Working Report 2012-04

Microbial Analyses of Cement and Grouting Additives

Lotta Hallbeck
Sara Jägevall
Anna Pääjärvi
Lisa Rabe
Johanna Edlund
Sara Eriksson

January 2012

POSIVA OY
FI-27160 OLKILUOTO, FINLAND
Tel +358-2-8372 31
Fax +358-2-8372 3709

Working Report 2012-04

Microbial Analyses of Cement and Grouting Additives

Lotta Hallbeck
Sara Jägevall
Anna Pääjärvi
Lisa Rabe
Johanna Edlund
Sara Eriksson
Microbial Analytics Sweden AB

January 2012

ABSTRACT

During sampling in the ONKALO tunnel in 2006, heavy growth of a slimy material was observed in connection with grouting. It was suggested to be microbial growth on organic additives leaching from the grout. Two sampling campaigns resulted in the isolation of several aerobic bacterial strains. Some of these strains were used in biodegradation studies of three solid cement powders, eight liquid grout additives, and six plastic drainage materials. Degradation was also studied using ONKALO groundwaters as inoculums. The isolated strains were most closely related to hydrocarbon-degrading microorganisms. The biodegradation of seven of the products was tested using microorganisms isolated from the ONKALO slime in 2006; none of these strains could degrade the tested products. When ONKALO drillhole groundwaters were used as inoculums in the degradation studies, it was demonstrated that Structuro 111X, Mighty 150, and Super-Parmix supported growth of the groundwater microorganisms. Structuro 111X is a polycarboxylate condensate while Mighty 150 and Super-Parmix are condensates with formaldehyde and naphthalene. Some of the isolated microorganisms belonged to the genus *Pseudomonas*, many strains of which can degrade organic molecules. None of the plastic drainage materials supported growth during the degradation studies. Microorganisms were present in two of the liquid products when delivered, GroutAid and Super-Parmix. The potential of the organic compounds in grout additives to be degraded by microorganisms, increasing the risk of biofilm formation and complexing compound production, must be considered. Microbial growth will also increase the possibility of hydrogen sulphide formation.

Keywords: Organic additives, grouting materials, plastic drainage materials, microbes.

SEMENTIN JA SEN LISÄAINEIDEN MIKROBIANALYYSIT

TIIVISTELMÄ

ONKALOsta vuonna 2006 otetuissa näytteissä havaittiin limamaisen materiaalin kasvua injektointimateriaalissa. Yhtenä selityksenä pidettiin sitä, että mikrobit kasvaisivat injektointimateriaalista liukenevissa orgaanisissa lisäaineissa. Kahdessa näytteenotto-kampanjassa pystyttiin eristämään useita aerobisia bakteerikantoja. Osaa näistä kannoista käytettiin kolmen kiinteän sementtipulverinäytteen, kahdeksan nestemäisen injektoinnin lisäainenäytteen ja kuuden muovisen putkinäytteen biohajoamiskokeissa. Eristettyjä bakteerikantoja tutkittiin myös käyttämällä ONKALOsta otettuja pohja-vesinäytteitä inokulaatteina. Eristetyt bakteerikannat olivat todennäköisesti hiilivetyä hajottavia mikro-organismeja.

Seitsemän näytteen biohajoamista tutkittiin käyttämällä ONKALOn limanäytteestä eristettyjä mikro-organismeja. Yksikään bakteerikannoista ei hajottanut testattuja tuot-teita. Kun ONKALOn kairanrei'istä otettuna pohjavesinäytteitä käytettiin inokulaatteina hajoamiskokeissa, havaittiin, että Structuro 111X, Mighty 150 ja Super-Parmix lisäsivät pohjaveden mikro-organismien kasvua. Structuro 111X on polykarboksylaattikonden-saatti, kun taas Mighty 150 ja Super-Parmix ovat formaldehydin ja naftaliinin konden-saatteja.

Osa eristetyistä mikro-organismeista kuului *Pseudomonas* -sukuun, joista useimmat hajottavat orgaanisia molekyylejä. Mikään muovisista putkinäytteistä ei lisännyt kasvua hajoamiskokeiden aikana. Alkuhetkellä kahdessa nestemäisessä näytteessä, Grout-Aid:ssa ja Super-Parmixissa, havaittiin mikro-organismeja. Injektointimateriaalissa olevien orgaanisten lisäaineiden mahdollisuutta hajota mikro-organismien takia kasvat-taen samalla riskiä, että biofilmejä ja kompleksoivia yhdisteitä muodostuu, on syytä harkita. Mikrobien kasvu lisää myös vetysulfidien muodostumisen todennäköisyyttä.

Avainsanat: Orgaaniset lisäaineet, injektointimateriaalit, muoviset putkimateriaalit, mikrobit.

TABLE OF CONTENT

1 INTRODUCTION

In 2006, heavy growth of a slimy material was observed in the ONKALO tunnel at locations where grouting was used. Samples for microbial analysis were taken from these places and analysed by Microbial Analytics Sweden AB on two occasions, October 2006 and October 2007. The results for these samples were summarised by Pedersen et al. (2008).

The project and results presented in the present report concern the microbiological investigation of some grouting products used in the tunnel. There are reports in the literature describing microbial growth problems, especially in drinking water systems constructed of cement and concrete products.

The project was performed in several steps. The experimental sequence started by determining the microorganisms present in the various cement powders and additives by means of growth studies in aerobic cultures. The tested products included four cement powders, eight liquid additives of different kinds, and six drainage materials. Thereafter, the potential of the materials to function as microbial growth substrates was studied using three bacterial strains isolated from ONKALO slime during previous sampling. This experiment was interrupted halfway through because very little growth had occurred on the products, and two other bacterial strains with different degradation capabilities were chosen. The degradation experiments with pure cultures continued with these strains. Furthermore, degradation experiments were performed using microorganisms from several Olkiluoto drillholes. Microorganisms isolated in the growth experiments examining additives and groundwater were identified by sequencing the 16S rRNA gene, a molecular biology method.

2 MATERIALS AND METHODS

The materials studied were sent by POSIVA to the Microbial Analytics laboratory in Gothenburg, Sweden. The first studies examined three solid cement powders and eight liquid additives. The products, manufacturers, and active components are presented in Table 1; information about the products comes from the product safety sheets and the manufacturers.

