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# Previously uncultured β-*Proteobacteria* dominate in biologically active granular activated carbon (BAC) filters

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ABSTRACT

Bacteria colonizing BAC filters used in drinking water purification from lake water were characterized by morphology, physiological tests, whole cell protein profiles and PLFA (phospholipid fatty acid) composition, and identified by partial 16S rRNA gene sequencing. Epifluorescence revealed prothecate bacteria to dominate in BAC. The majority of the isolates belonged to order *Burkholderiales* of β-*Proteobacteria*, a few to *Comamonadaceae* but the majority to an undescribed family and the related sequences belonged mainly to uncultured bacteria. Among the less common α-*Proteobacteria* the genus *Sphingomonas* and the genera *Afipia*, *Bosea* or *Bradyrhizobium* of the *Bradyrhizobiaceae* family were detected. The majority of cultured bacteria persisting in the BAC biofilter were *Burkholderiales*, which according to ecological information are efficient in the mineralisation of dissolved organic matter in BAC. The biotechnical potential of the previously uncultured dominant bacteria warrants to be further studied.



## 1. Introduction

The use of surface water as a water supply for drinking water necessitates efficient water treatment. BAC, as one of the treatment processes, has been successfully used in drinking water purification for the removal of assimilable organic carbon that facilitate regrowth of micro-organisms (Volk and LeChevallier, 2002, Chien et al., 2008) and organic compounds that cause odour, taste or colour, and precursor compounds that may produce harmful disinfection by-products (Stewart et al., 1990, Simpson, 2008). The combined use of ozonation and BAC enhances the removal of persistent organic compounds, such as cyanobacterial and algal toxins (Rositano et al., 2001, Orr et al., 2004). Biological activity of bacteria in BAC has been shown to be the predominant removal process (Ho and Newcombe, 2007). For example, Herzberg et al. (2006) showed that efficient phenol removal occurred in the activated carbon with simultaneous biofilm colonization of high biomass of viable bacteria.

Bacterial colonization of granulated activated carbon, GAC, is a result of the adsorptive properties of carbon. In addition, the porous nature of GAC provides protection from shear forces, and the functional groups of the GAC enhance microbial attachment (Stewart et al., 1990; Simpson, 2008). The composition of microbial species colonizing GAC and creating BAC is important for their capacity to biodegrade harmful and undesirable compounds. Because a stable, thin and active biofilm is desired for optimal purification via BAC, information on its composition and activity is needed for successful process control (Simpson, 2008). The composition of

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microbiota in BAC is also important as a possible source of water contamination in the distribution system.

Bacterial composition varies, e.g. with nutrient and dissolved oxygen levels and pH. Easily cultivable bacteria such as *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Bacillus*, *Acinetobacter*, *Aeromonas* and *Chromobacterium* have been isolated from the GAC product water (Stewart et al., 1990). In a recent study in Finland Berg et al. (2009, supplementary data at http://www.nature.com/ismej) isolated *Micrococcus*, *Chryseobacterium*, *Bosea*, *Brevundimonas*, *Phyllobacterium* and *Sphingomonas* strains from the finished water of several drinking water treatment plants that used GAC as one of the treatment processes. However, studies describing bacteria identified directly from GAC are rare. Stewart et al. (1990) identified *Pseudomonas* and *Flavobacterium* from GAC filters and carbon particles not exposed to disinfection. Magic-Knezev et al. (2009) showed that 68% of the isolated bacterial strains belonged to the *β-Proteobacteria* and 25% to the *α-Proteobacteria*. *Polaromonas*, *Hydrogenophaga* and *Methylibium* were the most common *β-Proteobacteria* in GAC filter beds, and *Sphingomonas*, *Afipia* and unclassified isolates dominated the *α-Proteobacteria*.

The aim of this study was to investigate the microbial colonization of BAC filters at a treatment plant that used lake water as the water supply, chemical coagulation, sand filtration and ozonation prior to BAC filtration and UV-disinfection. The dominating bacteria were isolated from BAC and characterized. Microbial colonization was observed by epifluorescence microscopy, ATP content and cultivation of bacteria. The isolated bacteria were characterized with morphological and physiological tests and clustered on the basis of SDS-PAGE. Physiological, phosholipid fatty acid (PLFA) analyses, partial sequencing of their 16S rRNA genes and a phylogenetic analysis of the strains representing different SDS-PAGE clusters was carried out.

## 2. Materials and methods

### 2.1. Pilot plant

The water purification process including the pilot plant processes has been described earlier (Vahala et al., 1999). Water from temperate climate Lake Päijänne (mesotrophic with moderate concentrations of humic substances) was transferred in a tunnel for 120 km to the treatment plant and water was treated in the full-scale process using chemicalisation ($CO_2$, lime and aluminium sulphate), flocculation, sedimentation and sand filtration. After the full-scale process the water was ozonated (0.43 mg $O_3$/mg TOC) and directed to the pilot plant process line consisting of a two-step GAC process. The first filter, BAC, contained a 1.7 m layer of used GAC. The second adsorption filter contained a 2 m layer of new charcoal-based activated carbon Filtrasorb 400 (CAS number 82600-58-6). The retention time was 13–15 min, and the filtration rate 8 m $h^{-1}$.

### 2.2. Sampling and sample treatment

Duplicate samples from BAC filters (whole content with water and BAC particles) were aseptically taken into 100 ml borosilicate bottles six times during the summer (representing 74 to 137 d from the onset of the experiment). The samples were transported to laboratory in insulation boxes and treated within 2 h of sampling. Dry weight of the BAC samples was measured according to the standard (SFS-EN 12880, 2000).

### 2.3. Measurement of ATP

ATP was extracted directly from BAC by transferring 1 g BAC into a sterile borosilicate bottle and adding 10 ml 20% trichloroacetic acid, 10 ml 8 mmol $l^{-1}$ EDTA and 212 μl 100-fold desorption buffer concentrate (Lechevallier and McFeters 1990). The extraction was carried out in an ultrasonic bath (Bransonic 32) for 20 min. After sedimentation the solution was filtered through filter paper (Whatman 150, No 1002150) and ATP was measured according to Vanhala and Ahtiainen (1994).

