Environmental Microbiology (2005) 7(8), 1248–1259 doi:10.1111/j.1462-2920.2005.00810.x

Bacterial dynamics in spring water of alpine karst aquifers indicates the presence of stable autochthonous microbial endokarst communities

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Summary

Spring water of two alpine karst aquifers differing in hydrogeology but of nearby catchments were investigated for their bacterial population dynamics. Dolomite karst aquifer spring 1 (DKAS 1) represents a dolomitic-limestone karst aquifer spring showing high average water residence time and relative constant flow. Limestone karst aquifer spring 2 (LKAS 2) constitutes a typical limestone karst aquifer spring with a dynamic hydrological regime and discharge. Dolomite karst aquifer spring 1 yielded constantly lower cell counts and biomasses (median of 15×10^6 cells l^{-1} and 0.22 μg C l^{-1}) as the LKAS 2 (median of 63×10^6 cells l^{-1} and 1.1 μg C l^{-1}) and distribution of morphotypes and mean cell volumes was also different between the considered systems, indicating the influence of hydrogeology on microbial spring water quality. Molecular bacterial V3 16S-rDNA profiles revealed remarkable constancy within each spring water throughout the investigation period. Time course analysis of a flood event in LKAS 2 further supported the trend of the temporal constancy of the microbial community. Except for one case, retrieval of partial and full length 16S rDNA gene sequences from the relative constant DKAS 1 revealed similarities to presently known sequences between 80% to 96%, supporting the discreteness of the microbial populations. The gathered results provide first evidence for the presence of autochthonous microbial endokarst communities (AMEC). Recovery of AMEC may be considered of relevance for the understanding of alpine karst aquifer biogeochemistry and ecology, which is of interest as many alpine and mountainous karst springs are important water resources throughout the world.

Received 1 November, 2004; accepted 21 January, 2005.
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Introduction

Groundwater resources from alpine and mountainous karst aquifers play an important role for public water supply in many areas throughout the world (Ford and Williams, 1996; Drew and Hötzl, 1999). Research on such karstic geo-ecosystems thus has a long-standing tradition with a focus towards hydrogeological, hydrochemical as well as more recently on zoological and vegetation sciences (Gibert and Danielopol, 1994; Ford and Williams, 1996; Drew and Hötzl, 1999; Dirnböck and Grabherr, 2000). In contrast, except a few reports from low-mountain karstic systems (Rusterholtz and Mallory, 1994; Griebler *et al*., 2001; Simon *et al*., 2001), currently no accessible information on microbial communities from alpine and mountainous karst aquifers exists (Ghiorse and Wilson, 1988; Gounot, 1994). Several studies have demonstrated, that aquatic habitats in alpine and mountainous areas, other than karst aquifers, such as lakes or streams, harbour abundant microbial communities (Haack and McFeters, 1982; Pernthaler *et al*., 1998; Batin *et al*., 2001). Consequently, significant numbers of microbes are also likely to occur in corresponding karst aquifers and respective spring waters.

As karst aquifers are relatively inaccessible, the investigation of such environments is restricted to natural access points such as springs and caves (Simon *et al*., 2001). Investigating microbial spring water quality under differing hydrological regimes thus represents a first cru-

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cial step, which is analogous to classical hydrographical spring analysis (Ford and Williams, 1996). Microbial spring water community dynamics may be assumed as a net result of all microbial community exchange processes of their interacting system compartments. This potentially includes (i) the direct surface runoff introduced into karst conduits, (ii) the soil–plant–vegetation compartment (i.e. the hydrotope) of the surface cover layer, (iii) the expected biofilm structures of the epikarst, (iv) the endokarst zones including the saturated karst aquifer and (v) the production/degradation of planktonic cells within the aquifer water itself. The resulting microbial exchange processes should thus vary according to the permeability of the considered karst system and their respective hydrological conditions. The karst aquifer itself may be conceptualized as a network of conduits of high hydraulic conductivity (K-values > 0.1 m s^{-1}) which is connected with a large volume of low permeability fractured and fissured rock (K-values between 10^{-3} and 10^{-7} m s^{-1}) (Doerfliger *et al*., 1999). At low water levels (base flow component), the spring outflow is thought to be mainly fed by the water from the low permeability volumes, whereas at high water levels most of the water from rainfall events is channelled directly through the karst conduit system (Doerfliger *et al*., 1999). However, in natural systems a very complex mixture of those situations may appear.

In order to enable a first insight into the occurrence of microbial communities in alpine karst aquifers a hydrographical spring water analysis, investigating hydrological, chemophysical as well as microbiological parameters, was performed. The study was based on spring water fed from two contrasting but nearby alpine karst aquifer catchments including various discharge regimes such as summer versus winter conditions, base flow versus increased discharge scenarios. Microbial communities were characterized by microscopic direct examination of bacterial cells, molecular bacterial 16S-rDNA profiling as well as conventional microbiological cultivation. In addition, a flood time course analysis from the dynamic limestone karst aquifer spring 2 (LKAS 2) during a summer thunderstorm was performed and furthermore full length and partial 16S-rDNA gene sequences were recovered from the more constant dolomite karst aquifer spring 1 (DKAS 1) environment. In this study, we present data which provide first evidence for the presence of stable autochthonous microbial endokarst communities (AMEC).

Results

Hydrological conditions and basic spring water quality characteristics

Recorded hydrological conditions during the investigation of the DKAS 1 and the LKAS 2 from April 2001 to March 2002 were in general agreement to hydrological conditions of former years although the mean discharge of DKAS 1 in 2001 after low winter precipitation was the lowest (280 l s^{-1}) since the beginning of the hydrogeological investigations in 1993 (cf. *Study area*). DKAS 1 yielded a rather constant discharge (Q) throughout the year, with a mean flow of 291.5 l s^{-1} and a ratio of Q_{min}/Q_{max} of 1:1.45 (Fig. 1A). In 2001 the snow break started in March and peaked in April. Intensive precipitation in autumn brought a rare second small peak in October. The snow break in 2002 started early in February and peaked in April with 350 l s^{-1} (Fig. 1A). The direct effect of precipitation very slightly concerned the aquifer characteristic. In contrast to DKAS 1, LKAS 2 showed a pronounced variation in flow conditions, with a mean discharge of 5412 l s^{-1} and a minimum to maximum ratio of 1:43 (Fig. 1B). Both, the snow break and the summer precipitation determined the aquifer characteristic. Comparison of the hydrological situation with the selected sampling regime (Fig. 1A and B) demonstrated that chosen sampling points matched the inherent hydrological variability in both aquifer systems covering base flow and increased discharge conditions as well as different seasonal aspects (e.g. winter cover, snow-break, summer conditions).

According to the investigated hydrological regime of DKAS 1, chemophysical parameters such as water temperature and conductivity revealed little variation throughout the investigation period (Table 1, Fig. 1A), suggesting stable temporal water quality characteristics. Furthermore no influence from microbial faecal indicators by surface water input could be observed (Table 1), except a negligible amount of 0.4 cfu and 0.2 cfu of faecal coliforms (FC) per 100 ml spring water was detected twice. Water quality characteristics in LKAS 2, such as conductivity, pH, turbidity and spectral absorbance coefficient at 254 nm (SAC), showed significant variations during sampling

Table 1. General spring water quality characteristics at DKAS 1 and LKAS 2 during the investigation period ($n = 14–15$).

		DKAS 1		LKAS 2	
	Unit	Median	Range	Median	Range
Q	l s^{-1}	278	260–301	3550	958–8930
TEMP	°C	6.7	6.6–6.7	5.2	4.5–5.6
CON	µS cm^{-1}	338	335–350	196	170–223
pH		n.d.	n.d.	8.0	7.4–8.2
TURB	ppm SiO_2	n.d.	n.d.	0.16	0.07–0.55
SAC	m^{-1}	n.d.	n.d.	1.1	0.6–5.3
FC	cfu/100 ml	0	0–0.4	1.0	0–128
ECOCI	cfu/100 ml	0	0–0	1.0	0–10
HPC	cfu ml^{-1}	1.1	0.7–3.3	25	3.0–1800

Q, discharge; TEMP, temperature; CON, electrical conductivity; SAC, spectral absorbance coefficient at 254 nm; TURB, turbidity; FC, faecal coliforms; ECOCI, enterococci; HPC, heterotrophic plate count; n.d., not determined.

