

# Molecular identification of an uncultured bacterium (“morphotype R”) in meromictic Lake Cadagno, Switzerland

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## Abstract

Comparative sequence analysis of almost complete 16S rRNA genes of members of the Desulfobacteriaceae retrieved from two gene clone libraries of uncultured bacteria of the chemocline of Lake Cadagno, Switzerland, resulted in the molecular identification of nine sequences, with a tight cluster of five sequences that represented at least three different populations of bacteria with homology values of 95% and 93% to their closest cultured relatives *Desulfomonile tiedjei* and *Desulfomonile limimaris*, respectively. In situ hybridization with probes DsmA455 targeting two subpopulations and DsmB455 targeting one subpopulation, detected bacteria with a peculiar morphology previously described as “morphotype R”. The individual probes detected about the same number of cells in all samples and together added up to represent all cells of “morphotype R” suggesting that the basic ecophysiological requirements of the subpopulations might be similar. In the monimolimnion, “morphotype R” cells accounted for up to 29% of all Bacteria and entirely represented the Desulfobacteriaceae, the most prominent sulfate-reducing bacteria. In the sediment, “morphotype R” was similarly prominent in the upper cm only where it represented all Desulfobacteriaceae and up to 50% of all Bacteria. Numbers and importance within the Desulfobacteriaceae and Bacteria declined significantly with depth in sediments suggesting potential effects of changing environmental conditions on the fate of “morphotype R”.

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## 1. Introduction

Meromictic lakes are characterized by a permanent stratification, in which, due to incomplete circulation, a portion of the water mass never mixes with the rest of the water body [1]. Lake Cadagno is a crenogenic meromictic lake located 1923 m above sea level in the Piora valley in the southern Alps of Switzerland (46° 33'N, 8° 43'E). The permanent stratification is stabilized by den-

sity differences between the mixolimnion characterized by electrolyte-poor surface water, and the monimolimnion containing water of high ionic strength (9–10 mM) constantly supplied by subaquatic springs [2–4]. Due to the infiltration of water through a dolomite vein rich in gypsum, Lake Cadagno contains approximately 10–20 times more sulfate than most freshwater lakes [3,5]. The high concentration of sulfate leads to a dominance of sulfur compounds in the chemistry of the lake [4,6] and thus favors the development of bacterial populations involved in transformations within the sulfur cycle.

With numbers of up to 10<sup>7</sup> cells ml<sup>-1</sup> [7–9], bacterial abundance is especially pronounced in the chemocline

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where high concentrations of sulfate, and steep, but relatively stable vertical gradients of oxygen, sulfide, and light are encountered [3,10,11]. Highest numbers of bacteria are generally found at a depth where sulfide becomes detectable and concentrations are increasing with depth to up to 240  $\mu\text{M}$  indicating the presence of both sulfide-producing as well as sulfide-consuming bacterial populations [12]. Most prominent numerically are large- and small-celled purple sulfur bacteria with numbers accounting for up to 35% of all bacteria, and sulfate-reducing bacteria that represent up to 23% of all bacteria [12]. More detailed analyses revealed a limited genetic diversity of numerically significant populations of these bacterial groups in the chemocline. Purple sulfur bacteria resemble *Chromatium okenii*, populations affiliated with the genus *Lamprocystis*, i.e., *L. purpurea*, *L. roseopersicina*, and populations D and F, and populations H and 448 of the genus *Thiocystis* [13]. Sulfate-reducing bacteria of the family Desulfovibrionaceae (recently reclassified to “Desulfobulbaceae” [14]) are almost entirely represented by one bacterium related to *Desulfocapsa thiozymogenes* that is generally associated to aggregates of four populations of small-celled purple sulfur bacteria belonging to the genus *Lamprocystis* [7,15–18].