### 2.4. DAPI staining

BAC samples were fixed by adding ice cold reagent (0.40 g $NaH_2PO_4$, 1.25 g $Na_2HPO_4$, 80 ml distilled water and 20 ml phosphate-buffered 50% glutaraldehyde) to a final concentration of 10% and stored in refrigerator for a maximum of three weeks. After addition of dispersant (0.1 mol $l^{-1}$ tetrasodium pyrophosphate), the samples were sonicated in a Bransonic 5200 ultrasonic bath for 30 min. Suitable sample aliquots were stained in a filter funnel with a final concentration of 0.0001% DAPI for 7 min and filtered through black Nuclepore polycarbonate membrane filters with 0.2 μm pore size. For microscopy, epifluorescence with 1250-fold magnification was used (Leiz Diaplan).

### 2.5. Enumeration and isolation of heterotrophic bacteria

The desorption from BAC and cultivation of heterotrophic bacteria was slightly modified from Camper et al. (1985). Duplicate aliquots of 1 g BAC were added to 100 ml of desorption buffer and the mixture was homogenized for 3 min at 16,000 rpm in an Omni Mixer Homogenizer in an ice bath. This $10^{-2}$ dilution was further diluted in phosphate buffer (ISO 8199, 2005) and 100 μl aliquots of serial dilutions were spread-plated on R2A agar. Plates were incubated at 25 ± 1 °C for 7 d. Colonies were counted using 16-fold magnification under a stereoscopic microscope. Bacterial strains were isolated on R2A plates from samples taken from the BAC at 74 d and 123 d from the onset of the experiment.

After further purification on R2A, isolated strains were stored in skimmed milk tubes at −70 °C. The tubes were prepared by weighing 20 g skimmed milk powder (MC27, LAB M™, UK) to purified water to a final volume of 100 ml and distributing as 1.5 ml portions to cryo-tubes, which were sterilised at 115 °C for 15 min. Bacterial strains were isolated from samples taken from the BAC at 74 d and 123 d from the onset of the experiment.

### 2.6. Characterization of the isolated strains

The strains were characterized by colony and cell morphology, Gram staining, oxidase (using Remel Pathotec*

CO Test Strips) and catalase tests (3% $H_2O_2$ added to colonies on R2A plates and gas bubbles recorded as a positive reaction).

### 2.7. SDS-PAGE

SDS-PAGE was carried out for 51 isolates from each date as described by Niemi et al. (1993) preparing the bacterial cell samples as described by Hantula et al. (1990). The gels were scanned using a laser densitometer (Molecular Dynamics) and the results were analysed using BioNumerics programs (version 5; Applied Maths). The very small size of colonies did not facilitate biomass estimation and limited the biomass available for protein analysis for several strains, even if cells from whole plates were collected, and this may have affected the protein patterns as well as the reliability of the physiological tests.

### 2.8. Characterization of selected strains

Eight representative strains from different protein profile groups were further characterized. API 20NE (bioMerieux®; incubated at $30 \pm 1$ °C for $44 \pm 4$ h) and APIZym test kits (bioMerieux®; incubated at $25 \pm 1$ °C for $20 \pm 2$ h) were used according to the manufacturer's instructions. The possible preference for low nutrient level was tested by growing the strains on the rich tryptone soy agar (TSA, Difco) using normal (100%), 50% and 10% nutrient concentrations (incubation at $25 \pm 1$ °C for 7 d).

The major fatty acids of the strains were identified with gas chromatography using MIS (Microbial Identification System; Microbial ID, Inc.) using cells grown on 10% TSA at 25 °C for 7 d. Because the strains grew poorly on TSA plates, the low biomass limited reliability of identification of the strains on the basis of the MIDI Sherlock program with an identification library. Thus, the identification library was not used, and only the major fatty acids of the strains were listed.

DNA was isolated from these strains using Fast DNA® Kit and Fast prep instrument (Qbiogene) according to the manufacturer's instructions. Partial sequences of 16S rDNA were amplified using the primers PRBA338f (Øvreås et al., 1997) and DS907r (Teske et al., 1996). For the PCR, 1 μl of the template or its 10- or 100-fold dilution, 5 μl 10-fold buffer (Finnzymes F-511), 3 μl bovine serum albumin (BioLabs 13900IS), 0.5 μl nucleotide mixture (dNTP 10 mM, Finnzymes F-560L), 0.5 μl DynaZyme II DNA Polymerase (Finnzymes F-501L), 35 μl sterile distilled water (Eppendorf) and 2.5 μl (10 μM) of each primer of the primers (TAG Copenhagen A/S) were mixed. PCR was run using a PTC-200 Peltier Thermal Cycler (Thermo Electron Corporation) with the program: once 95 °C for 3 min, then 29 times 94 °C for 30 s, 55 °C for 40 s, 72 °C for 1 min 30 s, and finally 72 °C for 3 min and cooling to 15 °C. Two negative controls and two positive controls, *Escherichia coli* (ATTC 11303) and *Aeromonas hydrophila* (SLV 81), were used. Sequencing was carried out at the Institute of Biotechnology (P.O. Box 56, 00014 University of Helsinki, Finland) with the DNA cycle sequencing system. BLAST searches were run according to Zhang et al. (2000) using the version 2.2.18+ and EMBL sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). CLUSTAL W version 1.81 was used for the sequence alignment and for the construction of a dendrogram with branch length (http://align.genome.jp). RDP Data release 10.3 and Seqmatch version 3 were used for evaluation of the taxonomic positions of the isolates (Cole et al., 2007; http://rdp.cme.msu.edu).

## 3. Results

### 3.1. Microscopy

DAPI-stained micrographs revealed apparently intact bacteria with variable morphology (Fig. 1). Cells with very long filamentous, sometimes branched outgrowths resembling *Hyphomicrobium* were common. Both single cells and rosettes of oval cells with very long filamentous outgrowth resembling *Planctomycetales* were also observed as well as oval cells with shorter (*Caulobacteraceae*-like) prostechates. Short straight and curved rods as single cells or aggregates were common. Instead of the blue fluorescence expected in DAPI-stained specimens, the cells were either yellow or green, which suggests that the cells were autofluorescent.