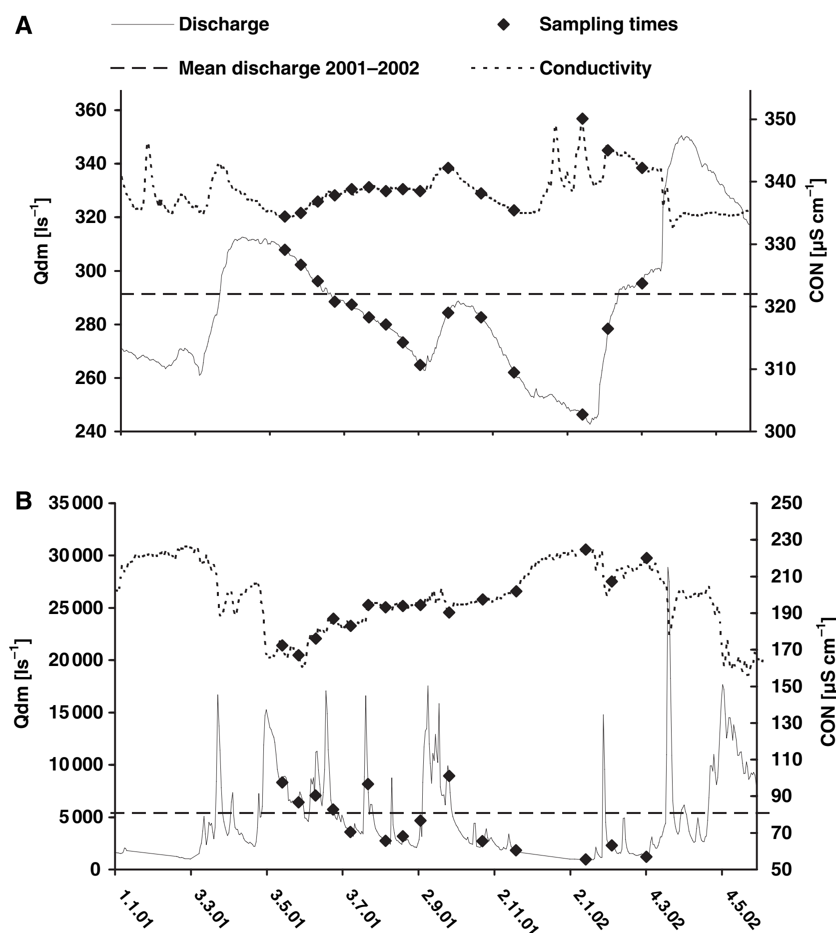

Fig. 1. Hydrological situation of the investigated alpine karst springs DKAS 1 (A) and LKAS 2 (B) and selected sampling points during the period 2000/2001. The upper lines show the electrical conductivity at 25°C (CON, right axes) the lower lines depict the daily mean discharge (Qdm, left axes); the mean discharges throughout the investigation period are marked by broken lines. Sampling points are shown as black diamonds. Note that different scales between aquifers are used.

(Table 1). For example, the SAC and turbidity showed values ranging from 0.6 m^{-1} up to 5.3 m^{-1} and 0.07 ppm SiO_2–0.55 ppm SiO_2, respectively (Table 1), indicating variable fluxes of dissolved organic carbon (DOC) and sediment particles in the LKAS 2 aquifer system. Also FC and enterococci (ECOCI) varied significantly, ranging from 0 up to 128 cfu and 10 cfu 100 ml^{-1} respectively. Heterotrophic plate count (HPC) values varied over three orders of magnitude (Table 1). Obviously, water quality was strongly influenced by the prevailing hydrological conditions as indicated by moderate to very high correlations ($\varphi = 0.52$–0.92) between discharge and SAC, turbidity (TURB), total bacterial numbers (BN-T) as well as faecal indicators (FC) (Table 2). Detection of faecal indicators

Table 2. Spearman rank correlation coefficient (φ) matrix between hydrographical and microbial parameters from spring water of the LKAS 2 aquifer ($n = 14$–15×10).

	Q	CON	SAC	TURB	BN-T	BN-R	BN-C	BN-V	HPC	FC
CON	–0.52									
SAC	0.52	–0.13								
TURB	0.92*	–0.63*	0.49							
BN-T	0.66	–0.16	0.50	0.81*						
BN-R	0.75	–0.28	0.43	0.86*	0.96*					
BN-C	–0.40	0.50	0.31	–0.26	0.11	–0.10				
BN-V	0.72	–0.22	0.51	0.84*	0.80*	0.73	0.04			
HPC	0.59	–0.10	0.46	0.40	0.25	0.30	–0.18	0.38		
FC	0.65	–0.17	0.54	0.72	0.81*	0.79*	0.37	0.76	0.24	
OTU	0.16	0.33	0.14	–0.04	0.23	0.18	0.31	0.15	0.53	0.47

Significance at 0.05 level is marked by one asterisk (significance corrected for multiple testing).
Q, discharge; CON, electrical conductivity; SAC, spectral absorbance coefficient at 254 nm; TURB, turbidity; BN-T, total bacterial numbers; BN-R, rod-shaped bacterial numbers; BN-C, coccus-shaped bacterial numbers; BN-V, vibrio-shaped bacterial numbers; FC, faecal coliforms; HPC, heterotrophic plate count; OTU, operational taxonomic units.

and increased levels of HPC in LKAS 2 spring water were limited to the warmer seasons and to increased water discharge situations. Thus immediate surface influence onto LKAS 2 could be assumed during spring and summer but not during winter where the surface was covered by ice and snow. For DKAS 1 no such significant influence from the surface could be detected.

Bacterial abundance, biomass and morphotypes in DKAS 1 and LKAS 2

The observed BN-T for both springs during the seasonal investigation were in the range of about 10^7–10^8 cells l^{-1}. Cell counts from DKAS 1 were always lower than in LKAS 2 and showed constant abundance throughout the whole investigation period according to the uniform discharge conditions at DKAS 1 (Fig. 2A). Limestone karst aquifer spring 2 revealed a more pronounced fluctuation of BN-T, which were correlated to discharge ($\varphi = 0.66$) and especially to turbidity ($\varphi = 0.81$) of the spring water (Table 2). Variation of bacterial numbers at LKAS 2 reflected thus the seasonal and hydrological conditions with high fluctuations during May to September and strictly decreasing from September to January (Fig. 2A). Rod (BN-R) and coccus (BN-C)-shaped cells generally comprised $\geq 90\%$ of the bacterial community in both spring habitats. Vibrio-shaped cells (BN-V) comprised the rest of the bacterial community (Table 3). In LKAS 2 the BN-R abundance clearly dominated over BN-C numbers (Mann–Whitney U, $P < 0.001$, $n = 2 \times 15$), whereas no differences could be detected for the DKAS 1 and the ratio between BN-R and BN-C appeared more or less balanced (Mann–Whitney U, $P = 0.67$, $n = 2 \times 15$) (Fig. 2B). Interestingly, from October to January the ratio between rod and coccus-shaped cells in LKAS 2 became also more balanced resembling that of the DKAS 1 situation (Fig. 2B), which can be explained by a decline of BN-R as total abundance variation within BN-T was highly correlated to BN-R ($\varphi = 0.96$) and not to BN-C ($\varphi = 0.11$) (Table 2). The observed biomass of BN-T for DKAS 1 and LKAS 2 was in the range of 0.16–0.29 μg C l^{-1} and 0.7–2.1 μg C l^{-1} respectively (Table 3). The mean cell volume for BN-T of 0.076 μm^3 cell^{-1} from LKAS 2 was significantly higher as the mean cell volume of 0.056 μm^3 cell^{-1} from

