Microbial communities in karst groundwater and their potential use for biomonitoring

Michiel Pronk · Nico Goldscheider · Jakob Zopfi

Abstract The structure, diversity and dynamics of microbial communities from a swallow hole draining agricultural land and two connected karst springs (Switzerland) were studied using molecular microbiological methods and related to hydrological and physicochemical parameters. Storm responses and an annual hydrological cycle were monitored to determine the short- and long-term variability, respectively, of bacterial communities. Statistical analysis of bacterial genetic fingerprints (16S rDNA PCR-DGGE) of spring water samples revealed several clusters that corresponded well with different levels of the allochthonous swallow hole contribution. Microbial communities in spring water samples highly affected by the swallow hole showed low similarities among them, reflecting the high temporal variability of the bacterial communities infiltrating at the swallow hole. Conversely, high similarities among samples with low allochthonous contribution provided evidence for a stable autochthonous endokarst microbial community. Three spring samples, representative for low, medium and high swallow hole contribution, were analysed by cloning/sequencing in order to identify the major bacterial groups in the communities. The autochthonous endokarst microbial community was mainly characterized of *δ-Proteobacteria*, *Acidobacteria* and *Nitrospira* species. A high percentage of unknown sequences suggested further that many karst aquifer bacteria are still undiscovered. Finally, the potential use of groundwater biomonitoring using microbial communities is discussed.

Received: 22 January 2008 / Accepted: 1 July 2008
Published online: 8 August 2008

M. Pronk · N. Goldscheider
Centre of Hydrogeology,
University of Neuchâtel,
Rue Emile-Argand 11, CP 158, CH-2009, Neuchâtel, Switzerland

J. Zopfi (✉)
Laboratory of Microbiology, Institute of Biology,
University of Neuchâtel,
Rue Emile-Argand 11, CP 158, CH-2009, Neuchâtel, Switzerland
e-mail: jakob.zopfi@unine.ch
Tel.: +41-32-7182260
Fax: +41-32-7182231

Keywords Subsurface microbiology · Ecology · Groundwater monitoring · PCR-DGGE · Cloning/sequencing · Switzerland

Introduction

Biomonitoring is widely used to evaluate the quality of surface waters and appears to be an interesting approach for groundwater. This reasoning is reflected in the Swiss Water Protection Ordinance (GSchV 1998), which demands, in order to improve water-quality standards, that the biocenosis in groundwater should be in a natural state and characteristic of water that is not or only slightly contaminated.

Unlike chemical analyses, biomonitoring may give indications about unknown contaminants and substances which are usually not analysed, and deliver temporally integrated information. Two main approaches to groundwater biomonitoring exist: active biomonitoring, where standardised organisms such as trout or the crustacean Daphnia, are introduced into the water and their responses are observed (Green et al. 2003); and passive biomonitoring, which uses naturally occurring organisms such as invertebrate fauna (e.g., Hahn and Friedrich 1999; Malard et al. 1996; Vervier and Gibert 1991).

Aquifers are increasingly considered as ecosystems harbouring their proper biocenoses (Danielopol et al. 2003; Griebler and Mösslacher 2003; Hancock et al. 2005) and the potential use of microbial communities for biomonitoring has been raised (Goldscheider et al. 2006; Mösslacher et al. 2001). This approach seems promising as bacteria are abundant, ubiquitous and thrive even in extreme habitats such as contaminated, anoxic, or thermal aquifers.

To date, most studies on karst microbiology have focused on caves within the unsaturated zone (e.g., Barton and Northup 2007; Chelius and Moore 2004), percolation water with extreme chemical compositions (e.g., Hose et al. 2000; Macalady et al. 2006, 2007), and on the role of specific bacteria in geochemical processes (e.g., Canaveras et al. 1999, Northup and Lavoie 2001). Few studies, however, have investigated the structure, diversity, and activity of microbial communities in pristine karst aquifers.

Karst systems are characterised by different bacterial habitats (Goldscheider et al. 2006) including the soil-vegetation compartment from where bacteria reach the aquifer by diffuse percolation or via swallow holes (i.e.,

 DOI 10.1007/s10040-008-0350-x

allochthonous origin), the unsaturated zone (fissures, fractures, cavities) and the saturated zone where bacteria live in groundwater, attached to rock surfaces or sediment particles (i.e., autochthonous origin). However, karst systems are rarely accessible, thus their investigation is often limited to spring water samples. Several studies have reported that groundwater samples from porous aquifers do not always accurately reflect aquifer microbiology due to a high ratio of attached versus suspended cells (Alfreider et al. 1997; Lehman 2007). However, the high variability of flow velocities in karst systems induces pronounced hydrological shear stress and leads to scouring and resuspension of autochthonous sediments and associated bacteria. Storm events appear not only to be key periods in scouring these aquifer biofilms, but also in transporting allochthonous soil bacteria into karst aquifers (Simon et al. 2001). Particle-size distribution (PSD) measurements at karst springs have allowed distinguishing between autochthonous and allochthonous particles. A relative predominance of finer particles, for example, suggests an allochthonous origin and is often associated with faecal indicator bacteria contamination (Pronk et al. 2007).

Personné et al. (2004) have studied bacterial colonisation of introduced substrates in a karst aquifer and concluded that enteric bacteria from periodical contamination with waste water were unlikely to persist in the system. Rusterholtz and Mallory (1994) have further described the bacterial community from Mammoth Cave (Kentucky, USA) sediments as highly diverse with no dominant species. Based on bacterial fingerprinting and sequencing of alpine karst spring water, Farnleitner et al. (2005) have provided first evidence for the presence of stable and autochthonous endokarst microbial communities. Most of the retrieved bacterial sequences were affiliated to *Proteobacteria* and the *Cytophaga-Flexibacter-Bacteroides* group. However, more than 95% of the 28 sequences revealed similarities lower than 97% to already known bacteria and were thus considered as new species.

The main objectives of this study were: (1) to explore structure, diversity and temporal variability of microbial communities in karst groundwater; (2) to differentiate between allochthonous bacteria from the land surface and autochthonous, endokarstic bacteria; and (3) to characterise the autochthonous microbial community by identifying the most abundant bacterial components. In order to achieve these goals, a karst system was investigated on short- (storm events) and long-term scales (annual hydrological cycle). Physicochemical monitoring at a swallow hole draining agricultural land and two connected springs was combined with PSD measurements and the genetic characterisation of bacterial communities by PCR-DGGE (polymerase chain reaction—denaturing gradient gel electrophoresis) and cloning/sequencing.

Study area

The Yverdon-les-Bains karst aquifer system is located in western Switzerland between two major geological units

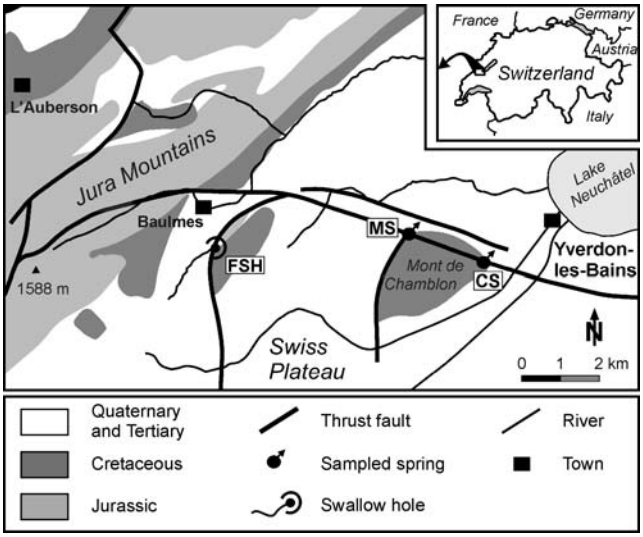

Fig. 1 Location and geological map of the Yverdon-les-Bains karst system. *FSH* Feurtille swallow hole; *MS* Moulinet spring; *CS* Cossaux spring