Table 1-1. *Materials tested microbiologically for growth, together with their manufacturers and active components.*

Manufacturer	Product	Component
Finnsementti Oy	1. SR-sementti	Cement
Scancem Chemicals	2. Mighty 150	Sulphonated naphthalene condensate
Elkem ASA	3. GroutAid	Silicon dioxide
Rescon Mapei	4. Mapequick AF-2000	2,2-iminodiethanol (5–10 %), aluminium sulphate (30–60 %) (and other inorganic aluminium salts)
Cementa AB	5. Ultrafin 16	Cement
Nedmag	6. $CaCl_2$	Calcium chloride 36 %
Cementa AB	7. SetControl II	Sulphonated naphthalene polymers (1–10 %), calcium nitrate (30–60 %)
Finnsementti Oy	8. Parmix silica	Amorphous SiO_2 (80–99 %)
Semtu Oy	9. Structuro 111X	Polycarboxylate polymer
Finnsementti Oy	10. Super-Parmix	Naphthalene formaldehyde polycondensate
Finnsementti Oy	11. Rapid sementti	Portland cement (75–90 %)
Scancem Chemicals (SIKA, Finland)	12. Scancem SP-40*	Sulphonated melamine condensate

* Not tested; unavailable.

2.1 Growth experiment 1 – Determination of cultivable aerobic bacteria in grouting products

This experiment sought to elucidate whether microorganisms were present in the products as delivered by the manufacturer. The method determined the number of aerobic, heterotrophic microorganisms.

2.1.1 Growth experiment setup

Petri dishes containing agar and nutrients were prepared as described elsewhere (Pedersen and Ekendahl 1990) for determining the numbers of cultivable heterotrophic aerobic bacteria (CHAB) in grouting product samples. Two 10× dilution series of grouting products were made in sterile analytical-grade water (Millipore Elix 3, Millipore, Solna, Sweden) containing 1.0 g L^{-1} of NaCl and 0.1 g L^{-1} of K_2HPO_4; 0.1-mL portions of each dilution plus the original solution were spread with a sterile glass

rod on the plates in triplicate, for a total of nine plates per product. The plates were incubated for seven to nine days at 20 °C, after which the number of colony-forming units was counted; plates with 10–200 colonies were used. Details of the cultivation method can be found in et al. Pedersen (2008).

The same method was used for the solid cement products, except that 0.05 g of the powder were initially transferred to 10 mL of sterile dilution solution, called the original concentration. The original concentration was then diluted in two 10× dilutions. The inoculation and incubation were done using the original concentration plus the two dilutions, totalling nine plates per cement product.

From the growth plates, microorganisms from the highest dilution series were isolated into pure cultures for identification using standard microbiological methods for isolating pure cultures on solid media (Kriegh and Gerhardt 1994).

2.1.2 DNA extraction

Total genomic DNA was extracted from one colony using the DNeasy Blood & Tissue kit (no. 69504; QIAGEN, Solna, Sweden) according to the manufacturer's protocol for Gram-positive bacteria. The DNA extractions were stored at –20 °C.

2.1.3 PCR, cloning, and sequencing

Eubacterial primers 27f and 1492r were used for the 16S rDNA PCRs (Lane et al. 1985; Lane 1991). The PCR mixtures for the bacterial 16S rRNA contained 0.5 μL of each primer solution (10 pmol μL^{-1}), 20 ng of DNA, and 12.5 μL × 2 of iProof Master Mix (no. 172-5310; Bio-Rad Laboratories, Sundbyberg, Sweden) in a final reaction volume of 25 μL.

Amplification was carried out on a thermal PCR cycler (MyCycler; Bio-Rad Laboratories). After initial denaturation for 3 min at 98 °C, 30 cycles were performed, each cycle consisting of 30 s at 98 °C, 90 s at 59 °C, and 90 s at 72 °C; a final extension step was carried out for 7 min at 72 °C. PCR products were visualised on 1 % agarose gel containing ethidium bromide (EtBr) (no. 161-0433; Bio-Rad Laboratories) and the bands were then cut out and purified using a gel extraction kit (no. 28604; QIAGEN) according to the manufacturer's protocol. The 16S rDNA gene sequences were then determined by the MWG Biotech sequencing team (Ebersberg, Germany) using the universal internal 16S rRNA primer 907r and then aligned with sequences from GenBank using the BLAST tool at NCBI (http://www.ncbi.nlm.nih.gov) based on high BLAST score similarities (Altschul and Erickson 1985).

2.2 Growth experiment 2 – Growth of three isolated groundwater microorganisms on sterile additives and cement

These experiments were performed to analyse whether the products contained any substances that could act as microbial growth substrates. Three strains of bacteria isolated from ONKALO slime in October 2007 were used as test organisms, i.e., *Hydrogenophaga taeniospiralis*, *Hydrogenophaga atypical*, and one *Flavobacteriaceae* strain (Pedersen et al., 2008). The experiment was divided into two parts because of its

size. Only growth experiment 2a was performed, since the bacterial strains chosen lacked the proper metabolism for the initial degradation of the complex organic substances in the tested products. The procedure for growth experiment 2a is described below.

2.2.1 Growth medium

The growth medium was a liquid medium of the same composition as that used for the CHAB plates in growth experiment 1, but without added agar. The 25-mL growth cultures were placed in sterile 50-mL Erlenmeyer flasks closed with cotton plugs.

2.2.2 Additions of test products

The liquid products were sterilised by filtration. The products were filtered directly into the medium together with 5 mL L^{-1} of medium through a sterile filter with a pore size of 0.2 µm. This procedure revealed that GroutAid could not be filter sterilised, so this product was instead autoclaved in a glass bottle. After cooling, 5 mL of the sterile product were added per litre of medium.

The solid products were treated as in growth experiment 1, i.e., 0.5 g of the product were added to 10 mL of sterile dilution solution. The tubes were shaken vigorously and then centrifuged. Five mL L^{-1} of supernatant was added to the medium, as in the case of the liquid products.

Cultures of the three organisms were prepared in duplicate for the products, giving six cultures of each. In addition, one sterile medium-containing flask was made for each product. As negative control, medium without added product was used for each organism; as positive control, the organisms were grown in nutrient broth (NB), a rich multi-purpose medium. The experimental setup is presented in Tables 1-2 and 1-3.

Table 1-2. *Test products and organisms used in growth experiment 2a.*

Product	***Hydrogenophaga taeniospiralis***	***Hydrogenophaga atypica***	***Flavobacteriaceae* strain**	Sterile	Total
SR-Sementti (cement)	2	2	2	1	7
Mighty 150 (liquid)	2	2	2	1	7
GroutAid (liquid)	2	2	2	1	7
Mapequick AF-2000 (liquid)	2	2	2	1	7
Ultrafin 16 (liquid)	2	2	2	1	7
$CaCl_2$ (liquid)	2	2	2	1	7
Cementa SetControl II (liquid)	2	2	2	1	7
Totals	14	14	14	7	49

Table 1-3. *Organisms and controls in growth experiment 2a.*

Bacterium	Medium (negative control)	NB (positive control)	Total
Hydrogenophaga taeniospiralis	2	2	4
Hydrogenophaga atypical	2	2	4
Flavobacteriaceae strain	2	2	4

The inoculums were washed cells of pre-grown cultures of the studied strains. The growth cultures and controls were incubated on shakers at room temperature in the dark for seven days.

