observed in particular with catabolic enzymes. Harder and Dijkhuizen (10) hypothesize that such a response is of ecological advantage for organisms living at sub-saturated substrate concentrations. It seems a paradox that at higher growth rates the K_s for the carbon and energy source is reduced, despite an increase of the steady state substrate concentration (Fig. 2). But high growth rates combined with high substrate affinity may give a maximum competitive advantage in the fast changing intestinal environment, the natural habitat of *E.coli*.

The data presented in this chapter suggest that besides aerobic denitrification (Robertson and Kuenen, 1984) also dissimilatory nitrate ammonification is possible in presence of oxygen. Thermodynamic considerations only, are seemingly not sufficient to predict the variety of possible microbially mediated processes under certain intermediary steady state conditions which can be obtained in a chemostat.

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CHAPTER 7

CONCLUDING REMARKS

The results described in the preceding chapters of this thesis may leave the reader with a kaleidoscopic impression. An apparent diversity of results has been obtained: bacterial growth and slime formation, inhibition of carbonate precipitation by fulvic acids, anaerobic iron oxidation and inhibition of sulphate reduction in seawater, ferro-nitrosyl complexes and aerobic nitrate ammonification in *E.coli*. The aim of this chapter is to discuss the results in brief retrospect in order to provide a framework for a more coherent understanding of this thesis.

In the first place it should be noted that the diversity of subjects is the result of rather broad formulated research objectives on natural pore water-sediment systems. The primary objective of this study was to obtain information on groundwater quality during aquifer thermal energy storage as a result of (i) bacterial growth and slime formation and (ii) biogeochemical precipitation reactions. Because quantitative data interlinking lab and field observations were scarce, a structured approach of the formulated research objectives was not immediately possible. As a consequence heuristics played a decisive role in the early orientating stages of this study. For instance, the data presented in Chapter 2 result from a typical heuristic trial and error approach. Taken into consideration the already available literature data, Chapter 2 basically provides knowledge rather than insight in the microbiological aspects of well clogging. Microbiological well clogging is complex process in which cause and effect cannot be fully separated. As a consequence the question is not why this process takes place but merely when it takes place. An unintended, yet decisive finding reported in Chapter 2 is the mobilization of dissolved organic carbon in sand columns operated at elevated temperatures. This phenomenon may significantly affect the mobility and precipitation of iron, calcium and magnesium in groundwater during thermal energy storage. Calcium and magnesium precipitation was studied in the presence of thermally mobilized dissolved organic carbon (Chapter 3). An important finding reported in Chapter 3 is that the mineralization rates of organic carbon in pristine anaerobic aquifers can be prevented by slow kinetics for several thousands of years. Following a temperature increase the mineralization rates of organic carbon are considerably accelerated. The concomitant mobilization of fulvic acids results in the complexation of calcium and magnesium. The subsequent super-saturation of the water phase with respect to calcite and

dolomite is an important parameter for the dimensioning of water treatment techniques. From a water treatment point of view it was also considered interesting to pursuit a study on the mobilization and the subsequent precipitation of iron. Sulphate reduction was studied because of anaerobic metal corrosion by hydrogen sulphide and the precipitation of ferrous sulphide. Since pore water from the sediment used in Chapter 3 was sulphate depleted it was decided to use common seawater instead in the experiments described in Chapter 4. In this chapter it is reported that nitrate acts as an inhibitor of iron and sulphate reduction. It is well established in the literature that in anaerobic aquifers and sediments ferrous iron is not found in the presence of nitrate. The data presented in Chapter 4 provide evidence that nitrite, as an intermediate of nitrate reduction and denitrification, may react spontaneously with ferrous iron to nitric oxide and ferric iron. The potant oxidant nitric oxide that results from this chemodenitrification reaction is responsible for the inhibition of sulphate reduction and may be the cause for commonly found spatial separation of denitrification, iron reduction and sulphate reduction in anaerobic habitats. The precise details of this reaction mechanism could not be elucidated with bacterial mixed cultures. *E.coli* was used as a model bacterium to study chemodenitrification. In Chapter 5 it is reported how nitric oxide is chemically formed in nitrate respiring cultures of *E.coli* E4 to which ferrous iron had been added. The use of electron paramagnetic resonance spectroscopy showed that in these cultures a ferro-nitrosyl complex was an important and thusfar unknown reaction intermediate during chemodenitrification. In the course of the experiments described in Chapter 5 it was found that *E.coli* E4 was able to grow aerobically on nitrate as the sole source of nitrogen. In aerobic continuous cultures of *E.coli* E4, nitrate can effectively compete with oxygen for electrons of the respiratory chain despite of its lower electron affinity (Chapter 6). As a consequence, aerobic nitrate ammonification allows *E.coli* E4 to grow on nitrate as the sole source of nitrogen. Although at first sight it would appear energetically unfavorable, it is now widely accepted that nitrate and oxygen can be simultaneously reduced under certain environmental conditions. Aerobic denitrification has indeed become rule rather than exception. In conclusion, it should be stated that from a biogeochemical point of view the data presented in Chapter 6 nevertheless must be interpreted with some caution. Data obtained with reactions occurring in chemostat cultures are