Members of the Desulfobacteriaceae (reclassified to “Syntrophaceae” [14]) that represent free cells or cells loosely attached to other cells and debris are less important in the upper part of the chemocline than Desulfovibrionaceae, but become more prominent numerically in the lower part of the chemocline and in the monimolimnion [17]. Similar to the Desulfovibrionaceae, the Desulfobacteriaceae are essentially represented by one population. This bacterium that can easily be identified by its distinctive morphology that resembles slightly curved rods with a length of 2–6  $\mu\text{m}$  and a width of 0.4–0.5  $\mu\text{m}$  and by its green fluorescence after acridine orange staining, has previously been described as “morphotype R” [19]. Based on its morphological features that include the presence of a central gas vacuole, and on habitat occurrence, a close relationship between “morphotype R” and other yet uncultured bacteria such as “morphotype T5” of anoxic waters was proposed [19,20]. Representatives of “morphotype T5” are *Desulfomonile tiedjei* and *Desulfomonile limimaris* that have been isolated and described recently [21,22]. Pure cultures of “morphotype R”, however, are not available yet and attempts to characterize “morphotype R” by molecular methods such as, e.g., by in situ hybridization with probes commonly used to analyze populations of sulfate-reducing bacteria on a lower taxonomic level [23,24] were not successful [17].

The aim of this study was therefore to take advantage of the availability of two gene clone libraries of almost complete 16S rRNA genes retrieved by PCR from DNA samples obtained from uncultured bacteria of

the chemocline [25] in order to phylogenetically characterize “morphotype R” unaffected by the limitations of culturability. After comparative sequence analysis of clones selected after hybridization with probes SRB385Db and DSS658 targeting members of the Desulfobacteriaceae, specific oligonucleotide probes were designed and used to evaluate the significance of the sequences in environmental samples. Probes that detected bacteria resembling “morphotype R” were subsequently used for the numerical analysis of “morphotype R” in the water column (i.e., the chemocline and monimolimnion) and in the anoxic sediment of Lake Cadagno.

## 2. Materials and methods

### 2.1. Gene clone library analysis and probe design

The first 16S rRNA gene library consisted of 470 clones of 16S rRNA gene fragments of approx. 1400 bp in pGEM<sup>®</sup>-T (Promega, Wallisellen, Switzerland) in *Escherichia coli* TOP-10 (Stratagene, Heidelberg, Germany). The rRNA gene fragments had been generated by PCR amplification using DNA of bacteria from water samples obtained in September 1994 from the chemocline as template, and primers UNI16-SRNA-8F (5'ATT CTA GAG TTT GAT CAT GGC TCA, pos. 8–26 according to the *E. coli* numbering [26]) and UNI16SRNA-1391R (5'ATG GTA CCG TGT GAC GGG CGG TGT GTA, pos. 1391–1411) [17,25]. The second library consisted of 96 clones of 16S rRNA gene fragments of approx. 1300 bp in pCR 2.1 TOPO<sup>®</sup>-T (Invitrogen, Carlsbad, CA) in *E. coli* TOP-10. Water samples obtained from a depth of 17 m in June 2000 were used for DNA extraction and subsequent PCR amplification of rRNA gene fragments using Taq Extender<sup>™</sup> PCR Additive (Stratagene) and primers 63F (5'CAG GCC TAA CAC ATG CAA GTC, pos. 43–63 according to the *E. coli* numbering [26]) and 1387R (5'GGG CGG WGT GTA CAA GGC), pos. 1404–1385) [27].

Ribosomal RNA gene fragments of four clones from the first and five clones from the second library were identified by the Clone-FISH technique as described previously but with higher hybridization and washing temperatures (53 and 55 °C, respectively) [28] using Cy3-labeled oligonucleotide probe SRB385Db [29] and probe DSS658 targeting bacteria of the Desulfobacteriaceae [23]. The cloned sequences were further re-amplified, amplification products purified with the QIAquick PCR Purification Kit (Qiagen), and prepared for sequence analysis with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing was carried out using the ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) as specified by the manufac-

turer instructions. The absence of chimeras was verified with the RDP program CHECK\_CHIMERA [30]. Sequence data were aligned initially with a subset of bacterial 16S rRNA sequences obtained from the Ribosomal Database Project [30] using the CLUSTALW service at EBI [31]. Phylogenetic relationships were estimated using MEGA, version 2.0 [32]. The sequences obtained were deposited in the EMBL/GenBank databases with accession numbers AJ831748 to AJ831750, AJ831753, and AJ316020 to AJ316024, respectively (Fig. 1).