(Fig. 1): the folded Jura Mountains reaching an altitude of 1,588 m above sea level near the test site; and the Molasse Basin forming the Swiss Plateau at an altitude of 430–600 m. The forested south-east slope of the Jura Mountains, where Upper Jurassic (Malm) and Cretaceous limestones crop out, is the most important autogenic recharge area of this karst system (Muralt 1999). Further to the south-east, due to a complex arrangement of folds and faults, limestone is exposed in two hydrogeological windows within the low-permeability sediments of the Swiss Plateau (Sommaruga 1997), which confine the aquifer. At the western hydrogeological window, a stream sinks into the Feurtille swallow hole (altitude: ~600 m). As the stream is draining an agricultural area, it frequently contains high levels of turbidity, organic matter, nitrate and faecal bacteria. About 4 and 6 km to the east, at the base of the second window, the Moulinet and Cossaux springs (altitude: ~450 m) are located at the contact zone between the low-permeable sediments and the Cretaceous limestone. The Moulinet spring consists of eight individ-

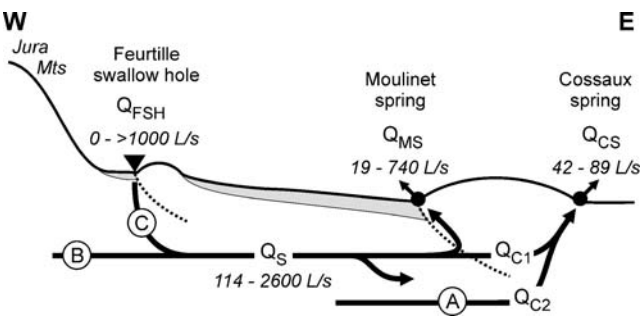

Fig. 2 Conceptual model (cross-section) of the Yverdon-les-Bains karst system (modified after Pronk et al. 2006) with minimum and maximum observed flow rates at different locations within the system. *A* deep and warm groundwater; *B* Malm limestone karst aquifer groundwater; *C* water from the swallow hole. *Dotted lines* represent major thrust faults and *grey shaded areas* represent the low-permeability sediments

 DOI 10.1007/s10040-008-0350-x

ual outlets showing nearly identical physical, chemical and microbiological characteristics. The Cossaux spring is captured by eight inclined drillings and contributes to the water supply of the city of Yverdon-les-Bains.

Previous studies based on hydrochemical and isotope data showed that the spring water consists essentially of three different components: deep, clean, and warm groundwater; clean and cold groundwater from the Malm limestone karst aquifer infiltrating on the forested slopes of the Jura Mountains; and cold and frequently contaminated water from the swallow hole (Muralt 1999). Further, monitoring of natural parameters allowed two important conclusions to be drawn (Pronk et al. 2006, 2007):

1. The swallow hole is the major source of nitrate, organic carbon and faecal indicator bacteria contamination occurring at the springs, particularly after heavy rainfall.
2. The two springs show similar temporal variations of physicochemical and microbiological parameters, whereas the absolute levels are differing. This is explained by additional inflow of clean and warm groundwater from greater depth to the Cossaux spring, resulting in lower contamination levels and a 2–3°C higher temperature.

Material and methods

Hydrological and physicochemical parameters

Discharge, water temperature, electrical conductivity, turbidity and total organic carbon (TOC) were monitored continuously (time step: 10 min) at the swallow hole and the springs. Discharge was measured by means of weirs and pressure probes (DL/N 64, STS, Sirnach, Switzerland). Temperature and electrical conductivity were recorded using conductivity probes (Cond 340i, WTW, Weilheim, Germany) coupled to data loggers (DT50, dataTaker, Rowville, Australia). Turbidity and TOC were measured with submersible flow-through field fluorometers (GGUN-FL30, University of Neuchâtel, Switzerland). The long-term stability of all instruments was checked on-site and in the laboratory twice-monthly. PSD was measured directly on-site with a portable particle counter (Abakus mobil fluid, Klotz, Unterhaugstett, Germany) and concurrent samples were collected for ion chromatography (DX-120, Dionex, Sunnyvale, USA). Daily precipitation data (MeteoSwiss) from three stations in the study area were considered: Yverdon-les-Bains (altitude: 433 m), Baulmes (642 m) and L'Auberson (1,110 m).

All dye-tracing tests were carried out by injecting 1 kg of uranine, dissolved in 10 L of water, at the swallow hole. Dye concentrations were continuously monitored with the above-mentioned flow-through field fluorometers. Additional water samples were collected with automatic samplers (6712C, Teledyne ISCO, Lincoln, USA) at time steps varying from 1 to 3 h and dye concentrations were determined in the laboratory using a spectral fluorometer (LS 50 B, PerkinElmer, Waltham, USA).

Sampling and sample processing for microbiological analyses

The swallow hole and the two springs were sampled twice-monthly during a complete annual hydrological cycle (March 2005 until February 2006), and daily during the April 2005 storm event. Water samples were collected in two sterile 1-L Nalgene bottles, transported to the laboratory in cooling boxes and processed within 6 h. Two volumes of 0.5-L water were filtered through 0.2-μm polycarbonate membranes (Cyclopore, Whatman, Kent, UK). Filters were immediately stored at –80°C until DNA extraction. Water quality indicator bacteria (including mesophilic aerobic bacteria, total coliforms, *E. coli*, and enterococci) were enumerated using standard cultivation techniques (APHA 2005).

Molecular analysis of microbial communities

Most bacteria do not grow on traditional culture media. Therefore culture-independent, molecular biological techniques are employed to bridge the disparity between cultivable and in-situ diversity. PCR-DGGE and cloning/sequencing of the 16S rDNA (a universal marker gene allowing the identification of bacteria and inferring their phylogenetic relationship) are two of these techniques and are widely used in microbial ecology but increasingly also in hydrogeology, contaminant hydrology and related disciplines. The different techniques including their potentials and limitations have been recently reviewed (Goldscheider et al. 2006; Nocker et al. 2007; Weiss and Cozzarelli 2008).

PCR-DGGE is a rapid fingerprinting technique that provides a simplified image of the bacterial community structure in a sample (e.g., Muyzer et al. 1993, Muyzer 1999, Nocker et al. 2007). Small PCR-amplified DNA products of about the same length are separated on a vertical acrylamide gel in a gradient of a denaturing agent. The direction of electrophoresis is parallel to the gradient; thus, as PCR products travel through the gel, increasing denaturing conditions start to separate the double-stranded DNA, resulting in a slower migration in the gel compared to the native conformation. The separation, or melting, of the double-stranded DNA occurs in discrete domains rather than in a zipper-like fashion and is strongly dependant on its GC (Guanine-Cytosine) content and nucleotide sequence. PCR-DGGE analysis of environmental samples containing different bacterial communities will result in a specific band pattern for each sample. Each band in the gel represents theoretically one bacterial species and the band intensity is about proportional to its relative abundance in the sample. This fingerprinting technique is thus well suited to detect community structure changes in groundwater in response to aquifer conditions (e.g., seasonal variations, rain events, contamination). However, it is estimated that PCR-DGGE only detects bacterial populations representing at least 1–2% of the microbial community (Muyzer et al. 1993).

In contrast, the direct cloning/sequencing approach is not a fingerprinting technique and rather laborious, but offers the highest phylogenetic resolution of all molecular

 DOI 10.1007/s10040-008-0350-x

methods. Identification of bacterial species or determination of similarities to already known species (or to sequences of uncultivated microorganisms) is possible through the use of sequence databases. Cloning/sequencing of environmental samples allows a more complete picture of the microorganisms present in a sample to be obtained and provides qualitative and quantitative data on the diversity (Chapelle 2001; Nocker et al. 2007).