The numbers of cultivable aerobic bacteria in the cultures were determined by plate count, as in the case of CHAB. Turbid cultures were diluted in a 10× series and the 100×, 1000×, and 10,000× series were inoculated on CHAB plates in triplicate. Clear cultures were also diluted in a 10× series, but only the 100× and 1000× series were spread on agar plates in triplicate. The plates were incubated at room temperature for up to one week or until clearly visible colonies appeared. The colonies were counted and the cell numbers in the growth cultures were calculated.

2.3 Growth experiment 3 – Growth of two isolated groundwater microorganisms on sterile additives and cement

This growth experiment followed the outline of growth experiment 2, but used two strains of *Pseudomonas* also isolated from ONKALO slime in October 2007. These strains were identified as *P. stutzeri*, denoted NRB 5-4 OL-KR40, and *P. fluorescens*, denoted NRB 1-2, OL-KR13 (Pedersen et al. 2008).

2.3.1 Growth medium

The growth medium was a liquid medium of the same composition as that used for the CHAB plates in growth experiment 1, but without added agar. The 25-mL growth cultures were placed in sterile 50-mL Erlenmeyer flasks closed with cotton plugs.

2.3.2 Additions of test products

All liquid products were sterilised by autoclaving in glass bottles; after cooling, 5 mL were added per litre of medium. The solid products were treated as in growth experiment 1, i.e., 0.5 g of the product were added to 10 mL of dilution solution. The tubes were shaken vigorously and then centrifuged. The supernatants were autoclaved and cooled before adding 5 mL L^{-1} of medium to the growth bottles, as in the case of the liquid products.

For each product, two cultures were prepared for each of the three organisms, giving four cultures for each product. In addition, one sterile medium-containing flask was made for each product. As negative control, medium without added product was used for each organism; as positive control, the organisms were grown in rich multi-purpose NB. The experimental setup is presented in Tables 1-4 and 1-5.

Table 1-4. *The experimental setup for growth experiment 3: growth with sterile cement and additive products.*

Product	*Pseudomonas stutzeri*	*Pseudomonas fluorescens*	Sterile (uninoculated)	Total
1. SR-Sementti (solid)	2	2	1	5
2. Mighty 150 (liquid)	2	2	1	5
3. GroutAid (liquid)	2	2	1	5
4. Mapequick AF-2000 (liquid)	2	2	1	5
5. Ultrafin 16 (Ultrafin 16) (fast)	2	2	1	5
6. $CaCl_2$ (liquid)	2	2	1	5
7. Scancem SP-40	2	2	1	5
8. Parmix silica	2	2	1	5
9. Structuro 111X	2	2	1	5
10. Super-Parmix	2	2	1	5
11. Rapid sementti	2	2	1	5
Total	22	22	11	55

Table 1-5. *Controls used in growth experiment 3.*

Bacterium	Medium, no additives (Negative)
Pseudomonas stutzeri	2
Pseudomonas fluorescens	2
Sterile	1
Total	5

The inoculums were pre-grown and washed cells of the studied strains. The growth cultures and controls were incubated on shakers at room temperature in the dark for seven days.

The numbers of cultivable aerobic bacteria in the cultures were determined by plate count as in the case of CHAB. Turbid cultures were diluted in a 10× series and the 100×, 1000×, and 10,000× series were inoculated on CHAB plates in triplicate. Clear cultures were also diluted in a 10× series, but only the 100× and 1000× series were spread on agar plates in triplicate. The plates were incubated at room temperature for up to one week or until clearly visible colonies appeared. The colonies were counted and the cell numbers in the growth cultures were calculated.

2.4 Growth experiment 4 – Growth of groundwater microorganisms on sterile additives and cement

In this experiment, the same growth medium and sterile additions of the cement and additives were used as in growth experiments 2 and 3. In growth experiment 4, groundwater microorganisms were used as inoculums.
The groundwater was collected from drillholes ONK-KR 1, ONK-KR 2, ONK-KR 3,

ONK-PVA 1, and ONK-PVA 2 in ONKALO. The water was sampled overnight (13–14 January 2009) by personnel from Microbial Analytics Sweden AB and brought back to the laboratory in Sweden on the 14 January. The samples were transported cooled and placed in a refrigerator immediately upon arrival. The cultures were inoculated as soon as the samples arrived at the lab.

2.4.1 Growth medium

The growth medium was a liquid medium of the same composition as that used for the CHAB plates in growth experiment 1, but without added agar (see Section 1.1.1). Sterile 50-mL Erlenmeyer flasks closed with cotton plugs and containing 25 mL of growth medium were used as growth vessels.

2.4.2 Additions of test products

The additions of test products followed the procedure used in growth experiment 3, as described above.

2.4.3 Inoculum

The groundwater samples were pooled to obtain three different test samples. To a sterile vessel, an equal volume of each groundwater was added as follows:

Sample 1 = ONK-KR 2 + ONK-PVA 1

Sample 2 = ONK-KR 3 + ONK-PVA 2 + ONK-KR 1

Sample 3 = ONK-KR1

One mL of each sample was added to each growth culture; the cultures were prepared in duplicate. The samples were pooled due to the results of previous molecular characterisations of microbial populations in the drillhole groundwaters (Pedersen et al. 2008). Groundwater samples with similar microorganism compositions were pooled to reduce the number of growth cultures used. As controls, growth cultures identical to the sample cultures, but without added products, were used. The growth cultures and controls were incubated on shakers at room temperature in the dark for seven days.

The numbers of cultivable aerobic bacteria in the different cultures were determined by plate count as in the case of CHAB. Turbid cultures were diluted in a 10× series and the 100×, 1000×, and 10,000× series were inoculated on CHAB plates in triplicate. Clear cultures were also diluted in a 10× series, but only the 100× and 1000× series were spread on agar plates in triplicate. The plates were incubated at room temperature for up to one week or until clearly visible colonies appeared. The colonies were counted and the cell numbers in the growth cultures were calculated.