### 3.2. ATP content and colony counts

ATP content was about twice as high in BAC (0.5–0.7 nmol/g GAC dw) than in adsorption filter (0.2–0.3 nmol/g GAC dw). Also colony counts of bacteria were higher in the BAC ($6 \cdot 10^7$–$10^8$ CFU/g GAC dw) than in the adsorption filter (2–4·$10^7$ CFU/g GAC dw).

### 3.3. Characteristics of isolates

Pigmented colonies dominated on the R2A plates. All the Gram-positive strains, ten isolates, grew as pink colonies, were oxidase-negative and catalase-positive and originated from day 74 samples (Table 1). Among the Gram-negative strains, pink colonies, oxidase- and catalase-positive oval cells in pairs were common. On the basis of SDS-PAGE these were clustered into a major group A with two subclusters. The cluster A isolates were represented equally on both of the sampling dates (43% from 74 d and 57% from 123 d; Fig. 2). The otherwise similar protein patterns between these subclusters differed slightly in the amount of protein. The other major cluster B was also subdivided into two subclusters. These subclusters consisted of strains isolated from yellow or grey colonies on both the sampling dates and displaying oval cells positive for oxidase and mainly also for catalase. The rest of the clusters were smaller and more heterogeneous.

Representative strains ($n = 8$) of the SDS-PAGE clusters (A1–E) were selected for physiological tests, PLFA analysis (as an independent chemotaxonomic method) and for partial sequencing of their 16S rRNA genes. All the strains grew better on diluted TSA, verifying the importance of using the low nutrient R2A (Table 2). All the strains yielded negative results for several physiological characteristics. The strains representing SDS-PAGE clusters A1 (VC138) and A2 (VC16) were similar. The strain from cluster B1 (VC93) differed from these only slightly. The two isolates representing cluster B2 (VC57 and VC101) were similar. The strain VC46 (cluster C) had the lowest number of positive physiological reactions. The strain VC40 (cluster D) showed several clearly positive reactions, and

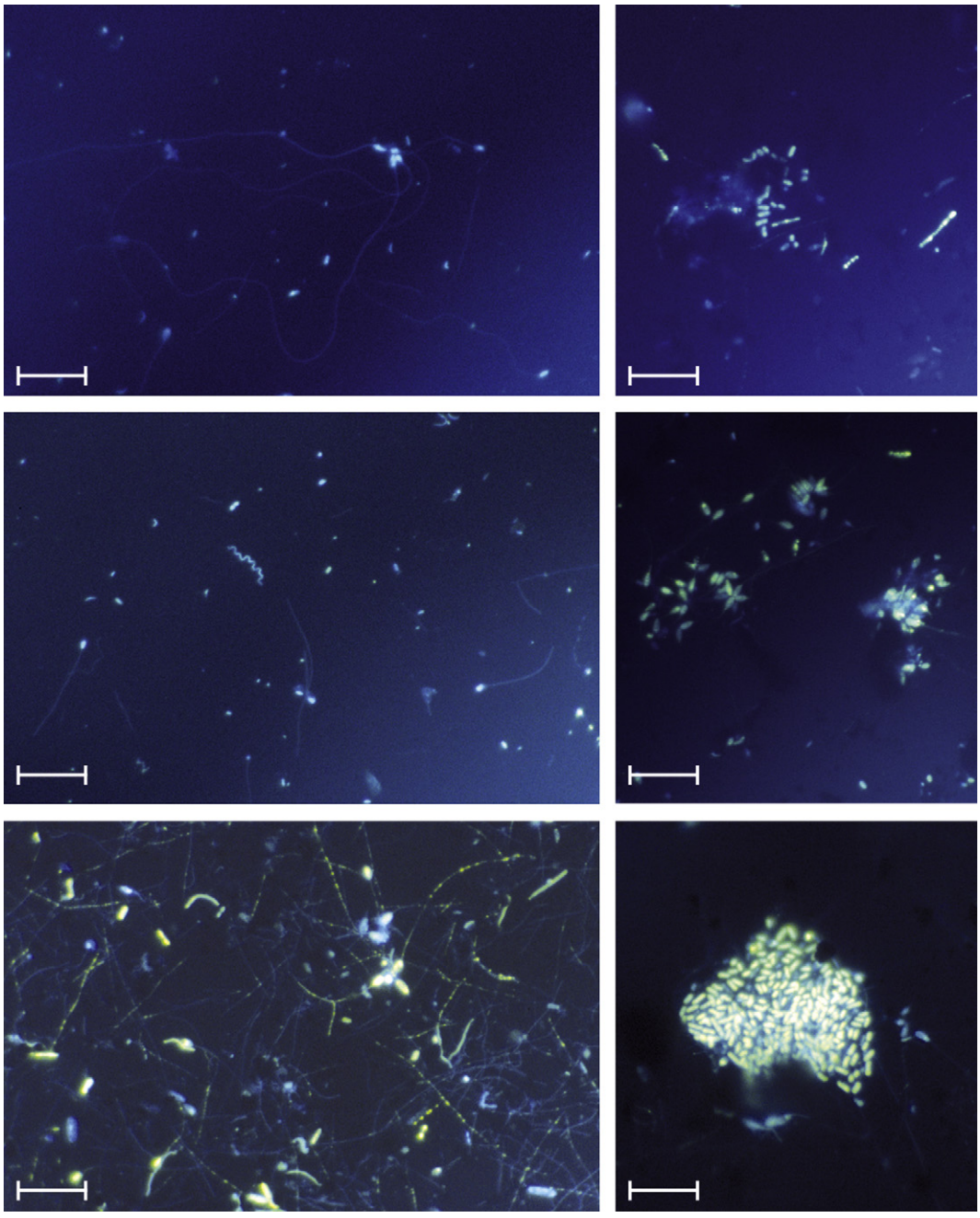

**Fig. 1 – Epifluorescence micrographs of DAPI-stained biofilter GAC samples. The line represents 10 μm.**

the strain VC98 (cluster) E was positive for the majority of the reactions. No isolates representing the heterogeneous clusters between B2 and C in Fig. 2 were further tested.