Technical procedures: Genomic DNA was extracted from the membrane filters with the FastDNA SPIN Kit (Bio101, Vista, USA) and a FastPrep bead-beating machine (Bio101) according to manufacturers' instructions. For DGGE analysis, a small DNA fragment (~180 bp) that covers the V3 region on the eubacterial 16S rDNA gene was amplified with the forward primer GC-Eub338f (5′-ACTCCTACGGGAGGCAGCAG-3′) carrying a GC-clamp at the 5′ end, and the reverse primer Univ518r (5′-ATTACCGCGGCTGCTGG-3′; Ovreas et al. 1997). DGGE was performed on the Dcode System (Bio-Rad, Vienna, Austria). A total of 600 ng of PCR products were loaded on 8% (wt/vol) polyacrylamide gels (acrylamide: 37.5:1; 40%; Bio-Rad) with a denaturing gradient from 30 to 60% (100% denaturant contained 7 M urea and 40% formamide). Electrophoresis was done for 4.5 h at 150 V and 60°C. After migration, gels were stained in a SYBR Green I bath for 20 min (1:10,000 dilution, Molecular Probes, Eugene, USA). Standardisation and analysis of the obtained DGGE banding patterns was performed with the Gelcompar II software (Applied Maths, Sint-Martens-Latem, Belgium). Comparison of DGGE profiles was done by using band-based (Jaccard similarity coefficient) and curve-based (Pearson similarity coefficient) procedures. For individual gel analysis, both procedures gave very similar results. However, Jaccard analysis was performed if several gels were analysed together, as variable staining from one gel to the other may influence the bands intensities used by the curve-based procedure.

For clone library construction, the nearly complete eubacterial 16S rDNA gene (~1,400 bp) was PCR amplified using the primers GM3f (5′-AGAGTTTGATC (AC)TGGC-3′) and GM4r (5′-TACCTTGTTACGACTT-3′; Muyzer et al. 1995). PCR products were purified using the Wizard SV clean-up system (Promega, Madison, USA) and ligated into pGEM-T vectors (Promega) according to manufacturers' instructions. The plasmid vectors were transformed into electrocompetent *E. coli* cells, which where then incubated overnight at 37°C on Luria-Bertani (LB) plates that also contained 50 mg/mL ampicillin, 125 µl of IPTG and 50 µl X-Gal. The presence of inserts of the expected size in the *E. coli* colonies was verified by PCR using T7 and SP6 primers (Promega). Colonies having the insert were grown overnight in 3 ml LB-broth containing 50 mg/mL ampicillin (37°C, 180 rpm agitation). Plasmids were extracted with Wizard plus SV miniprep kits (Promega) and then sequenced by MWG-Biotech (Germany). A total of 134 partial (~800 bp) and nearly full length 16S rDNA gene sequences have been deposited in the EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Database under the accession numbers AM991142 to AM991275. Sequences were analysed by tools available at the Ribosomal Database Project II (Cole et al. 2007; Wang et al. 2007). They were tested for PCR anomalies by using the chimera check program. Classification of the sequences was done by using the naïve Bayesian rRNA classifier.

Results and discussion

Quantification of the swallow hole contribution

Continuous flow measurements and results from five tracer tests conducted during different hydrological conditions (Table 1) allowed quantification of the flow rates inside the aquifer (Fig. 2) using water and mass balance equations (Eqs. 1–3). The calculation is based on the assumption that uranine behaves conservatively in saturated conduit systems, i.e., no adsorption or degradation loss (Käss 1998). Discharge and tracer recovery at the Moulinet spring (Q_{MS} and R_{MS} respectively) allowed the

Table 1 Summary of uranine tracer test results

Location	Property	Unit	Tracer injection date				
			1 Sept 2003	21 Mar 2005	2 June 05	22 Apr 2006	2 Nov 2007
Feurtille swallow hole (FSH)	Discharge Q_{FSH}	(L/s)	1	52	21	103	14
Moulinet spring (MS)	Discharge Q_{MS}	(L/s)	19	338	125	560	34
	Time of first detection	(h)	292.0	40.3	86.9	23.7	183.3
	Peak concentration	(µg/L)	27.9	21.8	25.2	20.2	24.6
	Recovery R_{MS}	(%)	16.8	27.5	26.1	28.8	21.1
Cossaux spring (CS)	Discharge Q_{CS}	(L/s)	43	61	52	81	44
	Time of first detection	(h)	260.0	43.5	81.9	27.8	159.1
	Peak concentration	(µg/L)	8.7	4.5	6.6	3.5	7.8
	Recovery R_{CS}	(%)	12.3	1.6	3.2	1.1	8.6
	Q_{C1}	(L/s)	14	20	15	21	14
	Q_{C2}	(L/s)	29	41	37	60	30
Both springs	Total recovery	(%)	29.1	29.1	29.3	29.9	29.7
Malm karst aquifer	System discharge Q_S	(L/s)	114	1,229	479	1,944	160

The two flow components contributing to the Cossaux spring (Q_{C1} and Q_{C2}) and the system discharge (Q_S) are defined in the text and Fig. 2

 DOI 10.1007/s10040-008-0350-x

system discharge Q_S (= discharge in the main karst aquifer between the swallow hole and the springs, as illustrated in Fig. 2) to be calculated by:

$$Q_S = Q_{MS}(100/R_{MS}) \quad (1)$$

On this basis, it was possible to establish a relation between the Moulinet spring discharge and the system discharge and to quantify the latter for any moment.

While the shapes of the breakthrough curves at both springs were identical, the tracer concentrations monitored at the Cossaux spring were systematically lower than those observed at the Moulinet spring (Table 1). The Cossaux spring consequently receives an additional inflow of groundwater not affected by the swallow hole, i.e., the deep and warm groundwater component (Fig. 2, component A). The amount of this inflow was calculated as follows:

$$Q_{C1} = Q_{MS}(R_{CS}/R_{MS}) \quad (2)$$

$$Q_{C2} = Q_{CS} - Q_{C1} \quad (3)$$

where Q_{CS}, Q_{C1}, Q_{C2}, and R_{CS} represent the discharge of the Cossaux spring, the two flow components contributing to the spring discharge (as illustrated in Fig. 2), and the tracer recovery at the Cossaux spring, respectively. Results showed that the Cossaux spring receives 66–75% of clean and warm groundwater (Table 1), diluting the frequently contaminated water from the swallow hole.

Tracer transit times from the swallow hole to the springs varied between 12 days during low-flow to 24 h during high-flow conditions for the Moulinet spring and 11 days to 28 hours for the Cossaux spring (Table 1). Relationships between transit times and system discharge were best fitted by power laws (Fig. 3). At the springs, the breakthrough of TOC entering the swallow hole following storm events further corroborated these discharge-time relationships. Based on these observations, it is thus possible to quantify the contribution of the swallow hole component to groundwater flow within the Malm limestone karst aquifer (Q_{FSH}/Q_S) at the moment of infiltration at the swallow hole.

Short-term monitoring: storm event

The physical, chemical and microbial response of two successive rainfall events was monitored in April 2005 (Fig. 4). The discharge of the swallow hole increased shortly after the rainfall events and showed two distinct peaks followed by rapid recession (Fig. 4a). High levels of turbidity (max. 60 NTU), TOC (max. 12 mg/L), faecal indicator bacteria (max. ~500 CFU/100 ml of *E. coli*) and nitrate (max. 55 mg/L) entered the swallow hole (data not shown).