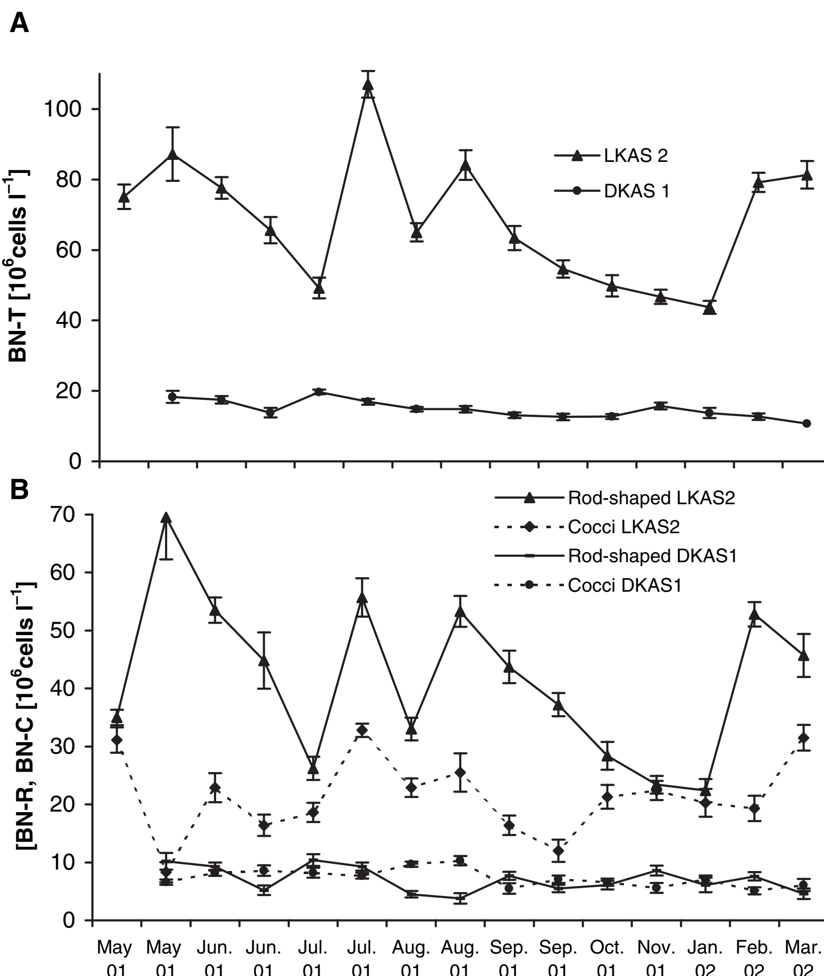

Fig. 2. Dynamics of total bacterial abundance (A) and abundance of bacterial morphotypes (B) in the alpine karst spring water of DKAS 1 and LKAS 2. BN-T, total bacterial numbers, BN-R, rod-shaped bacterial numbers, BN-C, coccus-shaped bacterial numbers; Values for BN represent the mean of 20 microscopic fields ± 1 standard error.

Table 3. General characterization of bacterial numbers, cell volume and total bacterial biomass in DKAS 1 ($n = 15$) and in LKAS 2 ($n = 15$) respectively.

	Bacterial numbers (10^6 cells l^{-1})		Cell volume (μm^3 $cell^{-1}$)		Bacterial biomass (μg C l^{-1})	
	Median	Range	Median	Range	Median	Range
In DKAS 1						
BN-T	14.8 (100%)	13–20	0.056	0.039–0.095	0.22 (100%)	0.16–0.29
BN-R	6.6 (45%)	4–10	0.093	0.078–0.110	0.16 (73%)	0.08–0.21
BN-C	7.7 (52%)	7–10	0.016	0.011–0.029	0.05 (22%)	0.02–0.0
BN-V	0.5 (3%)	0–2	0.119	0.095–0.173	0.01 (5%)	0.00–0.04
In LKAS 2						
BN-T	63 (100%)	44–107	0.076	0.060–0.098	1.1 (100%)	0.7–2.1
BN-R	38 (60%)	22–70	0.098	0.074–0.110	0.9 (82%)	0.5–1.6
BN-C	21 (34%)	8–33	0.022	0.013–0.033	0.1 (9%)	0.1–0.2
BN-V	4 (6%)	0–19	0.142	0.061–0.283	0.1 (9%)	0.0–0.6

Abbreviations according to Tables 1 and 2. Percentages of medians were linearly adjusted to 100%.

DKAS 1 ($P < 0.001$; Mann–Whitney U, $n = 2 \times 15$) (Table 3).

Bacterial V3 16S rDNA population dynamics and recovered 16S rDNA genes

Comparison of the bacterial V3 16S rDNA polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles recovered from the respective spring water samples demonstrated a temporal constancy of the observed band patterns throughout the investigation period with respect to the habitat type (Fig. 3A). Although 24 and 27 different band types could be detected in LKAS 2 and DKAS 1, only 5 band types in both systems, i.e. band types 1, 2, 3, 4, 5 in LKAS 2 and band types 1, 5, 6, 7, 8 in DKAS 1 (marked at Fig. 3A), accounted for more than half of the total observable 67 and 61 bands respectively. Cluster analysis using the total numbers of PCR-DGGE profiles revealed two significant and stable clusters, the DKAS 1 cluster and the LKAS 2 cluster (Fig. 3A). Determination of rePCR-DGGE resulted in more complex profiles but again proved of high temporal constancy (Fig. 3B). Cluster analysis, except for two profiles (DKAS 1, 28.05.01; and DKAS 1, 19.11.01) again resulted in two clusters of high overall similarity (> 90%).

Time course analysis of a flood in the LKAS 2 during a summer thunderstorm event revealed high constancy of the V3 16S rDNA PCR-DGGE profiles. The recovered profiles and band types apparently remained unaltered within an investigated period of 24 h, irrespectively of the analysed phases of the flood (i.e. I, IIa, IIb and III, see Fig. 4). It is remarkable that phase IIa and IIb could not be distinguished from each together by the DGGE profiles as the water masses were of different quality. Although spring water of phase IIa and IIb were both characterized by increasing levels of turbidity and BN-T, they significantly differed in their numbers of surface influence indicators, i.e. FC and ECOCI (Fig. 4). Whereas spring water of phase IIa was only very slightly effected by recent surface influence, phase IIb contained significant water masses of recent surface runoff.

Twenty-eight partial- or full-length 16S rDNA gene sequences were recovered by cloning and sequencing of DNA extracted from DKAS 1 from winter season 2001/02 (Table 4). A set of 12, 3 and 2 sequences of the 28 recovered sequences were identical, resulting in 14 unique sequence types (SDKAS 1_1 – SDKAS 1_14). Nine sequence types could be allocated with a high confidence (95%) to *Alpha-, Beta-*, and *Gammaproteobacteria* and to the *Flexibacter-Cytophaga-Bacteroides* cluster. SDKAS 1_10 could be affiliated with 70% confidence threshold to the *Deltaproteobacteria*. SDKAS 1_5, SDKAS 1_8, SDKAS 1_12, SDKAS 1_14 could be classified only to the domain of the *Bacteria* (95%) with high confidence; placement to a lower taxonomical level was not possible with a confidence higher than 50% (Table 4).