Clear differences were observed in the major fatty acids of the strains representing different SDS-PAGE clusters. The most common fatty acids for all the strain were 16:1 ω7c and 16:0, with the exception of strains VC 46 (cluster C) and VC 40 (cluster D) which had unsaturated 18:1 as the dominant fatty acids and differed from each other in relative concentrations of 16:1 ω7c and 16:0 (Table 3). The strains VC57 and VC101 from the cluster B2 were very similar, and the strain VC93 (cluster B1) differed only slightly from these. Also strains VC138 (cluster A1) and VC16 (cluster A2) differed only slightly from each other, the strain VC16 having 10:0 3-OH hydroxyl acid as a significant constituent in contrast to all other strains. The strain VC98 (cluster E) had a distinct PLFA profile.

### 3.4. *Phylogeny on the basis of 16S rRNA sequences*

The phylogenic relationship between our isolates and the most closely related database sequences were analysed for a 532 bp fragment corresponding to *Escherichia coli* 16S rRNA gene sites from 351 to 883 in CLUSTAL W analysis and a dendrogram with branch length was constructed (Fig. 3). Because the strain VC138 yielded indecisive basepairs due to multiple operons, the strain was omitted from the analysis.

**Table 1 – Characteristics of bacteria isolated from GAC.**

| No of identical | Colony colour | Gram reaction | Cell morphology | Oxidase | Catalase | Protein profile | Number in protein cluster (not tested) | Partial 16S rDNA sequence |
|---|---|---|---|---|---|---|---|---|
| 66 | Pink | – | Oval cells in pairs | + | +63/66[a] | A2 | 30 (15) | VC16 |
| | | | | | | A1 | 21 | VC138 |
| 2 | Grey | – | Oval cells in pairs | + | – | A1, A2 | 2 | |
| 23 | Yellow | – | Oval or rods in pairs, chains or clusters | + | +16/23[a] | B2 | 14 (9) | VC57, VC101 |
| 4 | Grey | – | Oval cells in pairs | + | + | B1 | 3 (1) | VC93 |
| 10 | Grey | – | Rods in pairs or clusters | + | +6/10[a] | E | 7 (3) | VC98 |
| 1 | Pink | – | Oval cells in pairs | + | + | E | 1 | |
| 13 | Grey | – | Oval or short rods in pairs or clusters | +7/10[a] | + | C | 6 (7) | VC46 |
| 2 | Grey | – | Oval or rods in pairs | + | – | C | 2 | |
| 2 | Yellow | – | Long rods in clusters | + | + | C | 2 | |
| 4 | Yellow | – | Rods in pairs | – | – | D | 3 (1) | VC40 |
| 3 | Yellow | – | Rods in pairs | + | + | D | 1 (2) | |
| 1 | Yellow | – | Short rods in pairs | – | – | D | 1 | |
| 1 | Grey | – | Oval cells in pairs | + | + | | 1 | |
| 2 | Grey | – | Oval cells in pairs | – | + | | 1 (1) | |
| 3 | Grey | – | Rods in pairs | – | + | | 3 | |
| 2 | Grey | – | Short or long rods in pairs or clusters | – | + | | 2 | |
| 2 | Orange | – | Oval to rods in pairs or clusters | + | + | | 2 | |
| 5 | Grey | – | Rods in pairs or chains | + | + | | (5) | |
| 1 | Yellow | – | Short rods in pairs | – | + | | (1) | |
| 1 | Yellow | – | Long rods in clusters | + | – | | (1) | |
| 10 | Pink | + | Oval cells in pairs or clusters | – | + | | (10) | |
| 158 | | | | | | | 102 (56) | |

a +/tested

Two major phylogenetic clusters were formed. According to RDP analysis members of the first main cluster (strains VC16 from SDS-PAGE cluster A2, VC93 from cluster B1, VC57 and VC101 from cluster B2 and VC98 from cluster E) were *β-Proteobacteria*. The other major phylogenetic cluster (strains VC46 from SDS-PAGE cluster C and VC40 from cluster D) were *α-Proteobacteria*.

The strain VC93 (from SDS-PAGE cluster B1) belonging to *β-Proteobacteria* (could be assigned to genus level, *Paucibacter* (Table 4). The strains VC57 and VC101 (both from cluster B2) and the strain VC16 (cluster A2) were related to order *Burkholderiales* and family Incertae sedis 5. Additionally, the strains VC57 abd VC101 were related to the genera *Leptothrix*, *Mitsuaria* and *Pelomonas*. The strain VC98 (cluster E) also belonged to the order *Burkholderiales*, but to the family of *Comamonadaceae*, which includes the genera *Comamonas* and *Curvibacter*.

The second major phylogenetic cluster included *α-Proteobacteria*. VC46 (SDS-PAGE cluster C) was related to order *Rhizobiales* and family *Bradyrhizobiaceae* that includes the genus *Bosea*. The strain VC40 (cluster D) belonged to the genus *Sphingomonas*.

Comparison of the partial 16S rRNA sequences of the *β*-proteobacterial isolates using BLAST searches revealed that the majority of the related sequences were derived from previously uncultured bacteria from environmental samples (Table 4). The strain VC16 (SDS-PAGE cluster A2), representing the most common isolates from BAC, was not closely related to any cultured and sequenced bacterium. Instead, it was relatively similar to uncultured bacteria inhabiting soil, aquatic habitats and occurring in arsenate-oxidizing biofilm. The strain VC138 (cluster A1) yielded indecisive basepairs in its sequence due to multiple operons, but four of the five closest hits were the same as for VC16, which supports the interpretation that these strains were closely related.

The isolate VC93 (cluster B1) was related to two cultured genera, *Paucibacter* and *Pseudomonas* but the majority of the closest hits were derived from uncultured bacteria from a wide range of habitats. For the strains VC57 and VC101 (both representing the SDS-PAGE cluster B2), one related strain from soil had been isolated, whereas the few other most closely related sequences of uncultured bacteria were from soil, sediment or bioreactor biofilm. The strain VC98 (cluster E) was only related to uncultured bacteria in sea water and in a drinking water distribution net.