The response at the Moulinet spring showed also two distinct events (Fig. 4b). A typical response to a storm event at the Moulinet spring is divided into three stages (Pronk et al. 2007). Stage I represents pre-storm conditions with low and stable values for all parameters and lasts until spring discharge increases (Fig. 4b). During stage II, characterised by the rising limb of the spring hydrograph, autochthonous particles are remobilised due to increasing flow velocities. TOC, nitrate and faecal indicator bacteria, which are typical natural tracers of the swallow hole component, remain at pre-event levels. During the two successive storm events of April 2005, turbidity increased from 0.7 to 1.4 NTU and from 0.9 to 4.6 NTU respectively (Fig. 4b), and was of typical autochthonous origin (Fig. 5, samples 18 April and 25 April). As stage I was not reached again before the second rainfall event, the 25 April sample was still affected by a minor allochthonous contribution (Fig. 5). Stage III is characterised by the arrival of allochthonous turbid and contaminated water, which entered the swallow hole during the rainfall events, and by the following storm event recession. PSD of samples of stage III exhibited a clear allochthonous signature (Fig. 5, samples 20 April and 27 April), while the 02 May sample, which was collected at the end of the recession, tended again towards the pre-storm PSD. During stage III, maximum values of all physical, chemical and bacterial parameters were reached (turbidity: 14 NTU, TOC: 4.2 mg/L, *E. coli*: 75 CFU/100 ml, nitrate: 26 mg/L).

The DGGE fingerprints of Moulinet spring water samples (Fig. 4c) revealed complex patterns with a large number of rather faint bands, indicating that the microbial communities are diverse and consist of about equally

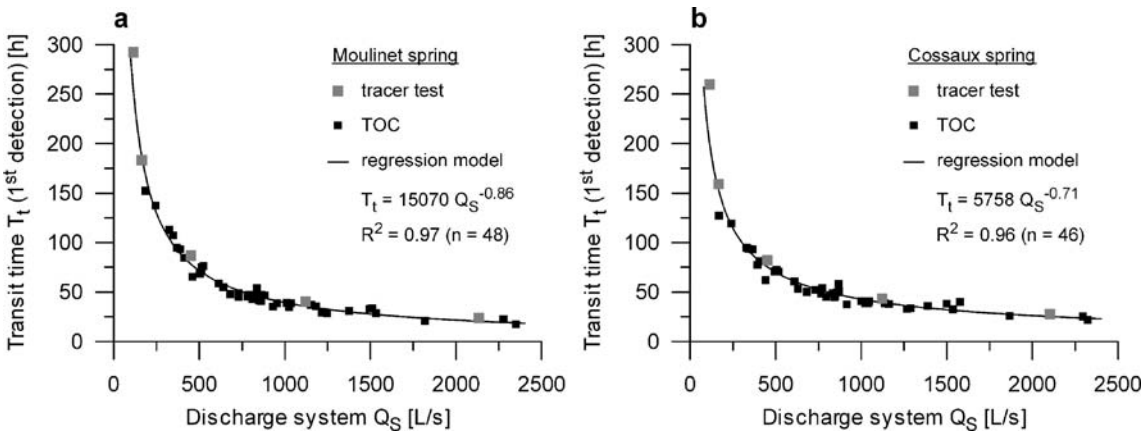

Fig. 3 Transit times between the swallow hole and the springs (**a** Moulinet; **b** Cossaux) as function of the system discharge. The relative average absolute errors of the regression models are 8.1 and 7.6% for the Moulinet and the Cossaux springs, respectively

 DOI 10.1007/s10040-008-0350-x

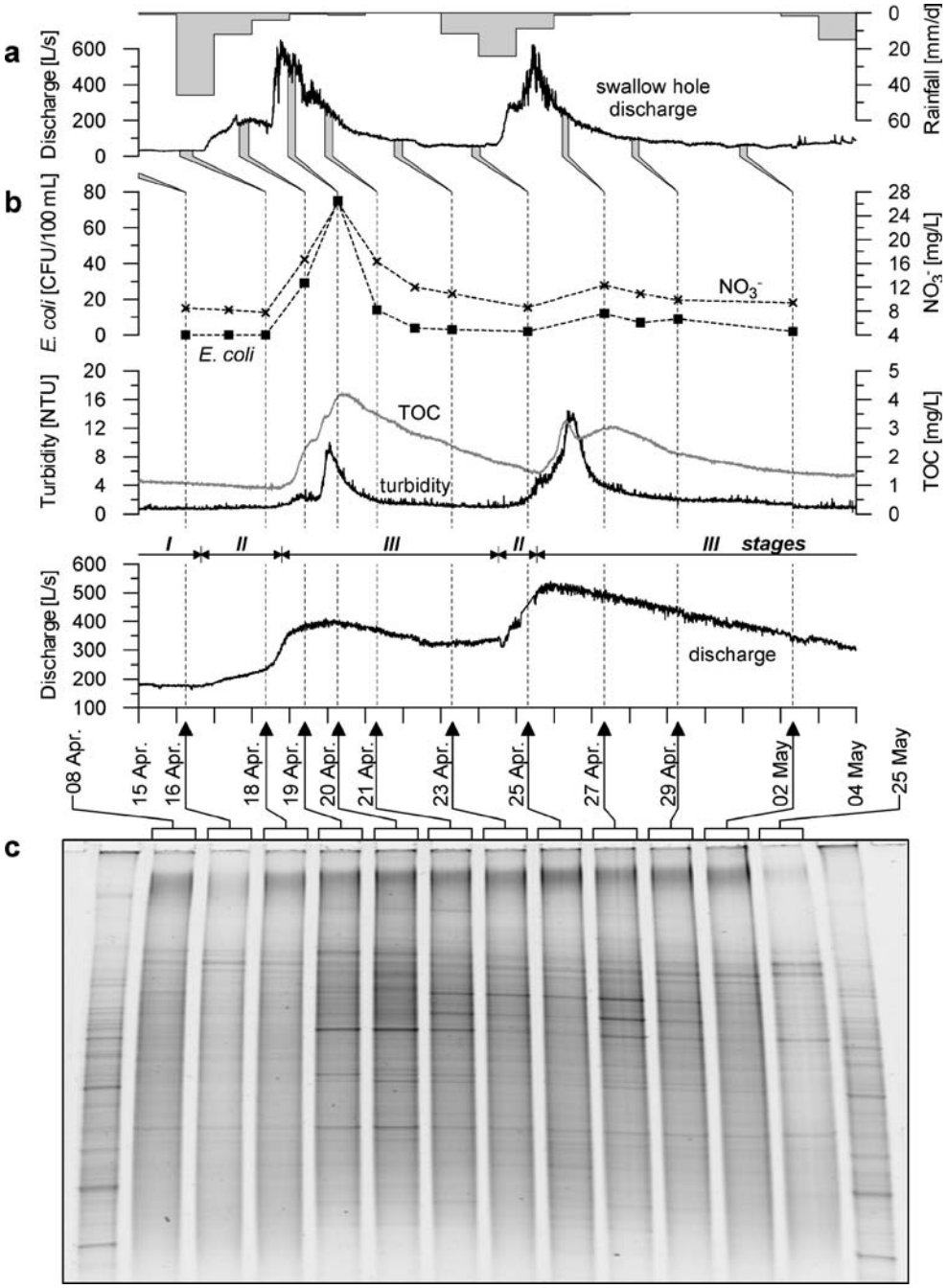

Fig. 4 Temporal variations of physical, chemical and microbial parameters during a storm event. **a** Feurtille swallow hole, **b** Moulinet spring, **c** 16S rDNA DGGE fingerprints of bacterial communities in Moulinet spring water samples (the two external profiles are reference lanes). The *grey areas* under the swallow hole hydrograph indicate the moments of infiltration at the swallow hole for each spring water sample. Stages *I*, *II*, and *III* are described in the text. *NTU* nephelometric turbidity units; *CFU* colony forming units

abundant species. Several of these bands were present in all stages of the storm event, which is consistent with an autochthonous endokarst microbial community as proposed by Farnleitner et al. (2005). In contrast, samples affected by the allochthonous swallow hole component (stage III) displayed several particularly thick bands, representing dominant populations of bacteria, which were not present in pre-storm event samples.