Table 1-6. *The experimental setup for growth experiment 4: growth with sterile cement and additive products with groundwater microorganisms.*

Product	Sample 1	Sample 2	Sample 3	Sterile	Total
1. SR-Sementti	2	2	2	1	7
2. Mighty 150	2	2	2	1	7
3. GroutAid	2	2	2	1	7
4. Mapequick AF-2000	2	2	2	1	7
5. Ultrafin 16	2	2	2	1	7
6. $CaCl_2$	2	2	2	1	7
7. Cementa SetControl II	2	2	2	1	7
8. Parmix silica	2	2	2	1	7
9. Structuro 111X	2	2	2	1	7
10. Super-Parmix	2	2	2	1	7
11. Rapid sementti	2	2	2	1	7
12. No addition	2	2	2	1	7
Total	24	24	24	12	84

Table 1-7. *Controls used in growth experiment 4.*

Sample	Medium, no additives (negative)
Sample 1	2
Sample 2	2
Sample 3	1
Total	5

Table 1-8. *Growth experiment 1 isolates selected for sequencing.*

Culture	Sample 1	Sample 2	Sample 3
2. Mighty 150 (liquid)	3:10,000× pink	1:10,000× white (pink)	3:1000× white
3. GroutAid (liquid)	2:10,000× pink	1:10,000× pink	3: 10,000× yellow
4. Mapquick AF-2000 (liquid)	1:10,000× black	-	-
7. Cementa SetControl II	1:1000× pink	3:10,000× pink	-
9. Structuro 111X	1:1000× white, smooth	2:10,000× white, smooth	-
10. Super-Parmix	1:10,000× pink-red	2:10,000× white	3:10,000× yellow

From the plates used for determining aerobic bacteria, pure cultures were obtained by isolation onto new agar plates. These bacterial strains were identified by sequencing the 16S rRNA gene. Table 1-8 above summarises the selected isolates.

2.5 Growth experiment 5 – Growth of groundwater microorganisms on solid drainage material

This experiment determined whether material used for drainage could enhance and support microorganism growth. The various drainage materials were cut into small pieces and added to the growth medium with no other carbon and energy source; the cultures were inoculated with microorganisms present in fresh Olkiluoto groundwater.

2.5.1 Growth medium

The growth medium was a liquid medium of the same composition as that used for the CHAB plates in growth experiment 1, but without added agar. The 25-mL growth cultures were placed in sterile 50-mL Erlenmeyer flasks closed with cotton plugs.

The solid materials used are listed in Table 1-6. The plastics were cut into small pieces. To each prepared growth-medium-filled Erlenmeyer flask, 0.5 g of plastic material was added. All samples were tested in duplicate. Growth cultures without added plastic materials were used as negative controls. The experimental setup is presented in Table 1-6.

Table 1-9. *The experimental setup for growth experiment 5: growth with drainage products and groundwater microorganisms.*

Product	Sample 1	Sample 2	Sample 3	*Total*
White cellular plastic	2	2	2	6
Grey cellular plastic	2	2	2	6
Yellow drainage pipe	2	2	2	6
Enkadrain TP	2	2	2	6
Enkadrain 10D	2	2	2	6
Enkadrain ST	2	2	2	6
No addition	2	2	2	6
Total	14	14	14	42

Table 1-10. *Controls used in growth experiment 5.*

Sample	Medium, no additives (negative)
Sample 1	2
Sample 2	2
Sample 3	1
Total	5

2.5.2 Inoculum

Groundwater samples were collected overnight by POSIVA personnel and sent to the laboratory in Gothenburg where inoculation was done.

The groundwater samples were pooled to obtain three different test samples. To a sterile vessel, an equal volume of each groundwater was added as follows:

Sample 1 = ONK-KR 2 + ONK-PVA 1

Sample 2 = ONK-KR 3 + ONK-PVA 2 + ONK-KR 1

Sample 3 = ONK-KR1

One mL of each sample was added to each growth culture; the cultures were prepared in duplicate. The samples were pooled due to the results of previous molecular characterisations of microbial populations in the drillhole groundwaters (Pedersen et al. 2008). Groundwater samples with similar microorganism compositions were pooled to reduce the number of growth cultures used. As controls, growth cultures identical to the sample cultures, but without added products, were used. The growth cultures and controls were incubated on shakers at room temperature in the dark for seven days.

The numbers of cultivable aerobic bacteria in the cultures were determined by plate count as in the case of CHAB. Turbid cultures were diluted in a 10× series and the 100×, 1000×, and 10,000× series were inoculated on CHAB plates in triplicate. Clear cultures were also diluted in a 10× series, but only the 100× and 1000× series were spread on agar plates in triplicate. The plates were incubated at room temperature for up to one week or until clearly visible colonies appeared. The colonies were counted and the cell numbers in the growth cultures were calculated.

3 RESULTS

3.1 Growth experiment 1 – Determination of cultivable aerobic bacteria in grouting products

3.1.1 CHAB

This experiment determined the aerobic bacteria present in the tested products. Bacterial growth was found in two products, GroutAid from Elkem ASA and Super-Parmix from Finnsementti Oy. The values are given as $>2 \times 10^5$ mL^{-1}, because all agar plates in all dilution series were overgrown, making it impossible to give a more exact value. No growth was observed in any other product.

Table 2-1. *Results from determining CHAB in the tested product.*

Product	Component	CHAB (mL^{-1})
1. SR-sementti	Cement	0
2. Mighty 150	Sulphonated naphthalene condensate	0
3. GroutAid	Silicon dioxide	$>2 \times 10^5$
4. Mapequick AF-2000	2,2-iminodiethanol (5–10 %), aluminium sulphate (30–60 %) (and other inorganic aluminium salts)	0
5. Ultrafin 16	Cement	0
6. $CaCl_2$	Calcium chloride (36 %)	0
7. SetControl II	Sulphonated naphthalene polymers (1–10 %), calcium nitrate (30–60 %)	0
8. Parmix silica	Amorphous SiO_2 (80-99 %)	0
9. Structuro 111×	Cement	0
10. Super-Parmix	Naphthalene formaldehyde polycondensate	$>2 \times 10^5$
11. Rapid sementti	Portland cement (75–90 %)	0

From the GroutAid and Super-Parmix growth plates, two different types of colonies of each product were isolated (Table 2-2). These were isolated and kept in pure cultures for further identification.

3.1.2 Molecular identification of microorganisms in tested products

From the CHAB agar plates, two types of bacteria were isolated for each product. The DNA sequences of the 16S rRNA gene were sequenced and the closest relative to these strains were identified by comparison with sequences deposited in the GenBank database.

Table 2-2. *Results from the molecular identification of bacterial strains isolated from the products.*

Product	Colony character	Number of base pairs	Accession number	Closest relative	Per cent identity	Comment
GroutAid	White, smooth	790	GU065305	*Nocardioides hankookensis*	97 %	Vinyl chloride degrading
	Yellow, smooth	809	GU065307	*Sphingomonas* sp. HI-K4	99 %	Degrades phenols, dioxins and other hydrocarbons
Super-Parmix	White, smooth	839	GU065304	*Brevibacterium sanguinis*	98 %	Degrades polyaromatic [or poly-aromatic?] hydrocarbons
	Yellow smooth	853	GU065306	*Brachybacterium* sp.	100 %	PAH degrading

More than half the total length of the 16S rRNA gene was sequenced for the four strains, i.e., 790–853 base pairs, and the obtained identities were 97–100 % (Table 2-2). The sequences were deposited in GenBank; for accession numbers, see Table 2-2.