## 2.2. Probe design and evaluation

Since morphological features and habitat occurrence had been used to propose a close relationship between

“morphotype R” and *D. tiedjei* and *D. liminaris* [19], sequences clustering with those of *D. tiedjei* and *D. liminaris* were assumed to represent “morphotype R”. Comparative analysis of sequences retrieved and those of reference organisms were used to design two oligonucleotide probes DsmA455 (5'AGT TCY CTG AGC TAT TTA CTC AAA GA, pos. 455–480 according to the *E. coli* numbering [26]) and DsmB455 (5'AGA TCC CTG AGC TAT TTA CTC AAG GA, pos. 455–480) specifically targeting the clones retrieved. Probe specificity with reference to available 16S rRNA sequences was checked with the ARB program [33] and in the EMBL/GenBank databases using FASTA [34] through the GCG package. To test probe specificity and to establish appropriate in situ hybridization

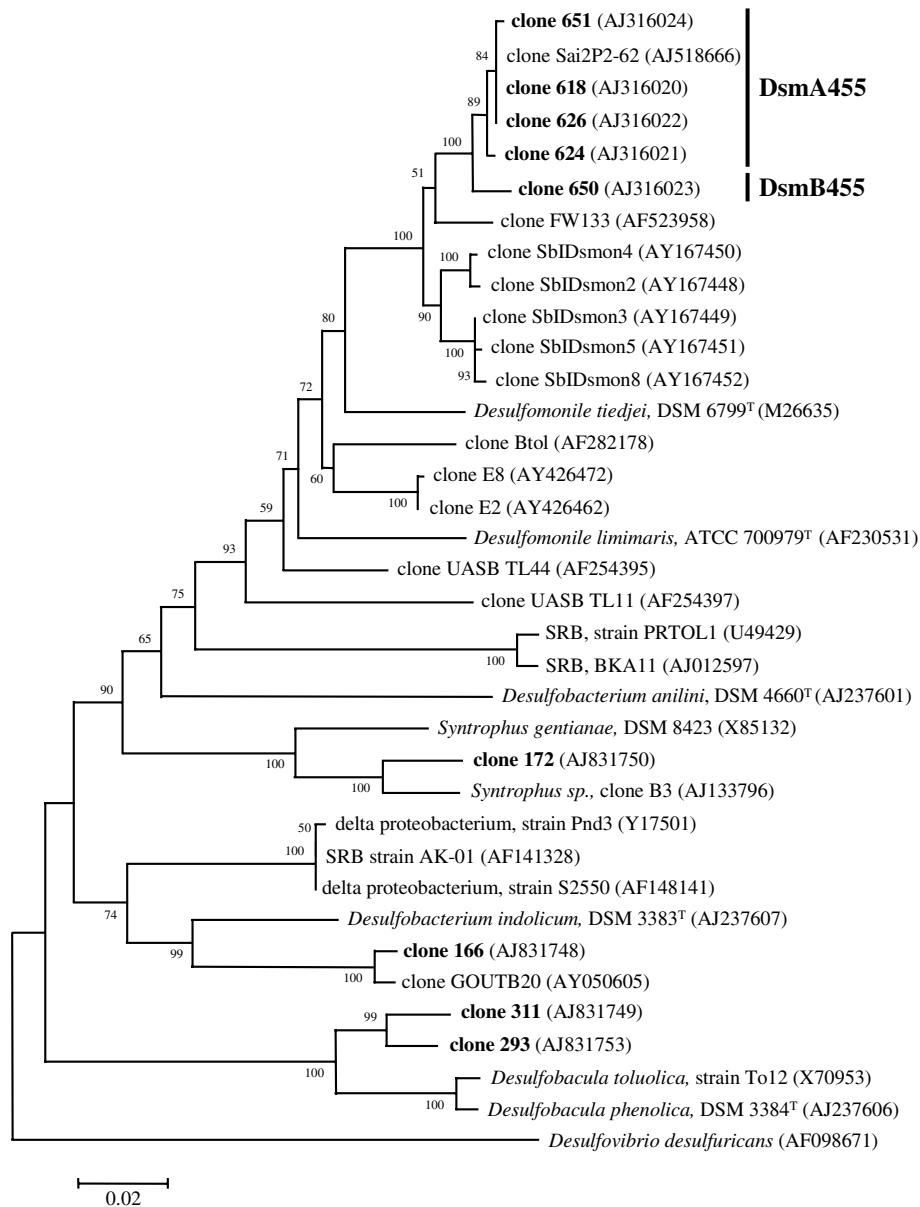


Fig. 1. Neighbor-joining tree showing the relationship of clones from the 16S rRNA gene clone library of lake Cadagno, to *D. tiedjei*, *D. liminaris* and other sulfate-reducing bacteria. The distance scale indicates the expected number of changes per sequence position.

conditions for the specific detection of “morphotype R”, samples from the chemocline, the monimolimnion and the sediment of Lake Cadagno were hybridized in the presence of increasing concentrations of formamide (0–35% in 5% steps) and results related to numbers obtained by morphological criteria indicative of “morphotype R”.

### 2.3. Sampling and physico-chemical analyses

A YSI 6000 profiler (Yellow Springs Inc., Yellow Springs, OH, USA) was used to retrieve basic physico-chemical parameters such as temperature, conductivity, pH, dissolved oxygen, turbidity and redox potential from the water column and to determine the position of the chemocline in October 2000 [7,17]. 100-ml-water samples were obtained from the chemocline located at a depth between 11 and 13 m with a resolution of 10 cm over a depth of 2 m with a thin layer pneumatic multisyringe sampler [7,17]. Additional water samples were collected from just above the chemocline at a depth of 10 m, and between chemocline and sediment down to a depth of 20 m in 1-m-intervals with a “Friedinger” type bottle (Zuellig AG, Rheineck, Switzerland). From these samples, 11-ml-subsamples were immediately