Statistical analysis of the storm event PCR-DGGE profiles was consistent with the above described observations and resulted in two separate clusters (Fig. 6a). Cluster I contained exclusively stage III water samples, which were characterised by a swallow hole contribution to the system of 6% to about 40% (Fig. 6b) and strictly allochthonous PSDs (Fig. 5). Cluster II comprised the remaining samples belonging to different stages (I–III), which were all only weakly affected by the swallow hole (4–5%). Of particular note is that samples collected before the swallow hole component arrival (16 and 18 April) and those collected towards the end of such events (25 and 02

 DOI 10.1007/s10040-008-0350-x

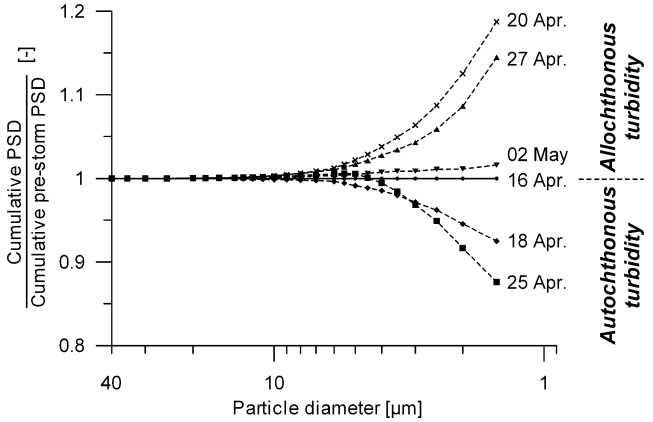

Fig. 5 PSD curves of Moulinet spring water during the April 2005 storm event. Cumulative PSDs, which represent the percent of particles smaller than a given diameter, were normalised to pre-storm conditions (sample 16 April, stage I)

May) fell into the same cluster. Therefore, it appears that the microbial community in spring water regains its pre-event structure. This is seen as evidence for the stability of the autochthonous endokarst microbial community and the non-persistence of allochthonous bacteria.

Long-term monitoring: annual hydrological cycle

Hydrological conditions of the Yverdon-les-Bains karst system showed high variations during the investigation period from March 2005 to February 2006 but were in general agreement with earlier years. The contribution of the swallow hole to spring water quality deterioration is clearly discernable from chemical (TOC, nitrate) and microbiological parameters (Table 2). Contamination was generally lower at Cossaux than at Moulinet spring. Moreover, water temperature was higher and all parameters showed less variations at the Cossaux spring, which is explained by a stable inflow of clean, warm groundwater from greater depth. Samples collected for molecular microbiological analyses covered the whole range of natural parameters shown in Table 2.

Swallow hole bacterial community profiles (i.e., 16S rDNA PCR-DGGE profiles) revealed about 20 intense bands indicating that these assemblages are dominated by a few abundant species. Furthermore, the banding pattern changed drastically from one sampling date to the other, showing that the microbial community composition in the swallow hole water is highly dynamic. This temporal variability is again reflected in the cluster analysis by the low similarities among swallow hole samples (Fig. 7a, cluster I).

In contrast, DGGE fingerprints of spring water samples were consistent with those of the storm event (Fig. 4c) showing complex patterns with mostly faint bands. Several bands were present in all samples, which is again consistent with the presence of a stable autochthonous endokarst microbial community. Only samples strongly affected by the swallow hole were characterised by a few intense bands of dominating bacteria.

Hierarchical cluster analysis of all DGGE profiles from the three observation points revealed six separate clusters (Fig. 7a): a cluster composed of the Feurtille swallow hole samples (I), a mixed cluster with Moulinet and Cossaux spring samples (II), two clusters (III and IV) of Cossaux samples, and two clusters (V and VI) of Moulinet samples. Cluster II was most closely related to the swallow hole cluster I, which fits nicely with the calculated high swallow hole contribution to total system discharge of at least 20% (Fig. 7b). A maximum of 60% was determined for the Moulinet spring after heavy rainfall (sample MS 13 Dec. 05). Moreover, all water samples of this cluster II were characterised by high contents of TOC, nitrate, faecal indicator bacteria, and strictly allochthonous PSD curves. The relatively low similarities among cluster II mirror the high temporal variability of the bacterial communities entering the swallow hole (cluster I).

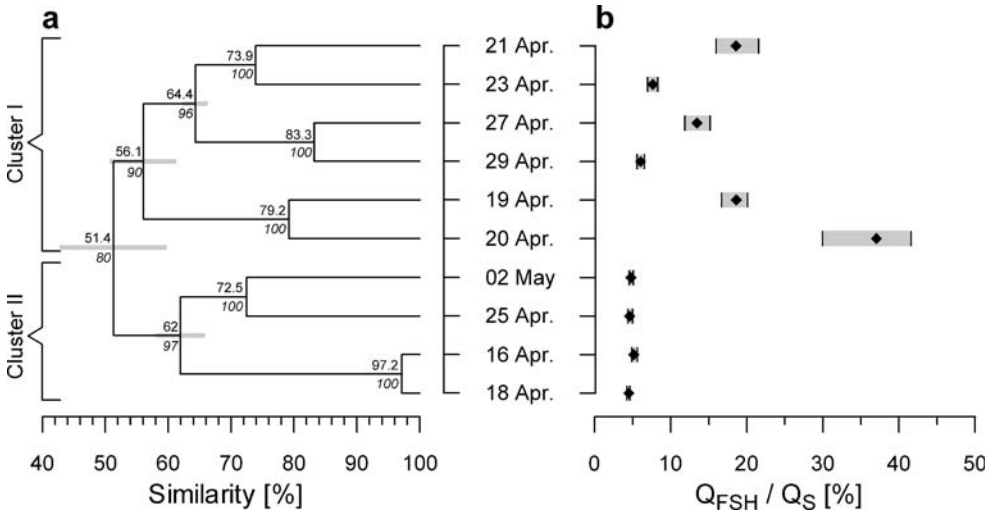

Fig. 6 **a** Hierarchical cluster analysis of storm event 16S rDNA DGGE profiles of Moulinet spring water samples (Jaccard correlation and unweighted pair group method with arithmetic mean). Error flags (*grey bars*), similarity (*numbers*), and cophenetic correlation coefficients (*italic numbers*) are shown. **b** Swallow hole contribution to the system discharge during the calculated infiltration time lag. Mean values (*diamonds*) with respective minimum and maximum values are shown. Dates of sampling are given between figures

 DOI 10.1007/s10040-008-0350-x

Table 2 General water quality characteristics of the karst system during the investigated period