in fact artificial and therefore they have a limited significance. As a consequence, reaction data obtained with chemostats have an important restriction because they do not necessarily reflect reactions occurring under natural conditions in aquifers.

In particular with such a heterogenous thesis, questions about what is new and what is applicable are inevitable. New is: (i) super-saturation of calcite and dolomite as a result of fulvic acid mobilization; (ii) inhibition of sulphate reduction by nitric oxide generated in a chemodenitrification reaction; (iii) formation of ferro-nitrosyl complexes during chemodenitrification; and (iv) aerobic growth of *E.coli* on nitrate as the sole source of nitrogen. The most important results for the application of aquifer thermal energy storage are as follows: (i) calcium and magnesium carbonate precipitation can be partly inhibited by thermally mobilized dissolved organic carbon; (ii) *in situ* treatment of iron rich groundwater can be improved when small amounts of nitrate are added for additional iron precipitation; and (iii) hydrogen sulphide formation and consequently anaerobic metal corrosion can be prevented with the addition of an appropriate amount of nitrate and ferrous iron.

SUMMARY

During the process of aquifer thermal energy storage the *in situ* temperature of the groundwater-sediment system may fluctuate significantly. As a result the groundwater characteristics can be considerably affected by a variety of chemical, biogeochemical and microbiological reactions. The interplay of these reactions may have a negative influence on the operational performance of ATES-systems. The objective of this thesis was to investigate bacterial clogging processes and the biogeochemical aspects of carbonate and iron precipitation in aquifer material obtained from ATES test facilities.

In the General Introduction presented in Chapter 1 the principle of aquifer thermal energy storage is briefly outlined first. This is followed by more detailed information on the thermodynamics of biogeochemical reactions and the conceivable changes in groundwater characteristics during aquifer thermal energy storage. Chapter 2 reports on some microbiological aspects of well clogging during aquifer thermal energy storage. In column experiments well clogging was simulated using aquifer material from a heat storage site and synthetic groundwater. The well clogging potential of oxic and anoxic column effluents was studied at 10° and 30°C using a hollow fiber membrane from which slime depositions were recovered. Only under oxic conditions a slight increase in slime deposition was observed after a temperature rise from 10° to 30°C. No significant difference in bacterial plate counts was measured in oxic and anoxic column effluents, despite the increase in dissolved organic material concentrations at elevated temperatures. This organic material was mobilized from the soil particular organic carbon fraction. The biologically available organic carbon concentration was less than 1% of the dissolved organic carbon concentration, which was not enough to allow excessive bacterial growth or slime formation. Chapter 3 reports on the thermal mobilization of dissolved organic carbon and the concomitant carbon dioxide production in aquifer material from a heat storage site. These processes have been quantified aerobically and anaerobically within a temperature range of 4° to 95°C in sediment samples containing either quartz-rich coarse sand or peaty clay. At temperatures above 45°C dissolved organic carbon compounds, including fulvic acids, were mobilized from both sediments resulting in a substantial increase in the chemical oxygen demand of the water phase. Complexation of calcium and