transferred to screw capped tubes containing 0.8 ml of a 4% zinc acetate solution. These were stored on ice and used to determine sulfide concentrations by colorimetric analysis [35] using the Spectroquant® kit of Merck (Switzerland) [7,17]. Sulfate was analyzed by isocratic ion chromatography with suppressed conductivity detection with a Dionex DX-500 ion chromatograph (Dionex, Olten, Switzerland) using a AG-14 pre-column, a AS-14 column, a ASRS Ultra suppressor, and a mixture of 3.5 mM Na-carbonate and 1.0 mM Na-bicarbonate as eluent at a flow of 0.6 ml min<sup>-1</sup> [36].

At the same day and at approximately the same position from which water samples were obtained, a diver collected cores of sediments (20 cm in depth with a diameter of 4.5 cm) as well as equilibrium diffusion plates [37] that had been pushed into the sediment and allowed to equilibrate for 10 days. Pore water was obtained through septum stoppers at the sides of the plates using syringes equipped with hypodermic needles and analyzed for sulfide and sulfate as described above.

### 2.4. Microbial analyses

For the microbial analysis of the water samples from chemocline and monimolimnion, 15-ml-subsamples of water obtained with the multi-syringe sampler were filtered immediately after sampling through 0.22 µm polycarbonate membrane filters (25 mm diameter; Millipore, Volketswil, Switzerland) [24]. Bacteria were fixed by overlaying the filters with 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.13 M NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, and 3 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2) for 30 min.

at room temperature [38]. The filters were subsequently rinsed twice with PBS by vacuum filtration and transferred into plastic bags with 0.6 ml of 50% ethanol in PBS. In sealed bags, the bacterioplankton was released from filters and resuspended by slightly massing the filter with thumb and forefinger [39]. The complete release of the bacteria from filters was checked microscopically after DAPI staining. Re-suspended bacterial cells were then transferred into Eppendorf tubes and stored at –20 °C until further use [7,38]. Sub-samples of sediment cores (approx. 0.5 g wet weight) taken at 1-cm intervals were fixed with 4% paraformaldehyde in PBS overnight, subsequently washed with PBS twice and stored resuspended in 50% ethanol in PBS until further use.