Discussion

Evidence for the presence of stable AMEC

Bacterial 16S-rDNA gene population dynamics during the one year investigation provided clear evidence for the presence of stable AMEC indigenous to the investigated alpine karst ecosystems. Irrespectively of the hydrological (i.e. base flow vs. increased discharge conditions) as well as seasonal conditions (e.g. surface protection by snow cover vs. surface runoff during rain events) the recovered DGGE band patterns reflected a high degree of temporal constancy. As the total catchment area of both investigated alpine aquifers covers a rather large mountainous and alpine region (over 80 km^2), results from this study are likely representative for mountainous and alpine karst aquifers of comparable altitude and environmental conditions. Whilst the results suggest the occurrence of suspended AMEC in spring water, attached AMEC in the

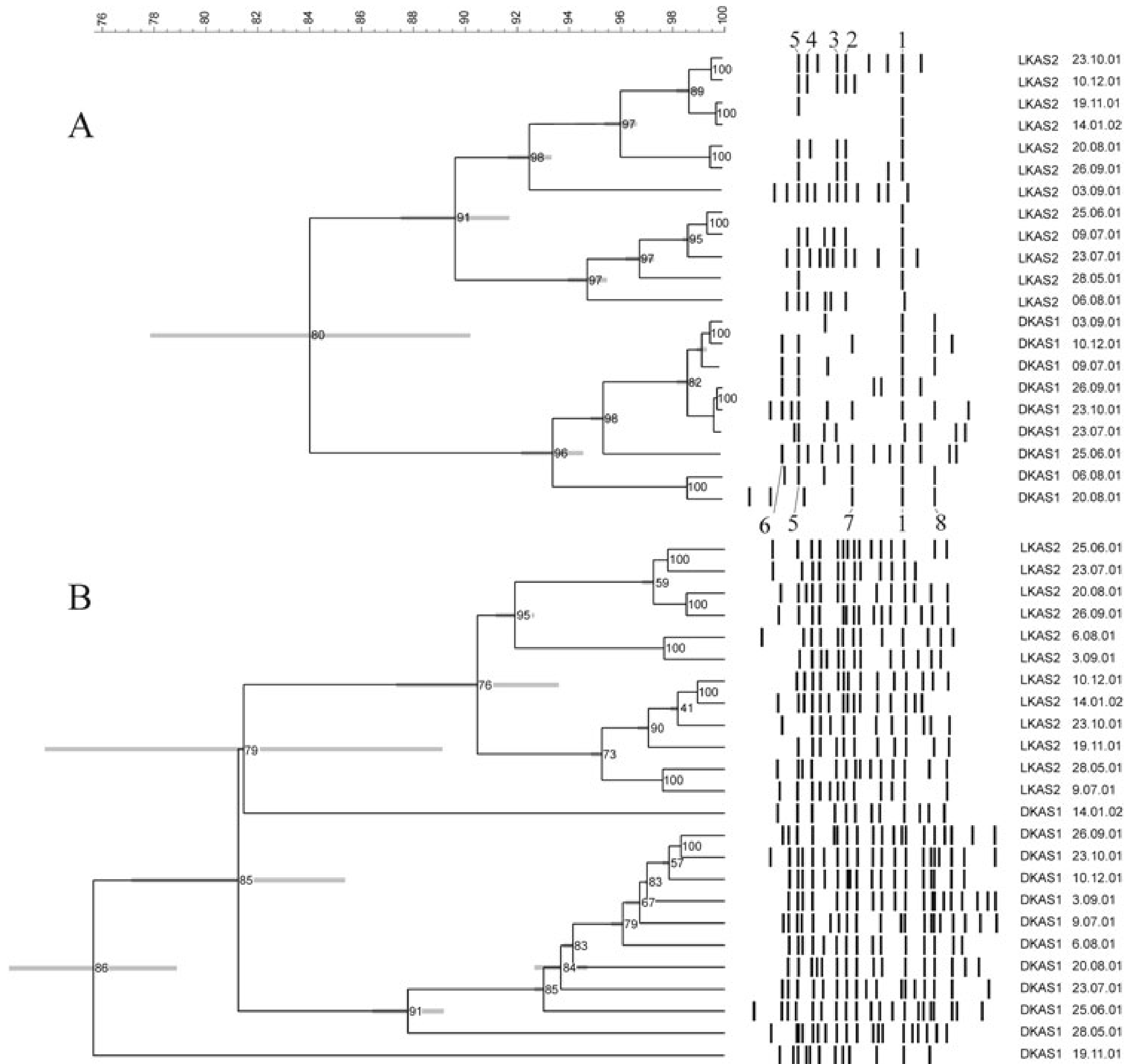

Fig. 3. A. Cluster analysis of 16S-rDNA PCR-DGGE profiles from LKAS 2 and DKAS 1 alpine spring water samples (Pearson correlation coefficient and UPGMA). Error flags and the cophenetic correlation coefficient are shown. Significance of the DKAS 1 and LKAS 2 subclusters was checked by Jack-knife analysis ($P < 0.01$, 100% correct re-classification). Marked band types 1–8 denominate dominant observed bands. Band types 1 and 5 occurred in both springs, whereas band types 2, 3, 4 and 6, 7, 8 were specific for LKAS 2 and DKAS 1 respectively.
B. Cluster analysis of the corresponding 16S-rDNA rePCR-DGGE profiles. Methods were applied as described above. Except for DKAS 14.1.02 and DKAS 19.11.01, re-classification by Jack-knife analysis was highly significant ($P < 0.01$).

aquifer itself were only reflected if an exchange between the rock surfaces and the surrounding water phase existed. For porous aquifers it has been concluded that well water normally does not accurately reflect attached microbial biofilm communities (Harvey *et al.*, 1984; Hazen *et al.*, 1991; Alfreider *et al.*, 1997) and planktonic cells may be inactive subsets of the attached organism (Hazen *et al.*, 1991) or differentiated communities (Lehman and O'Connell, 2002). For fractured, fissured or conduit aquifers only insufficient information exists to support a similar conclusion (Lehman *et al.*, 2001a,b). However, irrespective of the ratio of attached versus suspended microbes, attached communities at aquifer compartments showing high hydrological permeability may be exposed to pronounced hydrological shear stress. During periods of increased discharge conditions and floods, this is likely to lead to significant sloughing of attached cells into the water phase. In fact, this effect has been demonstrated for surface waters, as suspended bacteria during floods originated internally from scouring of stream and river sediment-biofilms (Blenkinsopp and Lock, 1994). Surprisingly, bacterial DGGE profiles remained constant through-