The *α*-proteobacterial strain VC46 (SDS-PAGE cluster C) was closely related to several isolated *Afipia, Bradyrhizobium* and *Bosea* species, an unidentified glacial ice bacterium and uncultured bacteria from various habitats including drinking water biofilms (Fig. 3 and data not shown). The other *α*-proteobacterial strain VC40 (SDS-PAGE cluster D) was closely related to *Sphingomonas* spp. and uncultured bacteria

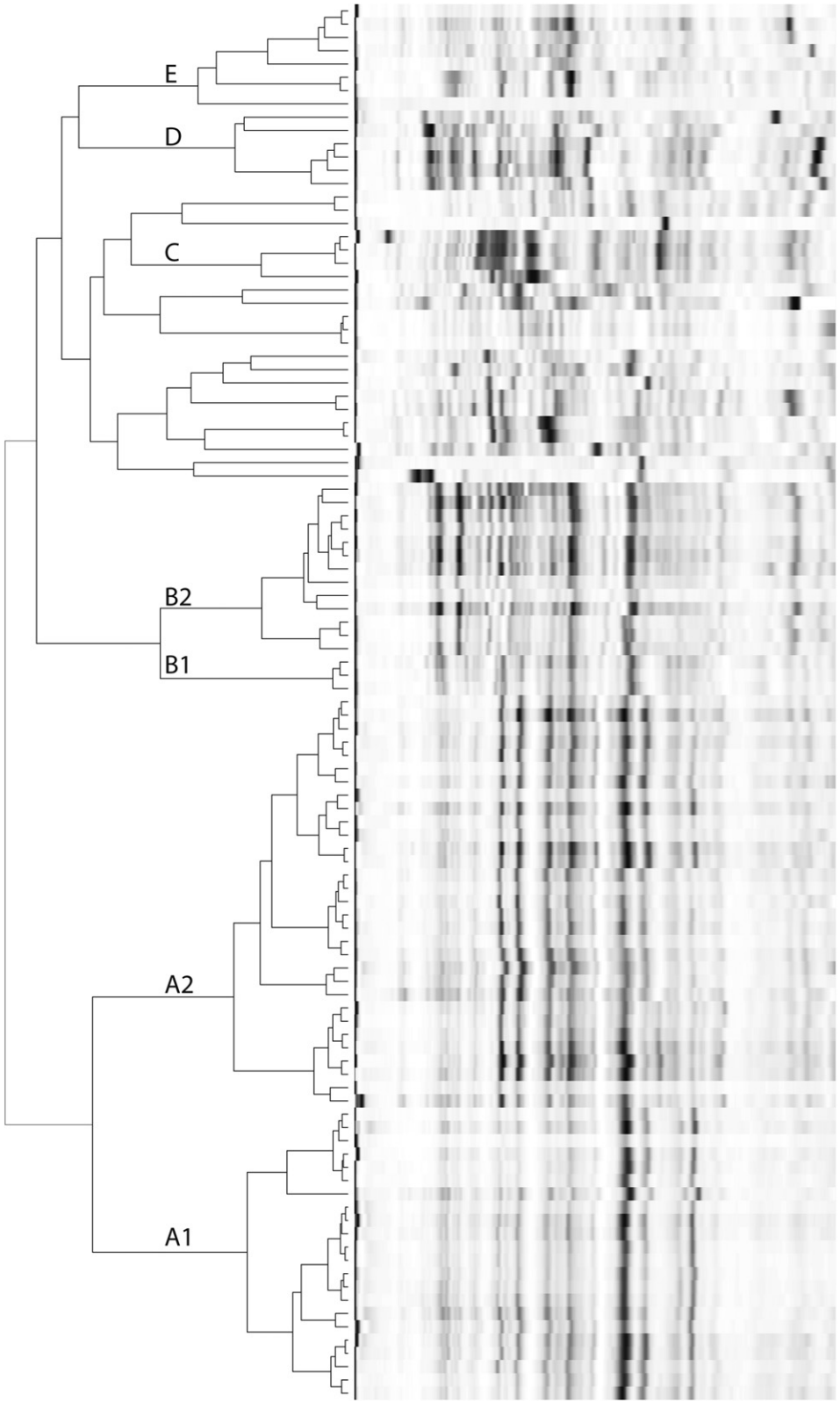


**Fig. 2 – Dendrogram and clusters on the basis of SDS-PAGE of GAC isolates.**

inhabiting diverse environments including water from cold environments such as Antarctica and Tibetan glaciers, soil, lake bacterioplankton, tropospheric clouds and oral noma lesions (Fig. 3 and data not shown).

The 16S rRNA gene sequencing confirmed the relatedness of protein group B2 isolates VC57 and VC101 and the distant relation of isolates VC40, VC46, VC 98 of groups D, C and E. VC16 of group A2 was related to VC93 of group B1.

**Table 2 – Characteristics of representative isolates from GAC. All the strains were Gram negative and yielded in the APIZym tests positive reactions for alkaline phosphatase and esterase-lipase and negative reaction for α- and β-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, and negative reaction in the API 20NE tests for nitrate reduction, indole production, glucose fermentation, activities of arginine dihydrolase, urease and β-galactosidase, assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, D-maltose, K gluconate, capric acid, adipic acid and trisodium citrate.**