3.2 Growth experiment 2a – Growth of three isolated groundwater microorganisms on sterile additives and cement

Table 2-3 shows the results of the plate counts of growth cultures from growth experiment 2a.

Table 2-3. *Results of growth experiment 2a, growth of three groundwater bacteria on sterile products.*

Product	*Hydrogenophaga taeniospiralis* (mL^{-1})	*Hydrogenophaga atypica* (mL^{-1})	*Flavobacteriaceae* strain TDMA-5 (mL^{-1})
1. SR-sementti	2×10^6	2×10^5	2×10^3
2. Mighty 150	1×10^4	0	0
3. GroutAid	5×10^4	3×10^5	2×10^3
4. Mapequick AF-2000	0	0	0
5. Ultrafin 16	8×10^4	2×10^5	1×10^3
6. $CaCl_2$	7×10^4	1×10^3	0
7. SetControl II	1×10^6	2×10^6	0
8. Parmix silica	n.t.	n.t.	n.t.
9. Structuro 111X	n.t.	n.t.	n.t.
10. Super-Parmix	n.t.	n.t.	n.t.
11. Rapid sementti	n.t.	n.t.	n.t.

n.t. = not tested

Growth of all three bacterial strains was obtained on two cement products, i.e., SR-sementti and Ultrafin 16, and on the liquid additive GroutAid. The *Flavobacteriaceae* strain grew or survived only on these three products; with all other products, no growth of this bacterium was detectable. Actual growth was only found in *Hydrogenophaga taeniospiralis* cultures with five products and in *Hydrogenophaga atypica* cultures with four products (see Table 2-3), i.e., SR-sementti, GroutAid, Ultrafin 16, and SetControl II. No growth was found when Mapequick AF-2000 was tested; this product has a low pH, and pH levels in the growth cultures were <5.

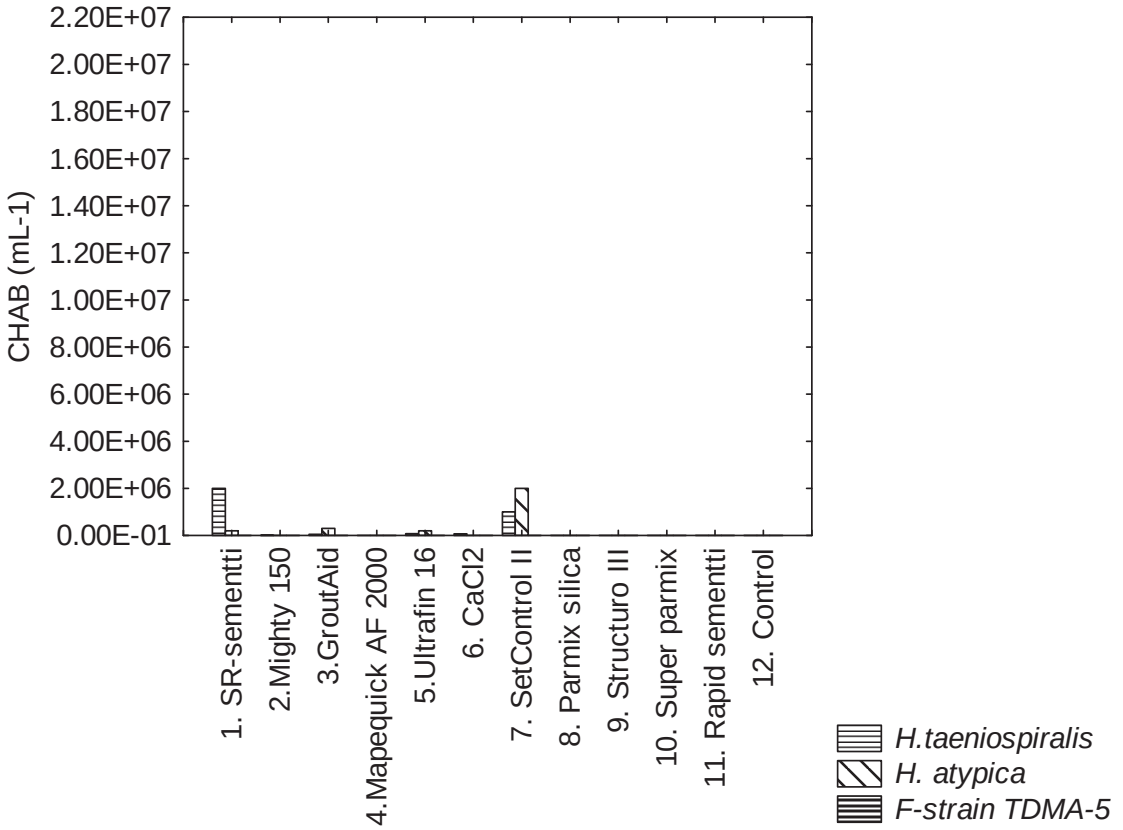

Figure 2-1. *Bar graph of the results of growth experiment 2a with microorganisms isolated from groundwater grown on sterile cement products and additives. Note that this experiment was done with products 1–7 only.*

3.3 Growth experiment 3 – Growth of two isolated aerobic groundwater microorganisms on sterile additives and cement

The results of growth experiment 3 are presented in Table 2-4. Sterile additives did not promote growth to any greater extent for either of the two strains used. The products that allowed growth of *P. stutzeri* to cell numbers higher than 5×10^6 mL^{-1} were Structuro 111X and Rapid sementti, with which numbers reached 6.9×10^6 and 5.6×10^6 mL^{-1}, respectively. *P. fluorescens* grew the best on Mighty 150 and Super-Parmix, with which numbers reached 6.6×10^6 and 7.7×10^6, respectively. The *P. fluorescents* cultures generally grew to higher cell numbers than did *P. stutzeri*.