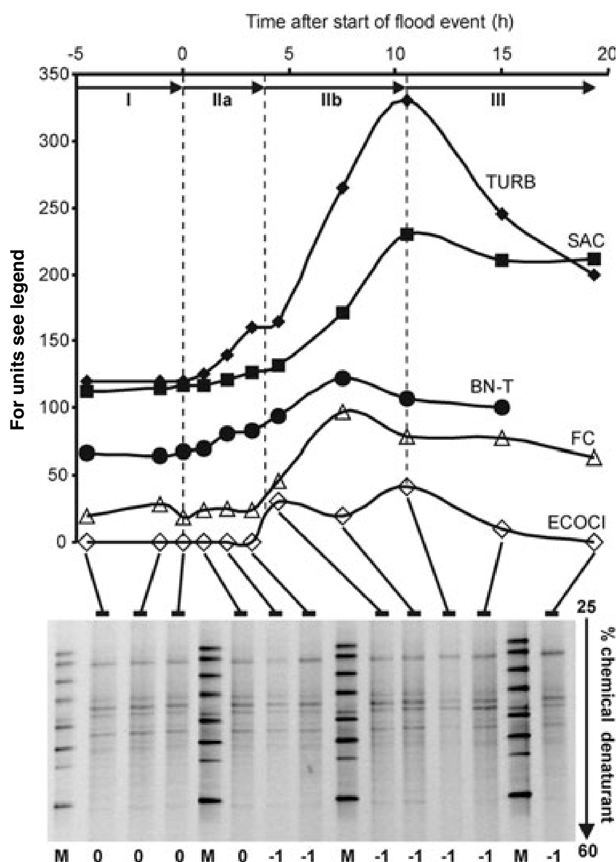

Fig. 4. Flood time course analysis of LKAS 2 in June 2002 after a summer thunderstorm event. Selected parameters of turbidity (TURB, ppm $SiO_2 \times 2 \times 10^{-3}$), spectral absorbance coefficient at 254 nm (SAC, $m^{-1} \times 10^{-2}$), total bacterial numbers (BN-T, cells $l^{-1} \times 10^6$), faecal coliforms (FC, cfu 100 $ml^{-1} \times 2$), enterococci (ECOCI, cfu 100 ml^{-1}) and the corresponding 16S-rDNA PCR-DGGE profiles are shown. Abbreviations for the DGGE profiles are: M = DGGE band pattern standard, 0 = no DNA template dilution applied, –1 = 10-fold DNA template dilution applied. According to the selected parameters distinct phases of the flood event could be distinguished (I-III). Phase I: before start of the flood event. Phase IIa: increase of discharge with increase of TURB, SAC and BN-T. Phase IIb: further increase of flood but with significant recent surface influence by sharply rising faecal indicators. Phase III: flood recession. The discharge (not shown) increased from 3900 l s^{-1} (start of the flood event) up to 6500 l s^{-1} (peak of the flood event).

out the whole flood periods which was encountered during the thunder storm event in the LKAS 2 system. Thus, data derived from the flood time course analysis suggest the presence of AMEC not only in the spring water itself but also for the compartment of attached communities which are able to interchange between the respective surfaces and the water column in the karstic aquifer. It should be pointed out, that these findings do not relate to metabolic activity of the microbial communities and refer only to the qualitatively corresponding AMEC community structure. In contrast, we speculate that any potential activity and metabolism with hypothetical importance for alpine karst biogeochemistry is likely mediated by surface associated AMEC, as it has already been proven for aquifers other than for karstic and fissured habitats (Alfreider *et al.*, 1997; Griebler *et al.*, 2001). Whether there exist metabolically active AMEC biofilms in alpine karstic environments with relevance for energy and matter flux, as previously suggested by Menne (1997,1999), has yet to be evaluated.

The variable part of the seasonal DGGE band patterns was most likely caused by varying drainage conditions within the endokarst

Analysis of the variable parts of the DGGE band patterns observed during the one year study did not reveal any conclusive results. Especially, no relationship between the numbers of bands per analysis (i.e. operational taxonomic units) in spring water of the dynamic LKAS 2 system to any of the observed hydrological parameters could be observed as there was no detectable correlation to discharge ($\varphi = 0.16$), turbidity ($\varphi = -0.04$), or SAC ($\varphi = 0.14$) (Table 2). Thus, variations of these band types could not be linked to surface runoff events probably adding soil-, vegetation- or surface-associated microbial populations into the LKAS 2 system. As the investigated springs are fed from large aquifer areas the variable part of the DGGE pattern may be explained simply by local population differences of AMEC in combination with varying contributions to the spring water because of differing hydrological drainage conditions. Furthermore, constancy of DGGE band patterns despite the proven immediate surface input in the LKAS 2 system by faecal indicators during the investigated flood event (Fig. 4, period IIb and III) can be explained by methodical constraints. Polymerase chain reaction-DGGE-based profiling approaches recover only the most dominating populations, and populations less than 1% in abundance are usually not detected (Muyzer *et al.*, 1993). Thus, temporal occurrence of transient microbial communities at subdetectable levels could not be detected. Occurrence of many subdetectable populations in the LKAS 2 may add up to a significant number of bacteria cells during warmer seasons where surface runoff existed, as indicated by the correlation between discharge and faecal indicators (Table 2). It should be mentioned that the used DGGE profiling approach targeted bacterial populations, whereas the applied bacterial direct count recovers bacterial as well as archaeal cells. As bacterial populations are thought to be the most significant fraction of prokaryotic populations in alpine aquatic systems (Pernthaler *et al.*, 1998; Batin *et al.*, 2001), this compartment was chosen in order to get a representative information about the genetic microbial population dynamics in alpine karstic spring water. A significant bias is thus not expect because of the methodical

Table 4. 16S rDNA sequences recovered from the DKAS 1 and its closest phylogenetic affiliations.

Sequence	Redundancy[a]	Accession No.[b]	bp	Classification[c]/Closest affiliation[d] (corresponding GenBank entry)	Similarity[e] (%)
SDKAS 1_1	2	AY734247	824	phylum *Bacteroidetes* (95%), genus *Flavobacterium* (95%)/*Flavobacterium succinicans* DSM 4002 (T)	99.3
SDKAS 1_2	3	AY734246	1302	class *Alphaproteobacteria* (95%), genus *Caulobacter* (95%)/*Caulobacter sp.* str. FWC38 (AJ227774)	96.6
SDKAS 1_3	1	AY742256	479	class *Alphaproteobacteria* (95%), family *Hyphomicrobiaceae* (90%)/clone BSV03 (AJ229177lg6273689)	96.4
SDKAS 1_4	1	AY734245	491	class unclassified *Betaproteobacteria*. (95%)/clone OPB37 (AF026985lg2746094)	93.8
SDKAS 1_5	1	AY734244	623	domain unclassified *Bacteria* (95%)/clone mb1228 (Z95733lg2329894)	94.9
SDKAS 1_6	1	AY734243	1346	class unclassified *Gammaproteobacteria* (95%)/*Coxiella burnetii* str. VR730. (D89798lg1742019)	91.7
SDKAS 1_7	1	AY734242	1359	class unclassified *Gammaproteobacteria* (95%)/*Rickettsiella* grylli (U97547lg2564110)	86.8
SDKAS 1_8	12	AY734239	1379	domain unclassified *Bacteria* (95%), family *Nitrospiraceae* (50%)/*Nitrospira marina* (X82559lg971567)	86.4
SDKAS 1_9	1	AY734238	1360	class unclassified *Gammaproteobacteria* (95%)/*Rickettsiella grylli*. (U97547lg2564110)	87.6
SDKAS 1_10	1	AY734237	1368	phyllum unclassified *Proteobacteria* (95%), *class Deltaproteobacteria* (70%)/clone Adriatic33 (AF030776lg2766457)	92.5
SDKAS 1_11	1	AY742257	685	class unclassified *Gammaproteobacteria* (95%)a/uncultured bacterium *DSSD65* (AY328763)	88.9
SDKAS 1_12	1	AY734236	821	domain unclassified *Bacteria* (95%), family *Nitrospiraceae* (50%)/clone C0209 (AF128668lg4761882)	85.0
SDKAS 1_13	1	AY734241	1381	class *Gammaproteobacteria* (95%)/*Rickettsiella grylli.* (U97547lg2564110)	85.0
SDKAS 1_14	1	AY734240	826	domain unclassified *Bacteria* (95%)/clone koll6 (AJ224539lg2916820)	80.0

a. Number of identical sequences recovered from the clone library.
b. Sequences are accessible at GenBank under the respective accession numbers.
c. Sequences were classified by the Naive Bayesian rRNA classifier algorithm (Version 1.0), Ribosomal Database Project – II, Release 9; The respective confidence threshold is given in brackets.
d. Closest affiliation was achieved by the sequence match algorithm, Ribosomal Database Project – II, Release 8.1.
e. Similarities were calculated to the closest affiliation by the similarity matrix function using the Kimura 2-parameter and empirical base frequency correction contained in the Ribosomal Database Project – II, Release 8.1.

constraints of the microscopic direct count versus the PCR-DGGE analysis.