Parameter	Unit	Feurtille swallow hole			Moulinet spring			Cossaux spring		
		n^a	Median	Range	n^a	Median	Range	n^a	Median	Range
Discharge	L/s	Cont	12	0–650	Cont	44	21–539	Cont.	45	42–73
Temperature	°C	Cont	9.5	3.4–23.9	Cont	11.1	9.2–11.6	Cont.	13.3	12.6–13.6
Electrical conductivity	µS/cm	Cont	729	76–970	Cont	444	345–608	Cont.	466	434–511
TOC	mg/L	Cont	4.8	2.7–15.6	Cont	0.8	0.2–6.4	Cont.	0.5	0.3–1.8
Turbidity	NTU	Cont	10.1	0.4–272	Cont	0.5	0.1–31.8	Cont.	0.2	0.1–2.2
Nitrate	mg/L	31	29.9	14.5–55.1	43	9.6	4.4–31.1	41	7.8	5.4–14.5
MAB[b]	CFU/ml	31	668	89–10,740	44	21	3–2,564	42	8	1–1,174
Total coliforms	CFU/100 ml	31	4,310	600–65,600	44	134	14–8,680	42	34	2–2,150
E. coli	CFU/100 ml	31	36	3–1,990	44	3	0–648	42	1	0–183
Enterococci	CFU/100 ml	31	32	2–890	44	2	0–480	42	1	0–123

[a] Number of samples (*cont* continuous monitoring)

[b] Mesophilic aerobic bacteria

Clusters III (Cossaux samples) and V (Moulinet samples) were characterised by an intermediate swallow hole contribution of about 3–10%, while clusters IV (Cossaux samples) and VI (Moulinet samples) received only small amounts of water from the swallow hole (Q_{FSH} about 0–1% of Q_S). However, for the spring water clusters III through VI no clear relation to the monitored natural parameters was observed.

The calculated similarities between clusters equally affected by the swallow hole (III and V; IV and VI) were low at just 42.8%. It is hypothesized that the additional inflow of deep, warm groundwater at the Cossaux spring could explain these differences. Nevertheless, the almost identical arrangement of Cossaux spring samples (II, III and IV) and Moulinet spring samples (II, V and VI) in the dendrogram suggests that the deep groundwater harbours a stable microbial community. Frequently high similarities among samples within low swallow hole affected clusters provide additional evidence for an autochthonous endokarst microbial community and suggest further a temporal constancy of this community.

Clone libraries and sequence analysis: identifying the microorganisms in the karst

Based on the PCR-DGGE results and hydrological parameters, three Moulinet spring samples were chosen for clone library construction and sequence analysis. The first one (A) being "MS 06 Dec. 05", collected during the rising limb of spring discharge following a rain event, characterised by a PSD typical for remobilised autochthonous particles, and the absence of any direct swallow hole contribution. This sample is considered as the most appropriate for probing the endokarst microbial community. The second sample (B) is "MS 09 Aug. 05", characterised by an intermediate swallow hole contribution, and a PSD indicating a minor impact of allochthonous particles and the third sample (C) is "MS 20 Apr. 05", characterised by a high swallow hole contribution and a PSD indicative for allochthonous particles that had entered the swallow hole during the preceding rain event. The library size was 150 clones for A, 104 clones for B, and 93 clones for C.

The relative abundances of different phylogenetic groups in the libraries were calculated for libraries A, B, and C (Fig. 8). The microbial communities in all three water samples were dominated by *Proteobacteria* by about 42 to 52%, which is comparable to other aquatic and terrestrial ecosystems (e.g., Janssen 2006; Roesch et al. 2007). However, the relative proportion of the *Proteobacteria* subgroups varied greatly. While the *α-Proteobacteria* were nearly absent in the low swallow hole affected sample (A) and were replaced by *δ-Proteobacteria*, both swallow hole affected samples (B, C) showed high abundances of *α-*, *β-* and *γ-Proteobacteria*. *α-Proteobacteria* are an important, sometimes even dominant, subclass of *Proteobacteria* in soils (Felske et al. 1998; Hartmann and Widmer 2006; Hugenholtz et al. 1998; Roesch et al. 2007). Their low abundance in library A, therefore, further supports evidence for a specific intrakarstic microbial community. No *ε-Proteobacteria* sequences were detected in the three libraries, although they were frequently observed in sulfidic springs and caves where they are believed to contribute to karstification by sulphide oxidation and concomitant sulphuric acid production (Engel 2007).

The relative abundance of *Firmicutes* in the clone libraries increased from 2 to 27% with an increasing swallow hole contribution, suggesting that they are of allochthonous origin. This phylum contains important soil bacteria such as *Bacillus* and *Clostridium* species but also gut bacteria (e.g., *Enterococcus* sp.) and facultative pathogens (e.g., *Staphylococcus* sp., *Streptococcus* sp.; Eckburg et al. 2005; Felske et al. 1998; Roesch et al. 2007).

The relative abundance of the aerobic ammonium oxidizing bacteria *Nitrospira* was highest in clone library A (5%) and decreased with increasing swallow hole contribution. Although *Nitrospira* sequences have been retrieved from soils, geothermal and subsurface ecosystems (Hugenholtz et al. 1998; Roesch et al. 2007), they seem to be part of the autochthonous microbial community in the Yverdon-les-Bains karst system. *Nitrospira* has also been detected in other karst or cave systems (Farnleitner et al. 2005; Macalady et al. 2007). *Acidobacteria* have been detected by molecular methods in diverse environments but seem to be particularly abundant in soils and sediments. Little is known about their physiology