In situ hybridization with fluorescent (Cy3-labeled) oligonucleotide probes was performed on aliquots (1 µl) of paraformaldehyde-fixed water or sediment samples ( $n = 3$ ) spotted onto gelatin-coated slides (0.1% gelatin, 0.01% KCr(SO<sub>4</sub>)<sub>2</sub>) [13] and hybridized and concomitantly stained with DAPI according to Zarda et al. [40] with an increase in hybridization time from 2 h to overnight. The analysis of water and sediment samples was performed in a top-to-bottom approach detecting initially members of the domain Bacteria (probe EUB338 [41]), followed by sulfate-reducing bacteria of the families Desulfovibrionaceae (probe SRB385 [38]) and Desulfobacteriaceae (probe SRB385Db [29]), and subsequently “morphotype R” using a combination of probes DsmA455 and DsmB455, respectively. Number obtained by the latter hybridization were compared to numbers based on morphology and the green fluorescence of “morphotype R” after acridine orange staining [19]. The slides were examined by epifluorescence microscopy using filter sets F31 (AHF Analysentechnik, Tübingen, Germany; D360/40, 400DCLP, D460/50) for DAPI, I2/3 (Leitz Ploemopak®, BP450-490, RKP510, LP515) for acridine orange, and F41 (AHF Analysentechnik, HQ535/50, Q565LP, HQ610/75) for Cy3. Microorganisms were counted at 1000× magnification in 40 fields covering an area of 0.01 mm<sup>2</sup> each [42]. Numbers were expressed as means ± SE.

## 3. Results and discussion

### 3.1. Gene clone library analysis and probe design

Comparative sequence analysis of nine clones hybridizing to probe SRB385Db that targets the Desulfobacteriaceae revealed the presence of four distinct groups of sequences (Fig. 1). Three of these groups were represented by one (clones 166 and 172, respectively) or two clones (293 and 311) each, with sequences displaying homology values of 93%, 94% and 95% to their closest cultured relatives *Desulfobacterium indolicum*, *Synthrophus gentianae*, and *Desulfobacula phenolica*, respectively

(Fig. 1). These clones were all retrieved from the first library representing bacteria of the chemocline. Sequences of the remaining five clones all retrieved from the second library representing bacteria of the monimolimnion formed a tight cluster together with an additional clone from the database (clone Sai2P2-62) [43]. Four of these clones (618, 624, 626, 651) and clone Sai2P2-62 harbored sequences with homology values between 99% and 100% to each other, and 98% to the remaining clone 650. This cluster showed homology values between 95% and 97% to sequences from other uncultured bacteria, e.g., clone FW133 retrieved from forested wetlands [44], or clones SbIDsm2-5/8 obtained from uncultured bacteria in low-sulfate, acidic fens [45]. The closest cultured relatives to our cluster were *D. tiedjei* and *D. limimaris* with homology values of 95% and 93%, respectively (Fig. 1). All sequences in our cluster displayed a mismatch to probe SRB385Db. Due to the location of this mismatch at the terminal base at the 3' end of probe SRB385Db, however, no effects were observed during in situ hybridization.