Table 2-4. *Results of growth experiment 3, growth of two groundwater bacteria on sterile products.*

Product	*Pseudomonas stutzeri*	*Pseudomonas fluorescens*
1. SR-sementti	3.5×10^5	2.8×10^6
2. Mighty 150	1.2×10^6	6.6×10^6
3. GroutAid	2.4×10^5	1.1×10^6
4. Mapequick AF-2000	0	0
5. Ultrafin 16	7.2×10^5	1.5×10^6
6. $CaCl_2$	2×10^6	4.3×10^6
7. SetControl II	6.9×10^5	1.7×10^6
8. Parmix silica	4×10^6	1.9×10^6
9. Structuro 111X	6.9×10^6	1.5×10^6
10. Super-Parmix	3.3×10^6	7.7×10^6
11. Rapid sementti	5.6×10^6	1.5×10^6
12. Control	7.5×10^5	1.6×10^5

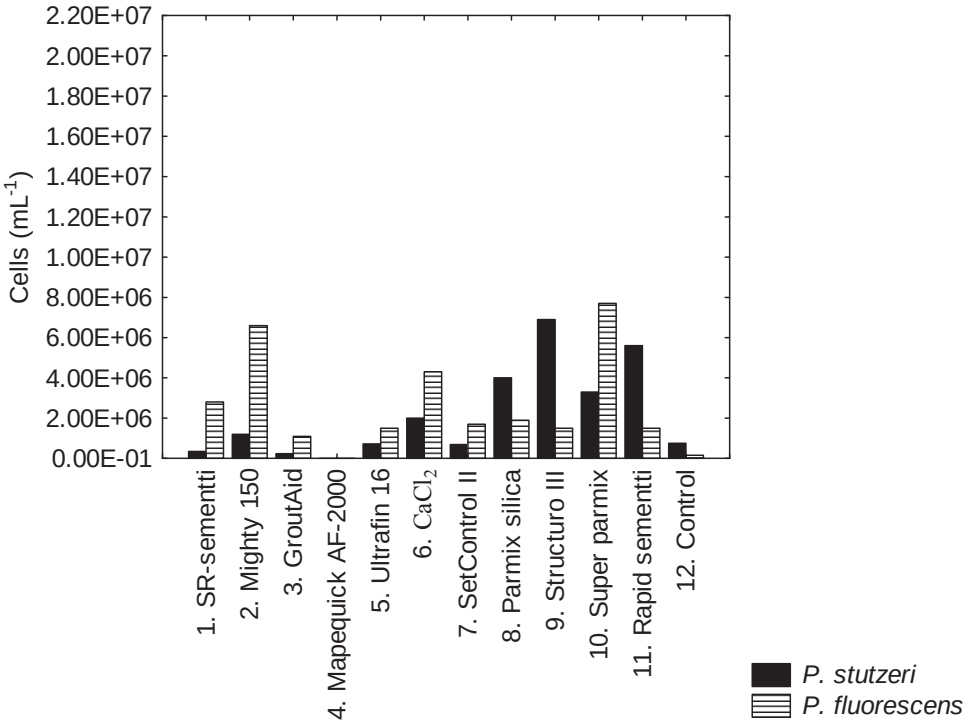

Figure 2-2. *Bar graph of the results of growth experiment 3 with microorganisms isolated from groundwater grown on sterile cement products and additives.*

3.4 Growth experiment 4 – Growth of groundwater microorganisms on sterile additives and cement

Table 2-5 shows the CHAB results for cultures grown on sterile products with inoculums from Olkiluoto groundwater; the results are further presented in bar graphs in Figure 2-3.

Table 2-5. *Results of growth experiment 4, growth of groundwater microorganisms on sterile products.*

Product	Sample 1 (mL^{-1})	Sample 2 (mL^{-1})	Sample 3 (mL^{-1})
1. SR-Sementti	4.4×10^5	7.7×10^5	4.3×10^5
2. Mighty 150	$>2 \times 10^7$	9.8×10^6	2.6×10^5
3. GroutAid	5.6×10^5	2.6×10^6	1.2×10^6
4. Mapquick AF-2000	6.9×10^4	0	0
5. Ultrafin 16	4.1×10^5	6.2×10^5	8.0×10^4
6. $CaCl_2$	5.4×10^4	1.3×10^5	8.2×10^5
7. Cementa SetControl II	5.0×10^5	1.9×10^6	7.2×10^4
8. Parmix silica	5.3×10	3.4×10^5	5.3×10^5
9. Structuro 111X	3.1×10^6	$>2.0 \times 10^7$	5.0×10^5
10. Super-Parmix	1.0×10^6	$>2.0 \times 10^7$	9.8×10^5
11. Rapid sementti	6.6×10^4	2.2×10^5	5.2×10^4
12. No addition	1.3×10^5	1.8×10^5	2.7×10^4

The products that supported the growth of the microorganisms from Olkiluoto groundwater were Mighty 150, Structuro 111, and Super-Parmix. The highest number of CHAB was found in the Mighty 150 culture inoculated with sample 1 and in Structuro 111 and Super-Parmix cultures inoculated with sample 2. All these cultures grew to cell numbers $>2 \times 10^7$ mL, the highest number reportable with the dilution series used in the growth determinations in Experiment 4; the actual cell numbers could have been higher.

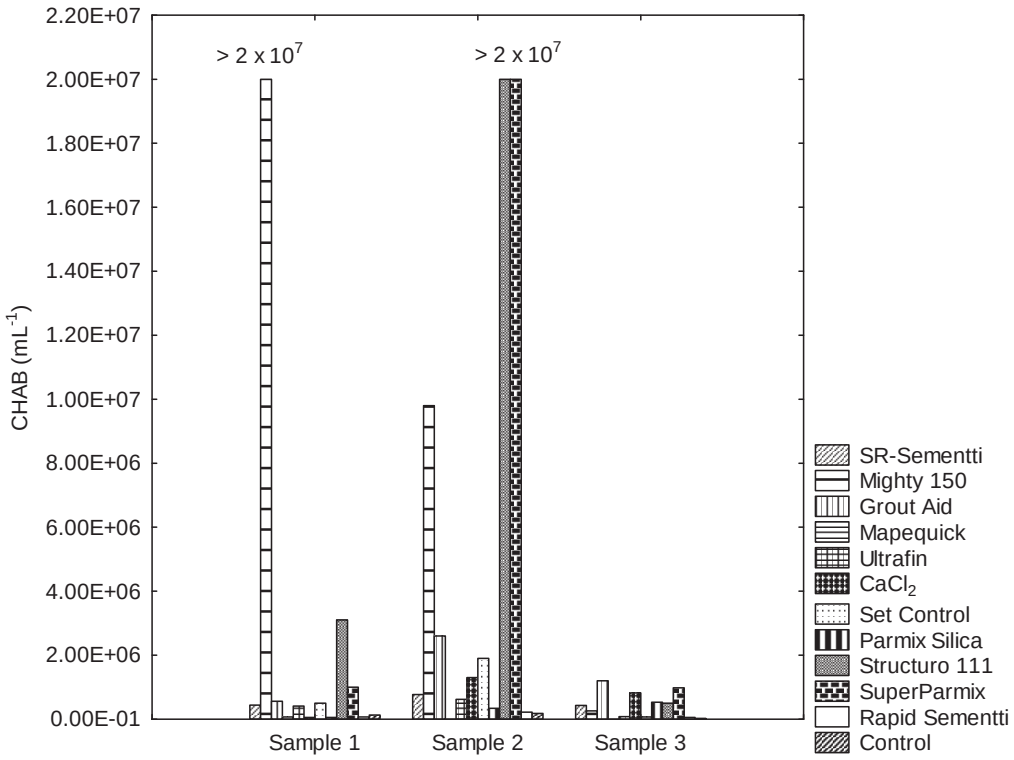

Figure 2-3. *Bargraph of the results of growth experiment 4 with groundwater microorganisms grown on sterile cement products and additives.*