 DOI 10.1007/s10040-008-0350-x

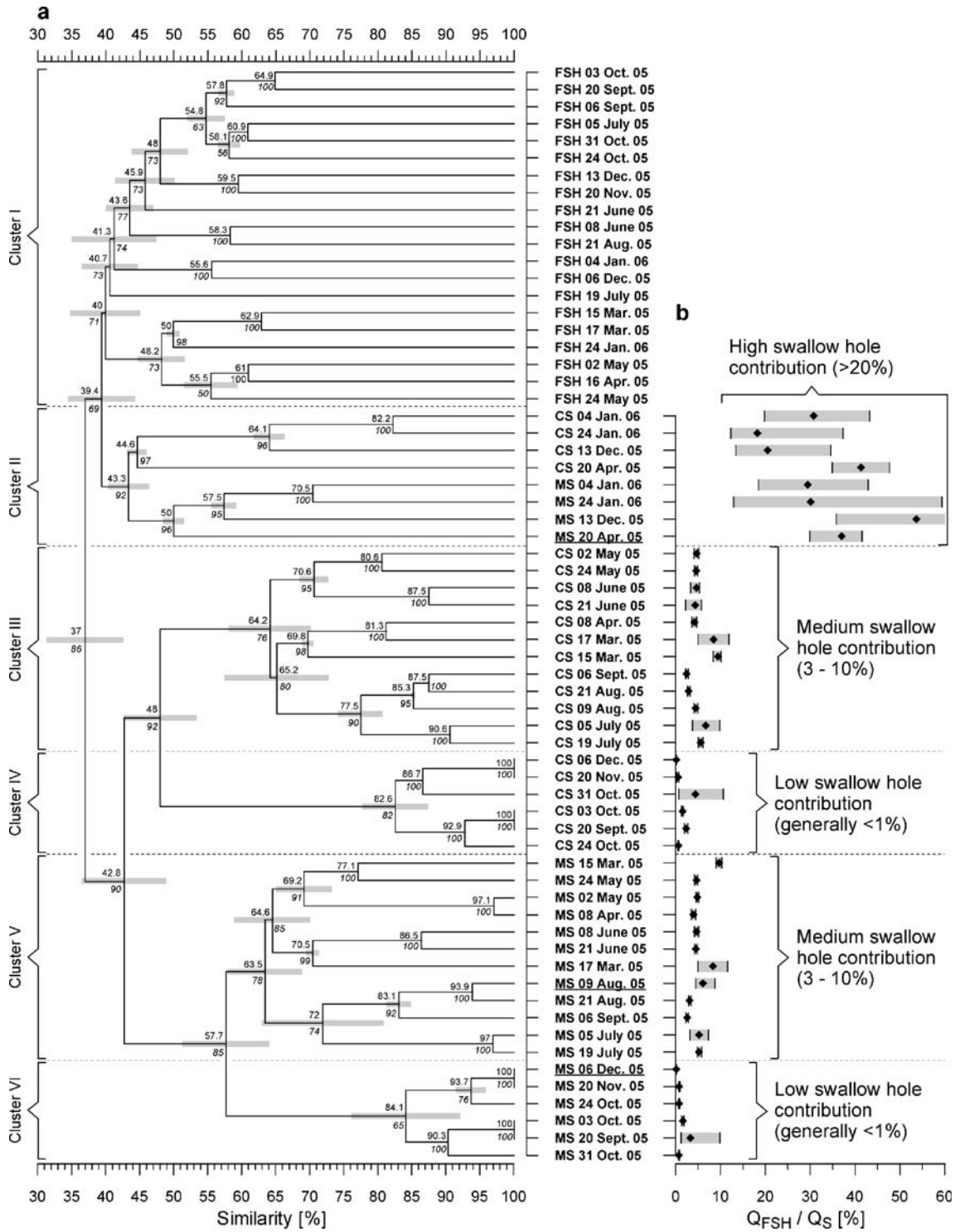

Fig. 7 **a** Hierarchical cluster analysis of bacterial community fingerprints (16S rDNA DGGE profiles) from swallow hole and spring water samples collected during an annual hydrological cycle (Jaccard correlation and UPGMA). Error flags (*grey bars*), similarity (*numbers*), and cophenetic correlation coefficients (*italic numbers*) are shown. **b** Swallow hole contribution to the system discharge during the calculated infiltration time lag. Mean values (*diamonds*) with respective minimum and maximum values are shown. Sample labels (*FSH* Feurtille swallow hole; *MS* Moulinet spring; *CS* Cossaux spring) and dates are given along the vertical axis. The *underlined samples* were chosen for clone library construction (see Fig. 8)

and ecology as only a few species have been isolated and characterised (Barns et al. 2007). *Acidobacteria* sequences were found in all three samples, but particularly in library A, suggesting that this little known group may also dwell in karst systems. Of particular note, 29–32% of the sequences retrieved from the low and the medium swallow hole affected samples remained unclassified, i.e., they were at least 5% dissimilar to all currently known bacterial sequences. This

 DOI 10.1007/s10040-008-0350-x

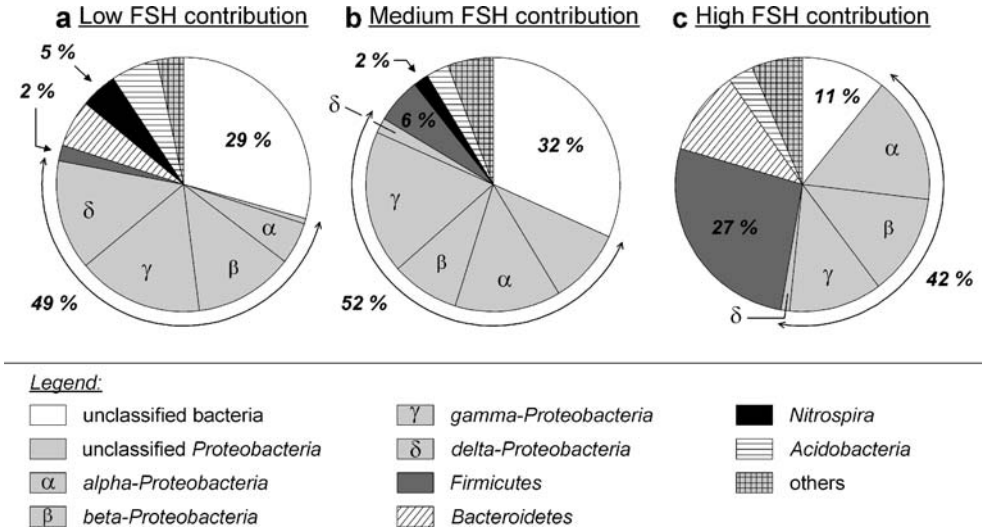

Fig. 8 Frequencies of phylotypes affiliated with major phylogenetic groups (confidence threshold ≥95%) in libraries from Moulinet spring water samples with **a** low (library A), **b** medium (library B), and **c** high (library C) swallow hole (*FSH*) contributions

high proportion of unknown sequences suggests that many karst bacteria are yet to be discovered, and illustrates the distinctiveness of the autochthonous endokarst microbial community.

Conclusions and perspectives

Evidence for a stable community of endokarst microorganisms

PCR-DGGE and cloning/sequencing allowed the structure, diversity and temporal variability of microbial communities in karst groundwater to be investigated. The autochthonous endokarst microbial community is characterised by a high diversity with only a few dominant species and a high temporal constancy. *δ-Proteobacteria*, *Acidobacteria* and *Nitrospira* species are important members of this community, but the high proportion of unclassified sequences (about 30%) suggests that many karst bacteria are still undiscovered. Moreover, it highlights that pristine karst aquifers are rarely studied, unique habitats and that sequences from shallow subsurface ecosystems are currently underrepresented in databases.

Both molecular techniques reveal a clear relation between the bacterial community composition in spring water and the swallow hole contribution. During periods of high input of water from the swallow hole, the autochthonous microbial community is overprinted by the allochthonous bacteria. Consequently, the high variability of bacterial communities in the spring water reflects the high fluctuations observed at the swallow hole. However, contamination of karst groundwater by allochthonous bacteria is unlikely to persist.

Particle-size distribution as tool to optimise sampling

Sampling strategy in karst aquifers should be based on hydrological conditions and focus on turbidity events. Autochthonous turbidity pulses at springs are the result of increasing flow rates in the aquifer. Due to higher shear stress, attached particles and cells are sloughed off the conduit surfaces and intrakarstic sediments are remobilised. Bacteria attached to particles are thereby brought into the water phase and transported to the spring. Particle-size distribution measurements allow distinction between autochthonous and allochthonous turbidity and represent an important tool for identifying water samples that harbour endokarst microorganisms.

Potential use for groundwater biomonitoring

Biomonitoring is a valuable supplementary tool for controlling groundwater quality, as it delivers more integrated—but less quantitative—information than chemical analyses. Bacteria are the dominant organisms in aquifers, and their occurrence and activity is related to the biogeochemical conditions. The structures of microbial communities, individual marker organisms, or even specific functional genes are therefore promising bioindicators. However, there is often a complex relation between water quality and microbial communities, and their response to specific contaminants is poorly understood. Short-term microbial contamination at karst springs such as those resulting from storm events, can be easily detected by in-situ monitoring of natural physical and chemical parameters. However, the long-term monitoring of autochthonous endokarst microbial communities during years or decades may be an interesting approach to assess the general water quality and to detect potential changes in ecosystem functioning due to chronic low level contamination or climate change. The applied molecular methodology does not have the required resolution and throughput for monitoring purposes yet, but new, promising techniques are continuously being developed (e.g., Chandler and Jarrell 2004; He et al. 2007; Roesch et al. 2007). This work represents therefore only a first step

 DOI 10.1007/s10040-008-0350-x

towards a better understanding of the microbial ecology in pristine karst aquifers and may serve as a starting point for developing biomonitoring tools.

Acknowledgements We are grateful to the water works of Yverdon-les-Bains for their cooperation, and for technical and logistical support. We thank the Swiss National Science Foundation (project BEKARST/KARSTDYN, grant No. 200020–113609/1) for funding and the three reviewers for their valuable comments.