magnesium by fulvic acids resulted in the super-saturation of the water phase with regard to calcite and dolomite and thus prevented the precipitation of these carbonates. The highest rates of carbon dioxide release were measured during the first four days of incubation. Aerobically, the maximum rate of CO_2 production varied between 35 and 800 (sand) or 15 and 150 (peaty clay) μmol CO_2 per gram volatile solids per day. Anaerobically, the rates were 25 and 500 (sand) or 10 and 110 (peaty clay) μmol CO_2 per gram volatile solids per day. At temperatures above 55°C, CO_2 was produced purely chemically. Chapter 4 deals with ferric iron precipitation in anaerobic Tris-HCl buffered seawater. In these incubations, 40 mM lactate was rapidly dissimilated to acetate by sulphate reducing bacteria after a lag period of three days. In presence of added nitrate or ferric iron (both 1 mM) or a combination of both, the initial lactate consumption rate was slowed down and sulphate reduction started after four days at a similar rate as was observed in the absence of nitrate and ferric iron. Nitrate in combination with ferrous iron totally inhibited sulphate reduction. Some lactate was initially oxidized, but its concentration did not change after day six of incubation. In these incubations ferrous iron was oxidized chemically to ferric iron with a concomitant reduction of nitrite to nitric oxide. In this so-called chemodenitrification process, nitrite was formed biologically from nitrate with lactate as a reductant. In Chapter 5 chemodenitrification was studied in details with *E.coli* E4 as a model bacterium. Both, L-lactate-driven nitrate and ferric iron reduction were investigated. Ferric iron reduction in *E.coli* E4 was found to be constitutive. Contrary to nitrate, ferric iron could not be used as an electron acceptor for growth. Ferric iron reductase activity of 9 nmol $Fe^{2+}.mg^{-1}$ protein.min^{-1} could not be inhibited by well known inhibitors of the *E.coli* respiratory chain. Active cells and the presence of L-lactate were required for ferric iron reduction. The L-lactate-driven nitrate respiration in *E.coli* E4 leading to the production of nitrite, was reduced to about 20% of its maximum activity with 5 mM ferric iron, or to about 50% in presence of 5 mM ferrous iron. The inhibition was caused by nitric oxide formed by a spontaneous chemical reduction of nitrite by ferrous iron. Nitric oxide was further chemically reduced by ferrous iron to nitrous oxide. With electron paramagnetic resonance spectroscopy, the presence of a free ferro-nitrosyl complex was shown. In presence of ferrous or ferric iron and L-lactate, nitrate was anaerobically converted to nitric oxide and nitrous oxide by

the combined action of *E.coli* E4 and spontaneous chemical reduction reactions. Chapter 6 reports on aerobic reduction of nitrate to ammonium in *E.coli* grown in continuous cultures, a novel feature of *E.coli*. Nitrate and nitrite was reduced by *E.coli* E4 in a L-lactate (5 mM) limited chemostat culture at dissolved oxygen concentrations corresponding to 90 - 100% air saturation. Nitrate reductase and nitrite reductase activity was regulated by the growth rate, oxygen and nitrate concentrations. At a low growth rate (0.11 h^{-1}) the measured nitrate and nitrite reductase activities were 200 and 250 $nmol.mg^{-1}protein.min^{-1}$, respectively. At a high growth rate (0.55 h^{-1}) both enzyme activities were considerably lower (25 and 12 $nmol.mg^{-1}protein.min^{-1}$). The steady state nitrite concentration in the chemostat was controlled by the combined action of the nitrate and nitrite reductase. Both enzyme activities were inversely proportional to the growth rate. The nitrite reductase activity decreased faster with the growth rate than the nitrate reductase. The chemostat biomass concentration of *E.coli* E4, with ammonium either solely or combined with nitrate as a source of nitrogen, remained constant throughout all growth rates and was not affected by nitrite concentrations. Contrary to batch, *E.coli* E4 was able to grow on nitrate as the sole source of nitrogen. When cultivated with nitrate as the sole source of nitrogen the chemostat biomass concentration is determined by the combined activities of nitrate and nitrite reductase and hence, inversely proportional to growth rate.