Recovered 16S rDNA sequences further support the presence of AMEC

The recovered 16S rDNA sequences from the DKAS 1, except SDKAS 1_1, revealed similarities lower than 97% to already known sequences (Table 4). Retrieved sequences can thus be proposed as representatives of new species (Stackebrandt, 2004) from an AMEC which has not been reported before. About two third of the sequence types aligned well to the division and subdivisions of *Proteobacteria and Flexibacter-Cytophaga-Bacteroides,* being in accordance to former studies in which representatives in several aquatic habitats were found (Hugenholtz *et al.*, 1998). The three identical clones of SDKAS 1_2 which matches the *Caulobacter group* (Table 4) corresponds quite well with the anticipated ecological characteristics, as many *Caulobacter* sp. are typical inhabitants of oligotrophic aquatic environments (Stahl *et al.*, 1992). One third of the retrieved sequence types could not allocated with higher confidence (> 50%) to a taxonomic hierarchy lower than the bacterial domain with the used Bayesian rRNA classifier algorithm, indicating the unique habitat and the originality of the AMEC sequences. In this respect, additional phylogenetic analysis of the dominant sequence type of SDKAS 1_8 and SDKAS 1_12 from the DKAS 2 system (i.e. 13 clones retrieved) by neighbour joining placed it in the vicinity of the *Nitrospira marina*/*Nitrospira moscoviensis* and the *Leptospirillum ferrooxidans* cluster (tree not shown), both well known to possess chemolithoautotrophic capabilities (Dworkin, 2004). Significant chemolithoautotrophic activities in alpine karst aquifers would be quite surprising, as karstic systems are believed to be mainly controlled and limited by allochthonous organic matter input (Culver, 1985). However, further detailed taxonomic as well as physiological investigations have to verify whether chemolithoautotrophic AMEC activities are of any significance in alpine karst aquifers.

Hydrogeology apparently influences the microbial ecology of the spring water and the karst aquifer

Direct cell counts of LKAS 2 were permanently higher by a median factor of 4.7 as compared with DKAS 1 (Fig. 2A). This may be explained by at least three components which are governed by hydrogeology. First, sloughing off and erosion of attached bacterial cells in the dynamic LKAS 2 aquifer is potentially more pronounced than in the DKAS 1 as indicated by hydrology and correlation analysis (Fig. 1, Table 2), thus resulting in higher numbers of suspended cells. Second, significantly increased input of transient microbial communities by surface runoff into the aquifer, although not detectable by the applied PCR-DGGE but proven by faecal indicators, is very likely. Third, because average water residence time in DKAS 1 is about on order of magnitude higher than for LKAS 2 (22 years vs. approximately 1 years) one could argue that external bacterial substrate supply is only available at a reduced level as compared with the LKAS 2 system (Culver, 1985). Thus reduced substrate supply in the conservative DKAS 1 may lead to a more pronounced bottom up control of bacterial abundance as compared with the dynamic LKAS 2 system. This scenario is supported by monthly determined DOC values during winter 2003/04 from December to June (A.H. Farnleitner and R.L. Mach, unpublished results). Dissolved organic carbon concentrations in LKAS 2 were with 0.9 mg C l^{-1} (0.5–1.2 mg C l^{-1}) approximately two times higher as DKAS 1 (0.4–0.5 mg C l^{-1}) ($n = 7$). This is in further agreement with the very low numbers of HPC in the DKAS (Table 1), indicating only low amounts of easily degradable organic material. The suggested higher substrate limitation of bacterial cells in DKAS 1 as compared with LKAS 2 also corresponds to the increased abundance of small cocci and a trend to decreased cell volumes. It is a well known fact that starvation of bacterial cells often results in changes in morphology and size. Upon starvation and degenerative division many bacterial populations become cocci from rod-shaped cells (Lappin-Scott and Costerton, 1990; Kjelleberg, 1993). However, other authors have reported that starved cells may retain their morphology to a certain degree (Morita, 1997). Nonetheless most authors agree that starvation results in a reduced cell size (Lappin-Scott and Costerton, 1990; Kjelleberg, 1993; Morita, 1997), which is likely a result of a smaller biochemical machinery, altered cell boundary composition leading to increased surface to volume ratios and less limitation by diffusion kinetics (Kjelleberg, 1993; Morita, 1997). Currently we cannot distinguish between a morphological differentiation on the community level or at the level of single populations. In conclusion, according to the hydrogeological factors mentioned, cell numbers decreased significantly in the LKAS 2 during winter season and snow cover which may have been an additive result of decreased external substrate supply, lower shear stress on attached communities and reduced external cell input (Fig. 2A). In contrast, bacterial abundance was more or less constant in DKAS 1, irrespective of seasonal and climatic conditions.

In conclusion, results from several investigated parameters provide first evidence for the presence of suspended AMEC in alpine karstic spring water. Furthermore, flood time course analysis suggests the presence of AMEC also for the compartment of attached communities which are able to interchange between the aquifer solids and the water column. Recovery of AMEC may be considered of relevance as they could provide a future basis for the understanding of alpine karst aquifer biogeochemistry. This is of interest as public water supply abstracting such groundwater resources may use improved knowledge on alpine karst water ecology for long-term water quality maintenance (White, 2002).

Experimental procedures

Study area

The studied alpine and mountainous karst systems are located in the so called Northern Calcareous Alps in Austria reaching altitudes up to approximately 2300 m. The selected contrasting alpine springs have nearby catchments areas and their spring discharges are directly accessible in the respective valleys at between 600 m to 800 m altitude (Stadler and Strobl, 1997; Stadler and Strobl, 2001). Dolomite karst aquifer spring 1 is a dolomitic-limestone spring type (Stadler and Strobl, 1997) according to the classification of D'Amore and colleagues (1983). The spring discharge shows very low dynamic variations with a mean discharge of 300 l s^{-1} (year 1993–2002) and a Q_{min}/Q_{max} discharge ratio of 1:1.59. The depletion coefficient α (d^{-1}) of the base flow is with 0.00164 characteristic for well stored dolomite karst aquifers (Stadler and Strobl, 2000). The storage dynamics of this spring is mainly affected by the snow break. The mean water residence time of about 22 years is rather high (Stadler and Strobl, 1997) and the aquifer shows a good water storage capacity. Dolomite karst aquifer spring 1 is dominated by fissured and porous media. The estimated catchment area and mean altitude is about 13.4 km^2 and 1175 m above sea level respectively (Stadler and Strobl, 1997). The plant cover is characterized mainly by natural and seminatural forests but also pasture grasslands at the mountain tops (Dirnböck *et al*., 1999). Limestone karst aquifer spring 2 is a typical limestone spring type according to D'Amore and colleagues (1983) having well developed karst conduits (Stadler and Strobl, 1997). The mean discharge was 4.836 l s^{-1} (year 1995–2000) showing high variations with a Q_{max}/Q_{min} discharge ratio of ≈ 40. The mean water residence was estimated between 0.8 and 1.5 years and the discharge response after precipitation is very quick (some hours). The estimated alpine catchment area and the mean altitude is about 70 km^2 of size and 1380 m above the sea level respectively (Stadler and Strobl,

2000). Vegetation comprises summer pastures, natural calcareous alpine swards with open krummholz and forests (Dirnböck *et al*., 1999).

Hydrological and chemophysical data

All hydrological and chemophysical data were recovered by in-field on-line sensors directly installed at both spring sites. Conductivity, water temperature and discharge related parameters (water pressure, current meters, inductive discharge measurements) were registered with the data collecting system GEALOG-S from Logotronic (Vienna, Austria). Used conductivity and pressure probes were WTW-Tetracon 96 A (WTW, Weilheim, Germany) and PDCR 1830 (Druck, London, UK). Impeller flow sensors were Peek 400 models (Peek, Houston, USA) and current meters were models from ELIN Water Technology (Vienna, Austria). Signals from these sensors were converted with algorithms from the manufacturers and from discharge stage relations (Stadler and Strobl, 2000). Data were stored every 15 min which comprised the data basis for all hydrographical investigations. For the herein given figures, daily mean values were used. All sensors were controlled with single measurements with an interval of one to four weeks, using instruments, which were part of a certificated quality management system. Turbidity, SAC_{254} and pH were measured with a Sigrist and an HL2200 device.