Table 2-6. *Information on the identity of strains isolated in growth experiment 4.*

Isolate (accession no)	Product	Sample no.	Number of base pairs	Closest-related strain	Identity (%)	Comment	Accession no.
Cement 7	GroutAid	2	394	*Pseudomonas mandelii* strain REG45	99	Close to many strains	EU586044
Cement 8 (GU65309)	GroutAid	3	854	*Arthrobacter bergeri*	100	Isolated from cheese	AJ609631
Cement 9 (GU65310)	Super-Parmix	3	624	*Microbacterium phyllosphaerae* isolate WP02-1-38	99	Isolated from alkaline environment	EF143430
Cement 4 (GU65307)	Mighty 150	1	812	*Aminobacter aminovorans*	100	Arsenic-resistant soil bacterium	AM285009
Cement 11 (GU65312)	Mighty 150	2	866	*Herminiimonas saxobsidens*	99	No information available	AM493906,
Cement 5 (GU65308)	Structuro 111X	3	762	*Pseudomonas veronii*	99	No information available	FM162562
Cement 6	Structuro 111X	2	346	*Pseudomonas migulae* strain IMER-B2-31	100	Close to a *P. fluorescens* strain	FJ715746
Cement 12 (GU65313)	Cementa SetControl II	1	861	*Sphingomonas melonis* strain 150	99	Isolated from water purifier in China	EU730903

Eight isolated strains were sequenced and compared with the GenBank database. Table 2-6 presents the results and closest-related strains. Many of the sequenced strains were difficult to obtain in pure cultures, so no sequence data are available for them. The sequences were deposited in GenBank; for accession numbers, see Table 2-6.

Close to half the total length of the 16S rRNA gene was sequenced for five of the strains, i.e., 762–866 base pairs, and the obtained identities were 99–100 % (Table 2-3). For the two strains closely related to the *Pseudomonas* group, only 394 and 346 base pairs, respectively, could be sequenced. This group of organisms was difficult to sequence with the direct sequencing method used in this experiment. The colonies on the agar plates seemed to be mixtures of strains. The short sequences were not deposited in the GenBank database.

3.5 Growth experiment 5 – Growth of groundwater microorganisms on solid drainage material

This experiment sought to determine whether some drainage materials could enhance groundwater microorganism growth. The CHAB cell numbers determined after growth for approximately one week are presented in Table 2-7.

Table 2-7. *Results of growth experiment 5, growth of groundwater microorganisms on drainage material.*

Product	Sample 1 (mL^{-1})	Sample 2 (mL^{-1})	Sample 3 (mL^{-1})
White cellular plastic	5.7×10^5	2.7×10^6	7.2×10^5
Grey cellular plastic	2.3×10^4	1.8×10^6	5.0×10^5
Yellow drainage pipe	8.0×10^5	6.3×10^5	3.5×10^6
Enkadrain TP	6.2×10^5	5.3×10^6	3.7×10^6
Enkadrain 10D	8.0×10^5	3.6×10^6	6.3×10^6
Enkadrain ST	3.7×10^6	6.6×10^5	5.8×10^6
Control	1.7×10^6	1×10^7	1.7×10^6

The additions did not enhance growth to cell numbers significantly higher than those of controls in any of the growth cultures. The highest cell number was found in the control growth culture for sample 2, in which it reached 1×10^7 mL^{-1}. The reason for such a high number for a control is unknown, and the result is remarkable compared with the numbers for the other controls, which are more reasonable in size; hence, this result should be regarded with caution.

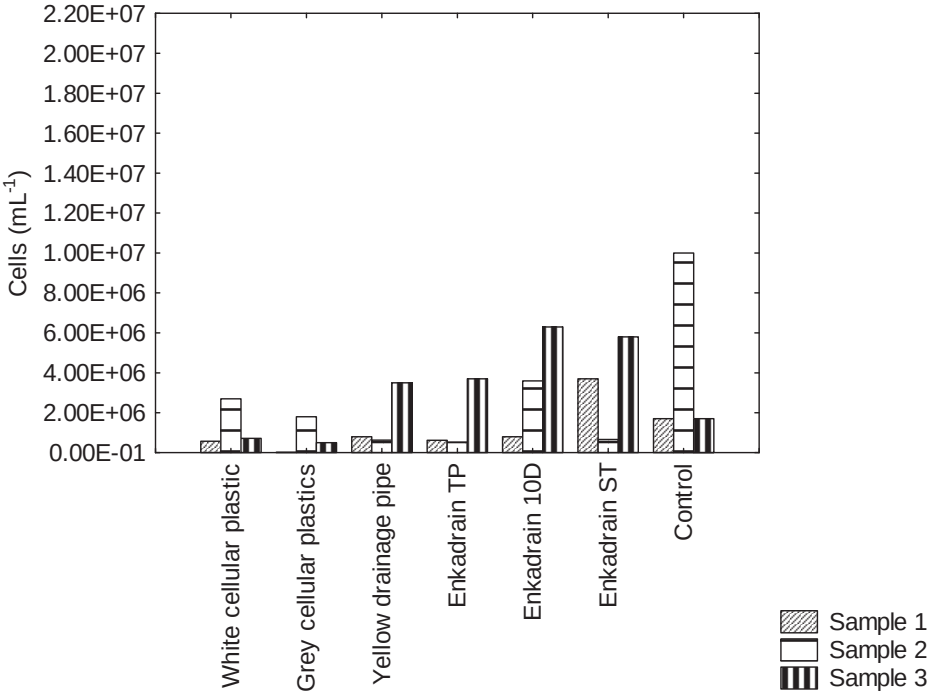

Figure 2-4. *Bar graph of the results of growth experiment 5 with groundwater microorganisms grown on drainage materials.*

4 DISCUSSION

Eight liquid grouting additives, three cement powders, and six drainage products were tested for their possible ability to support microorganism growth.