SAMENVATTING

Gedurende de opslag van warmte in ondiep grondwater kan de *in situ* temperatuur van het grondwater-sediment systeem aanzienlijk variëren. Als gevolg hiervan kunnen eigenschappen en samenstelling van grondwater worden beïnvloed door een scala aan chemische, biogeochemische en microbiologische processen. Afzonderlijk, maar ook door onderlinge interaktie, kunnen deze processen een verstorende invloed hebben op de bedrijfsvoering van installaties voor warmte-opslag. Het in dit proefschrift beschreven onderzoek was oorspronkelijk gericht op microbiologische en biogeochemische processen die slijmvorming, kalk-, magnesium- en ijzerneerslagen tot gevolg kunnen hebben en daardoor verstoring van de bedrijfsvoering veroorzaken.

In de Algemene Inleiding (Hoofdstuk 1) wordt eerst uitgelegd waar het bij warmte-opslag in ondiep grondwater om gaat. Vervolgens wordt meer gedetailleerd ingegaan op thermodynamische achtergronden van biogeochemische reakties en de mogelijke veranderingen in grondwaterkwaliteit tijdens warmte-opslag. Hoofdstuk 2 gaat over microbiologische processen die verstopping van onttrekkingsbuizen door slijmvorming kunnen veroorzaken. In kolomexperimenten is verstopping gesimuleerd gebruik makend van synthetisch grondwater en sediment afkomstig van een proefinstallatie voor warmte-opslag. Onder aërobe en anaërobe omstandigheden werd bij zowel 10° als 30°C kolomeffluent door een membraan geleid. Slijmafzettingen in de membraan konden eenvoudig door terugspoelen worden geïsoleerd en gekarakteriseerd. Wanneer de temperatuur van 10° naar 30°C werd opgevoerd kon alleen onder aërobe omstandigheden enige mate van verhoogde slijmafzetting worden gekonstateerd. Het aantal kolonie vormende eenheden in de verschillende effluenten bleek niet of nauwelijks toe te nemen. De concentratie assimileerbare organische koolstof was hierbij in alle gevallen minder dan 1% van de concentratie opgeloste organische koolstof; hetgeen te weinig is om bacteriële groei en slijmvorming te bevorderen. Bij 30°C bleek de concentratie opgeloste organisch koolstof door mobilisatie uit het sediment echter wel toe te nemen. Thermische mobilisatie van opgeloste organische koolstof en de gelijktijdige produktie van kooldioxide zijn de onderwerpen die in Hoofdstuk 3 aan de orde komen. Deze processen zijn met zowel zand als veen sedimenten in een temperatuurgebied van 4° tot 95°C aëroob en anaëroob gekwantificeerd. Bij temperaturen boven 45°C nam de thermische mobilisatie van opgeloste organische koolstof, waaronder fulvine zuren,