| Strain codes/Characteristics tested | Strains and their test results | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Isolation code | VC138 | VC 16 | VC 93 | VC 57 | VC101 | VC 46 | VC 40 | VC 98 |
| HAMBI code | 3038 | 3039 | 3040 | 3041 | 3042 | 3043 | 3044 | 3045 |
| Accession number FM | | 995602 | 995606 | 995605 | 995608 | 995604 | 995603 | 995607 |
| SDS-PAGE | A1 | A2 | B1 | B2 | B2 | C | D | E |
| Colony colour | Pink | Pink | Grey | Yellow | Yellow | Grey | Yellow | Grey |
| Cell morphology | Oval in pairs | Oval in pairs | Oval in pairs | Oval in pairs | Oval in pairs | Short rods in pairs or clusters | Rods in pairs or clusters | Short rods in pairs |
| Gram | − | − | − | − | − | − | − | − |
| Oxidase | + | + | + | + | + | − | − | + |
| Catalase | + | + | + | + | + | + | − | − |
| Growth on 100%/ 50%/10% TSA | −/(+)/+ | −/(+)/+ | −/(+)/+ | (+)/+/+ | −/+/+ | (+)/+/+ | (+)/+/+ | −/−/(+) |
| APIZym[a]: | | | | | | | | |
| Phosphatase alcaline | 40 | 40 | 40 | 30 | 40 | 10 | 40 | 40 |
| Esterase | 5 | 5 | 10 | 10 | 10 | 5 | 3 | 10 |
| Esterase-lipase | 20 | 20 | 20 | 10 | 20 | 10 | 20 | 20 |
| Lipase | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| Leucine arylamidase | 40 | 40 | 30 | 10 | 30 | 5 | 40 | 40 |
| Valine arylamidase | 10 | 20 | 3 | 0 | 3 | 0 | 40 | 40 |
| Cystine arylamidase | 3 | 3 | 3 | 5 | 3 | 0 | 5 | 20 |
| Trypsin | 3 | 3 | 10 | 0 | 3 | 0 | 0 | 3 |
| Chymotrypsin | 5 | 5 | 40 | 10 | 10 | 0 | 10 | 3 |
| Phosphatase acid | 10 | 5 | 5 | 10 | 20 | 3 | 40 | 20 |
| Naphthol-AS-BI-phospho-hydrolase | 10 | 5 | 3 | 3 | 1 | 5 | 40 | 3 |
| β-Glucuronidase | 0 | 0 | 0 | 0 | 0 | 0 | 10 | 0 |
| α-Glucosidase | 0 | 0 | 3 | 0 | 0 | 0 | 40 | 0 |
| ß-Glucosidase | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 |
| API 20NE: | | | | | | | | |
| Esculin hydrolysis | − | − | − | − | − | − | + | − |
| Gelatin hydrolysis | − | + | − | + | − | − | − | − |
| Assimilation of phenyl acetic acid | + | − | + | + | + | − | − | + |

a Reaction intensity read according to the manufacturer's instructions.

**Table 3 – The major fatty acids of representative isolates from GAC.**

| PLFA | VC138 | VC 16 | VC 93 | VC 57 | VC101 | VC 46 | VC 40 | VC 98 |
|---|---|---|---|---|---|---|---|---|
| 3-OH 10:0 | (8) | 14 | 3 | 4 | 3 | | | |
| 12:0 | (7) | (9) | 4 | 8 | 8 | | | |
| 3-OH 12:0 | | (5) | 2 | | | | | |
| 15:1 ω6c | | | 4 | (2) | (2) | | | |
| 15:0 | | | 4 | (2) | (2) | t[a] | 5 | 4 |
| 2-OH 14:0 | | | | | | | 8 | |
| 16:0 N alcohol | (27) | | | | | | | |
| 16:1 ω7c | 35 | 45 | 52 | 54 | 59 | 24 | 3 | 33 |
| 16:1 ω5c | | | | | | 5 | (t) | |
| 16:0 | 38 | 30 | 24 | 22 | 18 | 3 | 11 | 43 |
| 17:1 ω6c | | | (1) | (2) | (2) | | 16 | |
| 17:0 | | | | (1) | (1) | (t) | 3 | (2) |
| 3-OH 16:0 | | | | | | 3 | | |
| 18:0 | | | | | | | t | (4) |
| 19:0 CYCLO ω8c | | | | | | 4 | (t) | 6 |
| 20:0 | | | | | | | | (4) |
| Summed feature 5[b] | | | (3) | | | (1) | | |
| Summed feature 7[c] | (6) | (10) | (1) | (7) | 8 | 56 | 48 | 7 |

() high variation between replicates.
a less than 1%.
b 17:1 ISO i/ANTEISO B/i.
c 18:1 w7c/w9 t/w12t, 18:1 w12t/w9t/w7c.

However, PLFA patterns of VC93 are closer to VC57 and VC101 than VC16.

## 4. Discussion

### 4.1. β-Proteobacteria in BAC

The vast majority of BAC isolates were related to uncultured *β-Proteobacteria* of the order *Burkholderiales*, but the positions at the family level were less certain and the strains representing the most common isolates were designated to the family Incertae sedis 5. Clustering by SDS-PAGE was used as the preliminary grouping of the strains. Characterization of the representative strains of the two major clusters A and B (70% of the strains) and the cluster E (8% of the strains) showed that the isolates belonged to β-Proteobacteria. Three of the representative isolates had no cultured closely related genera. One of them was close to *Comamonadaceae*, possibly to the genera *Comamonas* and *Curvibacter*. *Comamonadaceae* are chemoorganotrophic or facultatively chemolitotropic bacteria exhibiting $H_2$ or CO oxidation (Willems and Gillis 2005). *Comamonas* strains are known for the biodegradation of *e.g.* polycyclic aromatic hydrocarbons and harmful chemicals. *Curvibacter* species have been isolated from well water (Ding and Yokota 2004).

Only one isolate could be assigned to genus level, *Paucibacter*, which was recently described as a degrader of cyanobacterial hepatotoxins in our earlier study (Rapala et al., 2005). Another strain was related to the genera *Leptothrix*, *Mitsuaria* and *Pelomonas* and to an unidentified rice paddy field isolate shown to display slow response metabolism and fitness in producing a maximum number of viable progeny generated from a limited allocation of resources (Dethlefsen and Schmidt 2007).

The predominating *β-Proteobacteria* isolated from BAC in this study differed from those isolated in previous studies in which predominantly identifiable bacteria have been detected. Camper et al. (1985) isolated common coliform bacteria (*E. coli*, *Kluyvera* spp., *Klebsiella oxytoga*, *Enterobacter* spp., *A. hydrophila*, *Citrobacter freundii*, *Serratia* spp. *and Hafnia* spp.) from GAC particles collected from finished drinking water. *Pseudomonas* and *Flavobacterium* were isolated from GAC by Stewart et al. (1990). Magic-Knezev et al. (2009) showed that *Polaromonas*, *Hydrogenophaga* and *Methylibium* were the dominant *β-Proteobacteria* in the GAC filter beds. The differences in the species composition are probably due to different kind of feed water, since available substrates, abiotic factors and competing microbiota affect the species composition. In addition, the age of the biofilm affects the species composition. In this study, the bacterial composition of the BAC was shown to be stabilized as the same bacteria could be isolated from samples taken at different days.