References

Alfreider A, Krössbacher M, Psenner R (1997) Groundwater samples do not reflect bacterial densities and activity in subsurface systems. Water Res 31(4):832–840

APHA, AWWA, WEF (2005) Standard methods for the examination of water and wastewater, 21st edn. Water Environment Federation, Alexandria, VA, USA

Barns SM, Cain EC, Sommerville L, Kuske CR (2007) *Acidobacteria* phylum sequences in uranium-contaminated subsurface sediments greatly expand the known diversity within this phylum. Appl Environ Microbiol 73(9):3113–3116

Barton HA, Northup DE (2007) Geomicrobiology in cave environments: past, current and future perspectives. J Caves Karst Stud 69(1):163–178

Canaveras JC, Hoyos M, Sanchez-Moral S, Sanz-Rubio E, Bedoya J, Soler V, Groth I, Schumann P, Laiz L, Gonzales I, Sainz-Jimenez C (1999) Microbial communities associated with hydromagnesite and needle-fiber aragonite deposits in a karstic cave (Altamira, northern Spain). Geomicrobiol J 16(1):9–25

Chandler DP, Jarrell AE (2004) Automated purification and suspension array detection of 16S rRNA from soil and sediment extracts by using tunable surface microparticles. Appl Environ Microbiol 70(5):2621–2631

Chapelle FH (2001) Groundwater microbiology and geochemistry. Wiley, New York

Chelius MK, Moore JC (2004) Molecular phylogenetic analysis of archaea and bacteria in Wind Cave, South Dakota. Geomicrobiol J 21(2):123–134

Cole JR, Chai B, Farris RJ, Wang Q, Kulam-Syed-Mohideen AS, McGarrell DM, Bandela AM, Cardenas E, Garrity GM, Tiedje JM (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res 35(Database issue):D169-D172. doi:10.1093/nar/gkl889

Danielopol DL, Griebler C, Gunatilaka A, Notenboom J (2003) Present state and future prospects for groundwater ecosystems. Environ Conserv 30(2):104–130

Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA (2005) Diversity of the human intestinal microbial flora. Science 308 (5728):1635–1638

Engel AS (2007) Observations on the biodiversity of sulfidic karst habitats. J Caves Karst Stud 69(1):187–206

Farnleitner AH, Wilhartitz I, Ryzinska G, Kirschner AKT, Stadler H, Burtscher MM, Hornek R, Szewzyk U, Herndl G, Mach RL (2005) Bacterial dynamics in spring water of alpine karst aquifers indicates the presence of stable autochthonous microbial endokarst communities. Environ Microbiol 7(8):1248–1259

Felske A, Wolterink A, van Lis R, Akkermans ADL (1998) Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands). Appl Environ Microbiol 64(3):871–879

Goldscheider N, Hunkeler D, Rossi P (2006) Review: microbial biocenoses in pristine aquifers and an assessment of investigative methods. Hydrogeol J 14(6):926–941

Green U, Kremer JH, Zillmer M, Moldaenke C (2003) Detection of chemical threat agents in drinking water by an early warning real-time biomonitor. Environ Toxicol 18(6):368–374

Griebler C, Mösslacher F (2003) Grundwasser-Ökologie [Groundwater ecology]. Facultas UTB, Vienna

GSchV (1998) Water Protection Ordinance, SR 814.201, Swiss Federal Law, Bern

Hahn HJ, Friedrich E (1999) Brauchen wir ein faunistisch begründetes Grundwassermonitoring und was kann es leisten? [Do we need a fauna-based groundwater monitoring, and what is its potential?]. Grundwasser 4:147–154

Hancock PJ, Boulton AJ, Humphreys WF (2005) Aquifers and hyporheic zone: towards an ecological understanding of groundwater. Hydrogeol J 13(1):98–111

Hartmann M, Widmer F (2006) Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. Appl Environ Microbiol 72 (12):7804–7812

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1(1):67–77

Hose LD, Palmer AN, Palmer MV, Northup DE, Boston PJ, DuChene HR (2000) Microbiology and geochemistry in a hydrogen-sulphide-rich karst environment. Chem Geol 169(3–4):399–423

Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180(18):4765–4774

Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Environ Microbiol 72(3):1719–1728

Käss W (1998) Tracing technique in geohydrology. Balkema, Rotterdam, The Netherlands

Lehman RM (2007) Understanding of aquifer microbiology is tightly linked to sampling approach. Geomicrobiol J 24(3–4):331–341

Macalady JL, Lyon EH, Koffman B, Albertson LK, Meyer K, Galdenzi S, Mariani S (2006) Dominant microbial populations in limestone-corroding stream biofilms, Frasassi cave system, Italy. Appl Environ Microbiol 72(8):5596–5609

Macalady JL, Jones DS, Lyon EH (2007) Extremely acidic, pendulous cave wall biofilms from the Frasassi cave system, Italy. Environ Microbiol 9(6):1402–1414

Malard F, Plenet S, Gibert J (1996) The use of invertebrates in groundwater monitoring: a rising research field. Ground Water Monit Remediat 16(2):103–113

Mösslacher F, Griebler C, Notenboom J (2001) Biomonitoring of groundwater systems: methods, applications and possible indicators among the groundwater biota. In: Griebler C, Danielopol DL, Gibert J, Nachtnebel HP, Notenboom J (eds) Groundwater ecology: a tool for management of water resources. Office for Official Publications of the European Communities, Luxemburg

Muralt R (1999) Processus hydrogéologiques et hydrochimiques dans les circulations profondes des calcaires du Malm de l'arc jurassien [Hydrogeological and hydrochemical processes in the circulation system in the Malm limestones of the Jurassic belt]. Beiträge zur Geologie der Schweiz, Geotechnische Serie, Lieferung 82, Schweizerischen Geotechnischen Kommision, Zurich, 236 pp

Muyzer G (1999) DGGE/TGGE a method for identifying genes from natural ecosystems. Curr Opin Microbiol 2(3):317–322

Muyzer G, Dewaal EC, Uitterlinden AG (1993) Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Appl Environ Microbiol 59(3):695–700

Muyzer G, Teske A, Wirsen CO, Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol 164(3):165–172

Nocker A, Burr M, Camper AK (2007) Genotypic microbial community profiling: a critical technical review. Microb Ecol 54(2):276–289

Northup DE, Lavoie KH (2001) Geomicrobiology of caves: a review. Geomicrobiol J 18(3):199–222

Ovreas L, Forney L, Daae FL, 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(9):3367–3373

Personné JC, Poty F, Mahler BJ, Drogue C (2004) Colonization by aerobic bacteria in karst: laboratory and in situ experiments. Ground Water 42(4):526–533

Pronk M, Goldscheider N, Zopfi J (2006) Dynamics and interaction of organic carbon, turbidity and bacteria in a karst aquifer system. Hydrogeol J 14(4):473–484

Pronk M, Goldscheider N, Zopfi J (2007) Particle-size distribution as indicator for fecal bacteria contamination of drinking water from karst springs. Environ Sci Technol 41(24):8400–8405

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1(4):283–290

Rusterholtz KJ, Mallory LM (1994) Density, activity, and diversity of bacteria indigenous to a karstic aquifer. Microb Ecol 28(1): 79–99

Simon KS, Gibert J, Petitot P, Laurent R (2001) Spatial and temporal patterns of bacterial density and metabolic activity in a karst aquifer. Arch Hydrobiol 151(1):67–82

Sommaruga A (1997) Geology of the central Jura and the Molasse Basin: new insight into an evaporite-based foreland fold and thrust belt. Mém Soc Neuchâteloise Sci Nat, XII edn., Société Neuchâteloise des Sciences Nat, La Chaux-de-Fonds, Switzerland

Vervier P, Gibert J (1991) Dynamics of surface-water groundwater ecotones in a karstic aquifer. Freshw Biol 26(2):241–250

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73(16):5261–5267

Weiss JV, Cozzarelli M (2008) Biodegradation in contaminated aquifers: incorporating microbial/molecular methods. Ground Water 46(2):305–322

 DOI 10.1007/s10040-008-0350-x