voor beide sedimenten aanzienlijk toe. De aldus gemobiliseerde fulvine zuren konden calcium en magnesium complexeren. Hierdoor ontstond een oververzadiging van de waterfase met betrekking tot calciet en dolomiet en werd neerslag van deze carbonaten voorkomen. De hoogste produktiesnelheden van kooldioxide werden gemeten gedurende de eerste vier dagen van incubatie. Onder aërobe omstandigheden varieerde de snelheid van CO_2-produktie tussen de 35 en 800 (zand) of 15 en 150 (veen) μmol CO_2 per gram organische stof per dag. Onder anaërobe omstandigheden waren deze produktiesnelheden 25 en 500 (zand) of 10 en 110 (veen) μmol CO_2 per gram organische stof per dag. Bij temperaturen boven 55°C was de produktie van CO_2 volledig chemisch. Hoofdstuk 4 gaat over de neerslag van ferri-ijzer in anaërobe incubaties met 25mM sulfaat als belangrijkste elektronenacceptor. Onder degelijke omstandigheden werd 40 mM laktaat binnen drie dagen omgezet tot acetaat door sulfaat reducerende bakteriën. Alleen, of gekombineerd, veroorzaakten nitraat of ferri-ijzer (beide 1mM) slechts een geringe vertraging (1 dag) van de laktaat dissimilatie en sulfaat reduktie. Echter, de kombinatie van nitraat met ferro-ijzer remde de sulfaat reduktie totaal. Enige mate van laktaat dissimilatie vond wel plaats maar deze stagneerde volledig na dag zes van inkubatie. In dergelijke inkubaties werd ferro-ijzer chemisch geoxideerd tot ferri-ijzer onder gelijktijdige reduktie van nitriet tot stikstofoxide. In dit zogenaamde chemodenitrifikatie-proces werd nitriet biologisch gevormd uit nitraat met laktaat als elektronendonor. In Hoofdstuk 5 wordt gedetailleerd verslag gedaan van chemodenitrifikatie met behulp van *E.coli* E4 als modelbakterie. L-laktaat gedreven nitraatrespiratie en ferri-ijzer reduktie werden in dit organisme bestudeerd. Het vermogen tot ferri-ijzer reduktie bleek in *E.coli* E4 konstitutief aanwezig te zijn. In tegenstelling tot nitraat kon ferri-ijzer echter niet worden benut als elektronenacceptor voor de groei. De maximaal waargenomen ferri-reduktase-aktiviteit van 9 nmol $Fe^{2+}.mg^{-1}$eiwit.min kon niet worden geremd door welbekende remmers van de ademhalingsketen van *E.coli*. Aktieve cellen en L-laktaat waren niettemin nodig voor de reduktie van ferri-ijzer. De L-laktaat gedreven nitraatrespiratie in *E.coli* E4 werd voor 50% geremd door Fe^{3+} maar voor 20% door Fe^{2+} (beide 5 mM). Deze remming werd veroorzaakt door stikstofoxide gevormd uit een spontane chemische reduktie van nitriet door ferro-ijzer. Stikstofoxide werd met ferro-ijzer chemisch verder gereduceerd tot lachgas. Met behulp van elektron paramagnetische resonantie (EPR) spectroscopie kon in aanwezigheid van Fe^{2+} een vrij

ferro-nitrosyl complex worden aangetoond. De vorming van een dergelijk complex kan het waargenomen verschil in remming van nitraatrespiratie door Fe^{3+} en Fe^{2+} goed verklaren. In aanwezigheid van ferro- of ferri-ijzer en L-laktaat werd nitraat anaëroob omgezet naar stikstofoxide en lachgas door een gekombineerde aktie van *E.coli* E4 en spontane chemische reduktiereakties. Hoofdstuk 6 gaat over een tot dusverre onbekende eigenschap van E.coli: aërobe groei met nitraat als enige stikstofbron. In een L-laktaat (5 mM) gelimiteerde kontinue kultuur van *E.coli* E4 werden zowel nitraat als nitriet gereduceerd bij opgeloste zuurstofconcentraties overeenkomend met 90-100% luchtverzadiging. De aktiviteit van nitraat- en nitrietreduktase werd niet alleen bepaald door de aanwezigheid van zuurstof en nitraat maar ook door de groeisnelheid. Bij lage groeisnelheden (0.11 h^{-1}) waren de gemeten aktiviteiten van nitraat- en nitrietreduktase respektievelijk 200 en 250 $nmol.mg^{-1}eiwit.min^{-1}$. Bij hoge groeisnelheden (0.55 h^{-1}) waren beide enzymaktiviteiten aanzienlijk lager (25 en 12 $nmol.mg^{-1}eiwit.min^{-1}$). De evenwichtsconcentratie van nitriet in de chemostaat werd bepaald door de gekombineerde aktie van nitraat-en nitrietreduktase. Beide enzymaktiviteiten waren omgekeerd evenredig met de groeisnelheid van *E.coli* E4. De nitrietreduktase-aktiviteit nam sneller af met de groeisnelheid dan de nitraatreduktase-aktiviteit. Met ammonium als stikstofbron, al of niet gekombineerd met nitraat, was de biomassaconcentratie in de chemostaat konstant bij alle groeisnelheden. Bij aërobe groei op nitraat als enige stiktofbron was de biomassaconcentratie in de chemostaat omgekeerd evenredig met de groeisnelheid; hetgeen een logische gevolg is van dalende nitraat- en nitrietreduktase-aktiviteiten.