4.1 Growth experiment 1 – Microorganisms in cement and grout additives

Two of the products, GroutAid and Super-Parmix, contained $>2 \times 10^5$ mL^{-1} aerobic bacteria. No other products displayed any growth of aerobic microorganisms. The strains isolated from these products were most closely related to organisms isolated from contaminated environments and had hydrocarbon-degrading abilities. The 16S gene sequence of the organism isolated from Super-Parmix, with smooth, yellow colony characteristics, was 100 % identical to a *Brachybacterium* sp., a poly-aromatic-hydrocarbon-degrading microrganism. Super-Parmix is a naphthalene-formaldehyde polycondensate. GroutAid is amorphous SiO_2 in water. That some products were contaminated with microorganisms is to be expected. The concentration of active substance in Super-Parmix is not too high for microorganism survival.

4.2 Growth experiment 2a – growth on products of isolated bacterial strains from ONKALO groundwater

This experiment revealed no heavy growth with the products tested. SetControl II was the only liquid addition that displayed any increase in cell number. This is a sulphonated naphthalene polymer (1–10 %) in 30–60 % calcium nitrate. Cell numbers above 10^6 mL^{-1} were also found in growth cultures of *H. taeniospiralis* on SR-sementti.

It would have been interesting also to test the remaining products, especially Super-Parmix, which is a naphthalene formaldehyde polycondensate and thus a product that might support growth of the three organisms used in growth experiment 2a.

4.3 Growth experiment 3 – Growth on products of isolated groundwater pseudomonades

The products that supported growth of the two pseudomonades *P. stutzeri* and *P. fluorescens* were Structuro 111X, Mighty 150, and Super-Parmix of the liquid additives and Rapid sementti of the cement powders. No cultures reached a cell number above 10^7 mL^{-1}.

Structuro 111X, Mighty 150, and Super-Parmix are all organic additives. Structuro 111X is a polycarboxylate polymer, Mighty 150 is a sulphonated naphthalene condensate containing formaldehyde, and Super-Parmix is a naphthalene formaldehyde polycondensate. It is difficult to determine what ingredients in these products were actually used by the microorganisms in this type of growth study, but both naphthalene and formaldehyde are compounds that can be used by microorganisms.

4.4 Growth experiment 4 – Growth on products of groundwater microorganisms

The products that clearly supported growth of groundwater microorganisms were Mighty 150, Structuro 111X, and Super-Parmix. These cultures reached cell numbers >2 × 10^7, above the numbers that could be enumerated with the dilutions used here. Importantly, the microorganisms present in the groundwater used the same products as did the earlier strain isolated from ONKALO groundwater.

The sequence data indicated that many of the isolated organisms belonged to *Pseudomonas* or other aerobic groups of organisms. The available information on the closest relatives did not imply that these organisms were involved in degrading any organic compounds. Interestingly, isolate 3 was most closely related to organisms isolated from an alkaline environment as stated in the annotation in Genebank. No further description is available regarding the type of environment.

4.5 Growth experiment 5 – Growth of groundwater microorganisms on drainage material

None of the drainage materials tested supported the growth of microorganisms from the various groundwater samples. The experimental growth period of approximately a week is probably too short for a microbial consortium to degrade solid plastic material to any greater extent. On the other hand, it can be concluded that the materials did not leak any substance that readily supported microbial growth.

4.6 Biodegradation of concrete additives and admixtures

Few published studies examine the degradation of concrete additives and admixtures. One group of microorganisms that could well be responsible for the degrading formaldehyde-containing products is the methylotrophs. These organisms can use one-carbon compounds, such as methanol and methane, and have formaldehyde as a metabolic intermediate. Some of these organisms can also use formaldehyde as a growth substrate, which is remarkable since formaldehyde is generally very toxic to living systems (Anthony 1982). One study describes a strain of *Methylobacterium* that could grow in formaldehyde up to 58 mM in concentration and even survived in a 100-mM solution (Chongcharoen et al. 2005). Slime sampled from ONKALO drillhole groundwater in October 2007 contained at least four 16S RNA gene sequences that were most similar to methylotroph sequences (Pedersen et al. 2008).

The microbial consortium growing on concrete additives is probably highly complex, containing several different types of microorganisms involved in the degradation processes.

The present study focused on aerobic organisms, but a number of anaerobic processes are likely involved as well. Since knowledge of this area of microbiology is limited and there is an increasing number of organic additives and admixtures available on the market, continued research into this subject is certainly required.

A considerable amount of additives would be used in concrete construction in a

repository. The potential of the organic compounds they contain to be used by microorganisms, thus increasing the risk of biofilm formation and the production of complexing components, must be taken seriously. The availability of energy and carbon would also enhance the potential for hydrogen sulphide formation. Increased availability of energy and carbon sources for the microbial community in the vicinity of a repository must be taken into account in safety analyses.

4.7 Conclusions

- Microorganisms were present in two of the tested products: GroutAid and Super-Parmix
- None of the tested products supported the growth of bacterial strains previously isolated from ONKALO slime to any greater extent. Some cultures grown on Structuro 111X, Mighty 150, and Super-Parmix displayed higher cell numbers than did cultures grown on the other products.
- Three products, i.e., Structuro 111X, Mighty 150, and Super-Parmix, supported the growth of microorganisms present in fresh samples of ONKALO groundwater.
- Two products, i.e., Mighty 150 and Super-Parmix, which supported growth were condensates with formaldehyde and naphthalene; the third product, Structuro 111X, was a polycarboxylate polymer.

REFERENCES

Anthony, C. 1982. *The biochemistry of methylothrophs*. Academy Press, New York.

Altschul, S. F. and Erickson, B. W. 1985. Significance of nucleotide sequence alignments: a method for random sequence permutation that preserves dinucleotide and codon usage. *Molecular Biology and Evolution*, 2(6), 526–538.

Chongcharoen, R., Smith, T. J., Flint K. P., and Dalton, H. 2005. Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. *Microbiology*, 151(8), 2615–2622.

Kriegh, N. R. and Gerhardt, P. 1994. Solid, liquid/solid and semisolid culture. In: Gerhardt, P., Murray, R. G. E., Wood, W. A., Krieg, N. R. (Eds.), *Methods for general and molecular bacteriology* (pp. 216–223. American Society for Microbiology, Washington, DC.

Lane, D. J. 1991. 16S/23S rRNA sequencing. In: Stackebrandt, E. and Goodfellow, M. (Eds.), *Nucleic acid techniques in bacteria systematics* (pp. 115–175). Wiley & Sons, Chichester, UK.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L., and Pace, N. R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Science of the United States of America*, 82(200), 6955–6959.

Pedersen, K., Arlinger, J., Eriksson, S., Hallbeck, M., Johansson, J., Jägevall, S., and Karlsson, L. 2008. Microbiology of Olkiluoto Groundwater. Results and Interpretations 2007. POSIVA Working Report 2008-34, POSIVA OY, Olkiluoto, Finland.

Pedersen, K. Ekendahl, S. 1990. Distribution and activity of bacteria in deep granitic groundwaters of southeastern Sweden. Microbial Ecology, 20, 37-52.