The isolated bacteria in BAC were also different from those observed in biofilms of water treatment using methods other than GAC, for example reverse osmosis (RO) membrane biofilms (Pang and Liu 2007, Bereschenko et al., 2008), sand filters (de Vet et al., 2009) or in experimentally established biofilms on polyethylene, glass or polycarbonate slides (Kalmbach et al., 1999, Keinänen-Toivola et al., 2006). Bereschenko et al. (2008) observed *β-Proteobacteria* of the genus *Burkholderia* dominating in the feed water but *α-Proteobacteria* were common in the membrane biofilm. Our dominant isolates from BAC belonged to the same order of *Burkholderiales* but to a different family.

### 4.2. α-Proteobacteria in BAC

*α-Proteobacteria* formed a minority of the BAC isolates. Because the influent water was disinfected prior to introducing to the BAC filter, this is in accordance with the results of Mathieu et al. (2009) who showed that *α-Proteobacteria* are relatively sensitive to disinfection. Contrary to the *β-Proteobacteria* isolated, the *α-Proteobacteria* isolated were related to cultured bacteria *Sphingomonas* spp. and *Bradyrhizobiaceae* (genera *Afipia*, *Bosea* or *Bradyrhizobium*). *Sphingomonas* species have been detected in a wide range of environments, including finished drinking water of several water treatment plants in Finland (Berg et al., 2009) and the RO biofilm used in drinking water treatment (Bereschenko et al., 2008). Magic-Knezev et al. (2009) showed that *Sphingomonas* was the dominant α-proteobacterial genus in GAC filters used in drinking water treatment. *Sphingomonas* is known to be able to degrade a range of recalcitrant compounds of environmental concern including various aromatic compounds (Yabuuchi and Kosako 2005). Our *Sphingomonas* isolate yielded more positive biochemical reactions than the other bacteria. Of the *Bradyrhizobiaceae* family, species of the genus *Bradyrhizobium* are nitrogen-fixing root nodule bacteria, species of the genus *Afipia* are prevalent in fresh water and have been isolated from potable water but are also known as the etiologic agent of cat

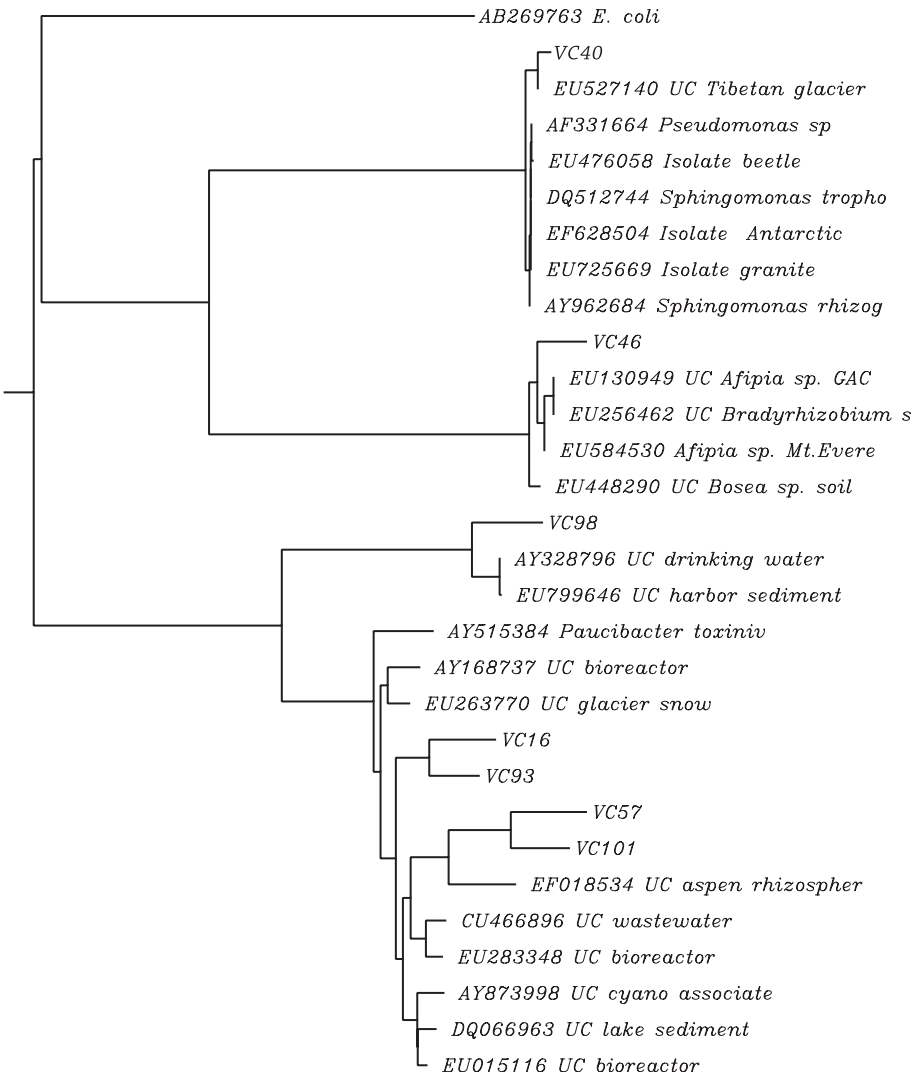


**Fig. 3 – Dendrogram with branch length of GAC isolates and closest 16S rRNA gene sequences on the basis of a clustal W run using EMBL nucleotide data. UC = uncultured.**

scratch disease (*A. felis*) and as opportunistic human pathogens, whereas representatives of the genus *Bosea* have been isolated from soil (Garrity et al., 2005). *Bosea* species have been repeatedly detected in fresh and drinking water.

*α-Proteobacteria* from the genera *Sphingomonas*, *Brevundimonas* and *Methylobacterium* have been shown to be common cultivable bacteria in the finished drinking water of surface water treatment plants of Finland (Berg et al., 2009). However, ozonation was used only at a limited number of the water treatment plants in that study, and ozonation seems to favour *β-Proteobacteria* over *α-Proteobacteria* (Magic-Knezev et al., 2009). Magic-Knezev et al. (2009) also showed, similar to the present study, that *Sphingomonas* and *Afipia* were the most commonly detected *α-Proteobacteria* in the GAC filter beds.