Sampling and sample processing

Dolomite karst aquifer spring 1 and LKAS 2 were sampled consecutively within 1 h for a 11-month period from May 2001 to March 2002. During June to September samples were taken fortnightly and for the rest of the period once a month. A 5-l volume of spring water per sample was taken in clean and autoclaved Nalgene sampling bottles, stored in dark cooling boxes at ambient spring water temperatures for transportation and processed within 6 h after collection. For microscopic cell analysis, 20 ml aliquot subsamples were put into sterile 25 ml glass vials and fixed with 1 ml buffered formaldehyde (33% w/v, pH = 8). Samples were stored at 4°C and analysed within 14 days. For molecular biological analysis a volume of 2 l spring water was filtered trough polycarbonate membrane filters (Isopore™, 45 mm diameter, 0.2 μm pore size, Millipore Corp. Bedford, MA). Immediately after filtration, filters were frozen and stored at −80°C until nucleic acid extraction. Enumeration of FC, ECOCI and HPC was performed as described elsewhere in detail (Standard, 1998; Standard, 2000; Farnleitner *et al*., 2002).

Microscopic determination of bacterial numbers, morphotypes, cell volumes and biomass

For microscopic examination, a slightly modified version of the acridine-orange direct count method after Hobbie and colleagues (1977) was applied. An amount of 5–10 ml of the fixed samples were mixed with an acridine-orange solution to a final concentration of 0.01%. After 1 min the mixture was filtered through a black 0.2 μm pore-size polycarbonate filter (Millipore, Vienna), which had been mounted onto a 0.45-μm pore-size cellulose-nitrate filter (Sartorius, Vienna) resulting in an even distribution of the cells. Filters were observed at a magnification of 1250× with a Leitz Diaplan microscope equipped with an HBO 50 W mercury lamp (excitation wavelength 450–490 nm, cut-off filter 515 nm). Bacteria were separated into three classes according to their different morphology: rods, cocci and vibrios (curved rods). Other forms were never observed. Bacteria were sized by eyepiece micrometer (Kirschner and Velimirov, 1997). Cell volume estimations were based on the assumption, that all bacteria are spheres or cylinders with two hemispherical caps. At least 10 microscopic fields per sample were counted and 100–150 cells were measured (> 30 per morphotype). Cellular carbon content in fgC $cell^{-1}$ (C) was calculated from estimated cell volumes (V; μm^3) assuming the allometric relation $C = 120 \times V^{0.72}$ according to Norland (1993).

Molecular 16S-rDNA analysis

Nucleic acid extraction was performed after Griffiths and colleagues (2000). Recovered DNA was stored at −80°C until all samples were collected. Polymerase chain reaction amplification of the bacterial 16S-rDNA and a subsequent DGGE of the amplicons was performed as previously described using an iCycler IQ™ and a D GENE Denaturing Gel Electrophoresis System according to manufactures instructions (Bio-Rad, Vienna, Austria) (Farnleitner *et al*., 2001). Primers PRBA 338f carrying an additional 5′GC clamp and PRUN 518r were used as oligonucleotide couples covering the hypervariable V3 domain of the bacteria (Ovreas *et al*., 1997). In order to further increase the likelihood to detect differences in the bacterial community composition, an additional rePCR-DGGE approach was applied as PCR is known for 'over' – amplification of less abundant target molecules under non-optimal conditions such as extended cycling numbers. (Wintzingerode *et al*., 1997). High cycle numbers also increase the likelihood of artificial products, thus only DGGE profiles as a whole, not single bands, were compared and only taken as indirect information on community changes. In order to promote such a levelling of targets irrespectively of the initial target ratio we significantly increased the cycling number to a total amount of 50 cycles. This was done by adding a second PCR (rePCR) with 20 amplification cycles to the initial one using 1 μl of a 10-fold dilution of the initial PCR amplicon. At the 15.12.2001 and the 20.1.2002 water samples from DKAS 1 was taken for recovery of 16S rDNA full and partial length sequences. Amplification, cloning and sequencing was performed after the protocol of Cho and Kim (2000). Two primer sets, 27F and 1492R (Cho and Kim, 2000) and 63f and 1387r (Marchesi *et al*., 1998) designed in order to target the bacterial domain were used. After amplification and gel electrophoresis equal amounts of the amplicons were pooled from both reactions and both sampling dates in order to decrease amplification bias (Wintzingerode *et al*., 1997). Sequencing of clones was performed until saturation of clone redundancy deviated more than a factor of 2 from the linear non-redundant theoretical distribution. Sequences were analysed by tools available at the Ribosomal Data Bases Project – II (http://www.rdp.cme.msu.edu/index.jsp). Sequences were checked by 'chimera check' and searched for closest affiliations by Sequence Match (RDP Version 8.1). Classification of sequences were achieved by the 'classifier' function

using a naive Bayesian rRNA classification algorithm (RDP Version 9). In addition, neighbour joining analysis was performed as included in the RDP package Version 8.1.

Statistical analysis

Statistical analysis was performed using SPSS (version 8.0) and non-parametric tests as described in the results. For standardization of the DGGE gels and subsequent band pattern analysis GelCompar (version 2) was used (Manual GelCompar 2, Applied Maths, Belgium). To enable DGGE standardization, sufficient DGGE-markers and sequencing were used (Farnleitner *et al.*, 2001). Comparison of DGGE profiles were performed by band-based (Jaccard) as well as curve-based (Pearson Similarity Coefficient) procedures (Farnleitner *et al.*, 2004) and cluster analysis applied single, complete, and UPGMA linkage. Significance of clusters was tested by Jack-knife resampling procedure.

Acknowledgements

This study was funded by a grant from the Austrian Academy of Sciences to A.H.F. [APART 10794 (Austrian Programme for Advanced Research and Technology)] and by the FWF project P15662-B07 of the Austrian Ministry of Sciences. Special thanks to Christian Beiwl and Hermann Kain for crucial technical assistance and to Dr Thomas Dirnböck for providing background information on the vegetation in the considered area.

References

Alfreider, A., Krössbacher, M., and Psenner, R. (1997) Groundwater samples do not reflect bacterial densities and activity in subsurface systems. *Water Res* **31:** 832–840.

Batin, T.J., Wille, A., Sattler, B., Psenner, R. (2001) Phylogenetic and functional heterogeneity of sediment biofilms along environmental gradients in a glacial stream. *Appl Environ Microbiol* **67:** 799–807.

Blenkinsopp, S.A., and Lock, M.A. (1994) The impact of storm flow on river biofilm architecture. *J Phycol* **30:** 807–818.

Cho, J.C., and Kim, S.J. (2000) Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. *Appl Environ Microbiol* **66:** 956–965.

Culver, D.C. (1985) Trophic relationships in aquatic environments. *Stygologia* **1:** 43–53.

D'Amore, F., Scandiffio, G., and Panichi, C. (1983) Some observations on the chemical classification of ground water. *Geothermics* **12:** 141–148.

Dirnböck, T., and Grabherr, G. (2000) GIS assessment of vegetation and hydrological change in a high mountain catchment of the Northern Limestone Alps. *Moun Res Dev* **20:** 172–179.

Dirnböck, T., Dullinger, S., Gottfried, M., and Grabherr, G. (1999) Die Vegetation des Hochschwab – Alpine und Subalpine Stufe. *Mitt Naturwissen Verein Stmk* **129:** 111–251.

Doerfliger, N., Jeannin, P.Y., and Zwahlen, F. (1999) Water vulnerability assessment in karst environments: a new method of defining protection areas using a multi-attribute approach and GIS tools. *Environ Geol* **39:** 165–176.

Drew, D., and Hötzl, H. (1999) Karst hydrogeology and human activities. In *International Conributions to Hydrogeology*. Drew, D., and Hötzl, H. (eds). Rotterdam, the Netherlands: A.A. Balkema.