NAWOORD

Op de omslag van een proefschrift staat doorgaans alleen de naam van de promovendus vermeld. De nietsvermoedende lezer zal zich wellicht niet afvragen wie er allemaal hebben meegewerkt om het geheel tot een goed einde te brengen. Ten onrechte, want voor U ligt het uiteindelijke resultaat van drie jaar onderzoek en intensieve samenwerking. Het is mij daarom een genoegen om op deze plaats een woord van dank te kunnen richten tot al diegenen die een bijdrage hebben geleverd aan dit proefschrift.

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In dit onderzoek werd ik verder geholpen door Jan van Doesburg (vakgroep Bodemkunde en Geologie) met röntgen diffraktiemetingen en Fred Hagen (vakgroep Biochemie) met EPR spectroscopie.

Door de interdisciplinaire samenwerking met het Instituut voor Aardwetenschappen van de Vrije Universiteit is het biogeochemische karakter van dit onderzoek goed tot uitdrukking gekomen. Speciaal wil ik hier bedanken Tony Appelo en Jasper Griffioen voor hun hydrochemische en modelmatige inbreng tijdens de vele discussies, voor, tijdens en vooral ook na de welbekende Expert Meetings. Voor de coördinatie in IEA-verband ben ik Guus Willemsen en Aart Snijders (IF Technology, Arnhem) erkentelijk voor hun inspanningen.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 21 maart 1954 geboren te Den Haag. Lager onderwijs genoot hij te Vlissingen, Singapore, Koudekerke en Renesse. Vervolgens bezocht hij de Rijksscholengemeenschap Professor Zeeman te Zierikzee en behaalde aldaar de diploma's mulo-b (1970) en hbs-b (1972).

In het daaropvolgende jaar werd begonnen met de studie Milieuhygiëne aan de Landbouwhogeschool te Wageningen. Het doctoraalpakket bestond uit een hoofdvak waterzuivering en een verzwaard bijvak microbiologie. Praktijkervaring met afvalwatertechnologie en proceskunde werd opgedaan bij de CSM suikerfabriek te Groningen.

Na het afstuderen, in september 1981, was de auteur tot december 1983 werkzaam bij de Landbouwhogeschool, vakgroep Waterzuivering. In die periode heeft hij binnen de werkgroep anaërobe zuiveringstechnologie onderzoek verricht naar de biologische afbreekbaarheid van zuiveringsslib en mest.

Van december 1983 tot oktober 1985 was de auteur met een ZWO-beurs werkzaam bij de vakgroep Microbiologie van de Landbouwhogeschool. In samenwerking met de vakgroepen Biochemie en Organische Chemie is gewerkt aan de toepassing van xanthine-oxidase uit *Arthrobacter* M4 bij de regiospecifieke oxidatie van heteroaromaten.

Van oktober 1985 tot juni 1989 was de auteur werkzaam bij de LUW vakgroep Microbiologie, werkgroep xenobiotica. In deze periode is met het Instituut voor Aardwetenschappen van de Vrije Universiteit samengewerkt inzake de biogeochemische aspekten van warmte-opslag in aquifers; hetgeen uiteindelijk heeft geresulteerd in dit proefschrift.

Van juli 1989 tot juli 1991 was de auteur werkzaam bij de firma EuroCetus te Amsterdam. Binnen de afdeling Process Development heeft hij gewerkt aan de inrichting en validatie van een proeffabriek voor de biotechnologische bereiding van farmaceutische produkten.

Thans is de auteur werkzaam bij het Laboratorium voor Bacteriële Vaccins van het RIVM te Bilthoven. Als hoofd van de afdeling Produkt- en Procesontwikkeling werkt hij aan de procesmatige opschaling van nieuwe vaccins.