*α-Proteobacteria*, especially *Sphingomonas* species, have also been shown to dominate the RO biofilms used in drinking water treatment without ozonation (Bereschenko et al. 2008). Pang and Liu (2007) also observed, both for the bacterial isolates from R2A and for the total DNA based clones, that a clear majority of bacteria on RO membranes constituted of *α-Proteobacteria*. These included the genera *Bradhyrhizobium* and *Bosea* also detected in the present study. The ability for the reduction of nitrate in the nitrate rich system was postulated to promote persistence of *Bosea* and production of exopolysaccharides and versatile carbon sources to favour *Bradhyrhizobium* and other *Rhizobiales* species.

### 4.3. *Other microbes and colonization of the BAC*

DAPI staining of the BAC samples revealed variegated cell morphology. Oval to short straight or curved rods dominated. Putative *Hyphomicrobium* or *Planctomycetales* cells with long filamentous outgrowths were the most common. *Planctomycetales* form rosettes that were common in the micrographs of the BAC. Either stalk or flagellum containing *Caulobacteraceae*-like cells were also observed. The genus *Hyphomicrobium* is common in fresh waters. Caulobacters together with hyphomicrobia have been postulated to be responsible for the bulk of mineralisation of dissolved organic matter in oligotrophic and low temperature aquatic environments (Poindexter 2006).

**Table 4 – The closest BLAST hits for the partial 16S rRNA gene sequences of the strains belonging to the protein profile groups A, B and E.**

| Isolate | Species and source | Max ident | Accession |
|---|---|---|---|
| VC 16 | Uncultured | | |
| | Trembling aspen rhizosphere | 99% | EU015116 |
| | Cyanobacterial phycospheres (*Gloeotrichia echinulata*) | 99% | AY873998 |
| | Sediment of Lake Washington | 99% | DQ066963 |
| | Membrane bioreactor suspension and biofilm | 98% | EU283348 |
| | Wastewater treatment plant anoxic basin | 98% | CU466896 |
| | Arsenite-oxidizing biofilm, Hot Creek | 98% | AY168737 |
| VC138 | Uncultured | | |
| | *Comamonadaceae* bacterium, trembling aspen rhizosphere | (92%) | EF018476 |
| | Membrane bioreactor suspension and biofilm | (91%) | EU015116 EU283348 |
| | Cyanobacterial phycospheres (*Gloeotrichia echinulata*) | (91%) | AY873998 |
| | Sediment of Lake Washington | (91%) | DQ066963 |
| | Wastewater treatment plant anoxic basin | (91%) | CU466896 |
| VC93 | *Paucibacter toxinivorans*, lake sediment, cyanotoxin degrader | 98% | AY515384 + 11 others |
| | *Pseudomonas*, grass rhizosphere soil, California | 98% | EU723148 |
| | Uncultured | | |
| | *Roseateles* sp., soil in Michigan | 98% | EF662774 |
| | Surface snow in the Kuytun Glacier 51 | 99% | EU263770 |
| | Cyanobacterial phycospheres (*Gloeotrichia echinulata*) | 98% | AY873998 |
| | Membrane bioreactor suspension and biofilm | 98% | EU015116 |
| | Arsenite-oxidizing biofilm, Hot Creek | 98% | AY168737 |
| | Wastewater treatment plant anoxic basin | 98% | CU466896 |
| | Activated sludge from membrane bioreactor | 98% | EU283348 |
| | Lake Vida ice cover, 4.8 m, Antarctica | 98% | DQ521527 |
| | Nam Co Lake water, Tibetan Plateau | 98% | EU443091 |
| | *Danio rerio* (zebra fish) digestive tract | 98% | AY537350 |
| VC57 | Rice paddy soil isolate | 98% | AY337603 |
| | Uncultured | | |
| | Trembling aspen rhizosphere | 98% | EF018534 |
| | Rhizosphere soil from rice field, China | 98% | AM909893 AM909877 |
| | Membrane bioreactor suspension and biofilm | 98% | EU015116 |
| | Sediment of Lake Washington | 98% | DQ066963 |
| VC101 | Uncultured, trembling aspen rhizosphere | 98% | EF018534 |
| VC98 | Uncultured, sea water | 99% | EU799646 + 2 others |
| | Uncultured, drinking water distribution system | 99 % | AY328796 + 23 others |

*Planctomycetales*, aquatic bacteria commonly associated with phytoplankton and known to produce enzymes for the degradation of polysaccharides, were also common in the RO membrane biofilm even if they were not detected in the feed water (Bereschenko et al., 2008). However, hyphomicrobia, caulobacteria and *Planctomycetales* were not detected among the isolated strains. Either they did not survive the water treatment or the cultivation conditions did not enable their isolation even if actively growing.

The same dominating cultured populations persisted for at least 50 d in BAC. LeChevallier and McFeters (1990) reported that R2A efficiently reveals pigmented bacteria in BAC. Our results confirmed this, because 75% of random isolates formed either pink or yellow colonies.

Both colony counts and ATP measurements showed that more active biomass was present in BAC than in the absorption filter which confirmed that the two-step GAC process functioned as expected. Hammes et al. (2008) showed that ozonation caused chemical destruction of bacterial cells and that GAC filtration after ozonation facilitated significant regrowth of the microbiota. They cultivated heterotrophic bacteria on a rich medium (plate count agar), used pour plate technique, incubated the plates at +30 °C for 72 h. They detected 0.8–6.5% of bacteria by using heterotrophic colony counts when compared with flow-cytometric total bacterial counts in GAC. It is plausible that also our heterotrophic colony counts underestimated the total number of viable bacteria even if the low nutrient level, avoidance of heat shock, extended incubation time and counting of microcolonies probably improved the yield. At quite similar incubation conditions than ours, Magic-Knezev et al. (2009) also reported that the colony counts were 0.2–9% when compared to total direct counts. The observation by Hammes et al. (2008) that ozonation released ATP from microbial cells and that this extracellular ATP caused overestimation of active biomass in the subsequent process phase is plausible in our case as well.

## 5. Conclusions

1. The present study provided evidence of colonization of BAC with well adapted, persistent bacteria of *Burkholderiales* belonging to different, mainly hitherto undescribed genera. These or closely related bacteria have been detected as 16S rRNA gene sequences in fresh water and soil environments and in bioreactors.
2. The present study gave insight to the stable composition of BAC including undescribed genera, even at family level, and offers a starting point for further studies on growth requirements and catabolic capabilities of dominant strains and process monitoring using, e.g. the FISH technique.

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