Dworkin, M. (2004) The prokaryotes: an evolving electronic resource for the microbial community [WWW document]. URL http://141.150.157.117.8080/prokPUB/index.html

Farnleitner, A.H., Reischer, G.H., Zibuschka, F., Lindner, G., and Mach, R.L. (2001) Comparison and differentiation of heterotrophic plate count communities in raw and drinking water by eubacterial 16S-rDNA amplicon profiling. *Vom Wasser* **97:** 167–180.

Farnleitner, A.H., Hocke, L., Beiwl, C., Kavka, G.G., and Mach, R.L. (2002) Hydrolysis of 4-methylumbelliferyl-beta-D-glucoronide in differing sample fractions of river waters and its implication for the detection of fecal pollution. *Water Res* **36:** 975–981.

Farnleitner, A.H., Zibuschka, F., Burtscher, M.M., Lindner, G., Reischer, G., and Mach, R.L. (2004) Eubacterial 16S – rDNA amplicon profiling: a rapid technique for comparison and differentiation of heterotrophic plate count communities from drinking water. *Int J Food Microbiol* **92:** 333–345.

Ford, D.C., and Williams, P.W. (1996) *Karst Geomorphology and Hydrology*. London, UK: Chapman & Hall.

Ghiorse, W.C., and Wilson, J.T. (1988) Microbial ecology of the terrestrial subsurface. *Adv Appl Microbiol* **33:** 107–172.

Gibert, J., and Danielopol, D.L. (eds) (1994) *Groundwater Ecology.* San Diego, CA, USA: Academic Press.

Gounot, A.M. (1994) Microbial ecology of groundwaters. In *Groundwater Ecology.* Gibert, J., and Danielopol, D.L. (eds). San Diego, CA, USA: Academic Press, pp. 189–215.

Griebler, C., Danielopol, D.L., Gibert, J., Nachtnebel, H.P., Notenboom, J. (2001) *Groundwater Ecology: A Tool for Management of Water Resources.* Griebler, C., Danielopol, D.L., *et al.* (eds). Luxembourg: Office For Official Publications of the European Communities.

Griffiths, R.I., Whitely, A.S., O'Donnel, A., and Bailey, M.J. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* **66:** 5488–5491.

Haack, T.K., and McFeters, G.A. (1982) Microbial dynamics of an epilithic mat community in a high alpine stream. *Appl Environ Microbiol* **43:** 702–707.

Harvey, R.W., Smith, R.L., and George, L. (1984) Effect of organic contamination upon microbial distributions and heterotrophic uptake in a Cape Cod, Mass., aquifer. *Appl Environ Microbiol* **48:** 1197–1202.

Hazen, T.C., Jimenez, L., de Victoria, G.L., and Fliermans, C.B. (1991) Comparison of bacteria from deep subsurface sediment and adjacent groundwater. *Microb Ecol* **22:** 293–304.

Hobbie, J., Dayley, R., and Jasper, S. (1977) Use of nuclepore filters for counting bacteria by epifluorescence microscopy. *Appl Environ Microbiol* **33:** 1225–1228.

Hugenholtz, P., Goebel, B., and Pace, N.R. (1998) Impact of culture-independent studies on the emerging phyolge-

netic view of bacterial diversity. *J Bacteriol* **180:** 4765–4774.

Kirschner, A., and Velimirov, B. (1997) Seasonal study of bacterial community succession in a temperate backwater system indicated by variation in morphotype numbers, biomass and secondary production. *Microb Ecol* **34:** 27–38.

Kjelleberg, S. (1993) *Starvation in Bacteria*. New York, NY, USA: Plenum Press.

Lappin-Scott, H.M., and Costerton, J.W. (1990) Starvation and penetration of bacteria in soils and rocks. *Experentia* **46:** 807–812.

Lehman, R.M., and O'Connell, S.P. (2002) Comparison of extracellular enzyme activities and community composition of attached and free-living bacteria in porous medium columns. *Appl Environ Microbiol* **68:** 1569–1575.

Lehman, R.M., Colwell, F.S., and Bala, G.A. (2001a) Attached and unattached microbial communities in a simulated basalt aquifer under fracture- and porous-flow conditions. *Appl Environ Microbiol* **67:** 2799–2809.

Lehman, R.M., Roberto, F.F., Early, D., Bruhn, D.F., Brink, S.E., O'Connel, S.P., *et al.* (2001b) Attached and unattached bacterial communities in a 120-meter corehole in an acidic, crystalline rock aquifer. *Appl Environ Microbiol* **67:** 2095–2106.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., *et al.* (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* **64:** 795–799.

Menne, B. (1997) Mikrobiologische Prozesse im Karstwasserköper. In *Proceedings of the 12th International Congress of Speleology*, La-Chaux-de-Fond, France, Vol. 3. Basel, Switzerland: International Union of Speleology, pp. 289–292.

Menne, B. (1999) Myxobacteria in cave sediments of the French Jura Mountains. *Microbiol Res* **154:** 1–8.

Morita, R.Y. (1997) *Bacteria in Oligotrophic Environments.* London, UK: Chapman & Hall.

Muyzer, G., De Wal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified genes encoding for 16S rRNA. *Appl Environ Microbiol* **59:** 695–700.

Norland, S. (1993) The relationship between biomass and Volume of bacteria. In *Handbook of Methods in Aquatic Microbial Ecology.* Kemp, P.F., Sherr, B.F., Sherr, E.B., and Cole, J.J. (eds). Boca Raton, FL, USA: Lewis, pp. 303–307.

Ovreas, L., Forney, L., Daae, F.L., and Torsvik, V. (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* **63:** 3367–3373.

Pernthaler, J.F., Glöckner, F.O., Unterholzner, A., Alfreider, A., Psenner, R., and Amann, R. (1998) Seasonal community and population dynamics of pelagic bacteria and archea in a high mountain lake. *Appl Environ Microbiol* **64:** 4399–3306.

Rusterholtz, K.J., and Mallory, L.M. (1994) Density, activity and diversity of bacteria indigenous to a karst aquifer. *Microb Ecol* **28:** 79–99.

Simon, K.S., Gibert, J., Petitot, P., and Laurent, R. (2001) Spatial and temporal patterns of bacterial density and metabolic activity in a karst aquifer. *Arch Hydrobiol* **151:** 67–82.

Stackebrandt, E. (2004) Defining taxonomic ranks: Systematics and Classification. In *The Prokaryotes: an Evolving Electronic Resource for the Microbial Community* [WWW document]. URL http://141.150.157.117.8080/prokPUB/index.html

Stadler, H.E., and Strobl, E. (1997) *Karstwasserdynamik und Karstwasserschutz Zeller Staritzen*. Graz, Austria: Institute of Hydrogeology and Geothermics, Joanneum Research.

Stadler, H.E., and Strobl, E. (2000) *Karstwasserdynamik und Karstwasserschutz Hochschwab.* Graz, Austria: Joanneum Research.

Stadler, H., and Strobl, E. (2001) *Karstwasserdynamik und Karstwasserschutz Hochschwab*. Graz, Austria: Institute of Hydrogeology and Geothermics, Joanneum Research.

Stahl, D.A., Key, R., Flesher, B., and Smit, J. (1992) The phylogeny of marine and freshwater caulobacters reflects their habitat. *J Bacteriol* **174:** 2193–2198.

Standard (1998) Enumeration of culturable micro-organism – colony count by inoculation in a nutrient agar culture medium. Brussels, Belgium. prEN ISO 6222.

Standard (2000) Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organisation for Standardisation. ISO/FDIS 7899–2:2000 (E).

White, W.B. (2002) Karst hydrology: recent developments and open questions. *Engin Geol* **65:** 85–105.

Wintzingerode, F., Göbel, U., and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. *FEMS Microbiol Rev* **21:** 213–229.

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