Since morphological features and habitat occurrence supported the assumption of a close relationship between “morphotype R” and *D. tiedjei* and *D. limimaris* [19,20], we focused on the latter cluster and designed probe DsmA455 targeting four clones (618, 624, 626, 651) as well as clone Sai2P2-62 and probe DsmB455 targeting clone 650 (Table 1). Probe DsmA455 was a mixture of two probes with one T/C exchange necessary to detect the sequence in clone 651 (Table 1). It differed from probe DsmB455 in two additional bases indicating

the presence of at least three distinct, but closely related populations within this cluster in Lake Cadagno, represented by sequences in clones 618/624/626, clone 650, and clone 651, respectively (Table 1). Clone FW133 harboring the closest non-target sequence with 97% homology to our clones, displayed at least two differences in the probe–target region, while the next more distantly related sequences (*SbIDsm2-5/8*, clones Btol and UASB TL44, *D. tiedjei* and *D. limimaris*) displayed already three or more differences (Table 1). For both probes, the specificity of the hybridization was adjusted by the addition of 20% formamide to the hybridization buffer and by a reduction of NaCl in the washing buffer to 225 mM [40]. In addition, hybridizations were carried out over night instead of for 2 h which increased the signal intensity and the number of cells detected significantly.

Both probes detected straight and slightly curved rods in the chemocline, monimolimnion and sediment that resembled “morphotype R” (Fig. 2) [19]. No cells were detectable in the mixolimnion after hybridization with probes DsmA455 and DsmB455. Numbers retrieved by the individual probes in the same sample added up to an amount that corresponded to all cells representing “morphotype R” (data not shown). Both probes detected about the same number of cells throughout the water column and the sediment suggesting that the basic ecophysiological requirements of these populations might be similar. Since we were interested in bacteria resembling “morphotype R” rather than in the individual subpopulations targeted by our probes, further

Table 1  
Target sites for probes DsmA455 and DsmB455

Probe DsmA455	AGAAACTCATTATCGAGTCYCTGA
Probe DsmB455	AGGAACTCATTATCGAGTCCCTAGA
Clone 624 (AJ316021)	UCUUUGAGUAAAUAGCUCAGGGAACU
Clone 626 (AJ316022)	UCUUUGAGUAAAUAGCUCAGAGAACU
Clone 618 (AJ316020)	UCUUUGAGUAAAUAGCUCAGAGAACU
Clone 651 (AJ316024)	UCUUUGAGUAAAUAGCUCAGAGAACU
Clone Sai2P2-62 (AJ518666)	UCUUUGAGUAAAUAGCUCAGAGAACU
Clone 650 (AJ316023)	UCCUUGAGUAAAUAGCUCAGGGAUCU
Clone FW133 (AF523958)	UCUUUGAGUGAAUAGCUCAGGGAACU
Clone Btol (AF2822178)	UCCCCAGGUAAAUAGCCUGGAGAACU
<i>Desulfomonile tiedjei</i> <sup>T</sup> (M26635)	UUUGCGGACGAAUAGUCCUGAAACU
Clone UASB_TL44 (AF254395)	-CCGAGAGCGAAUAACUCUUGGAACU
<i>Desulfomonile limimaris</i> <sup>T</sup> (AF230531)	ACCUAUGGUGAAUAGUCAUGGGUGCU
Clone UASB_TL11 (AF254397)	GGUGGGGGUGAAUUCGUCCACCCUU
Unidentified SRB strain PRTOL1 (U49429)	CCC GGACUGAACAGGUUUUGGAGU
Unidentified SRB strain BKA11 (AJ012597)	CCC GGAAUUGAACAGGUUUUGGAGU
<i>Desulfobacterium anilini</i> <sup>T</sup> (AJ237601)	UUCAUC-GUAAACAGCGCUGAAGACU
delta proteobacterium strain S2550 (AF148141)	GCUUCUGAUUAAUAUCUCAGGAGUACU
Unidentified SRB AK-01 (AF141328)	GCUUCUGAUUAAUAUCUCAGGAGUACU
delta proteobacterium strain Pnd3 (Y17501)	GCUUCUGAUUAAUAUCUCAGGAGUACU
Clone SbIDsm4 (AY167448)	UCUUCUAGUGAAUAGCUAGAGGAACU
Clone SbIDsm5 (AY167449)	UCCAUGAGUAAAUAGCUCUAGGAAAU
Clone E2 (AY426462)	UCAAUAGGUGAAUAGUCUUAUUGAACU

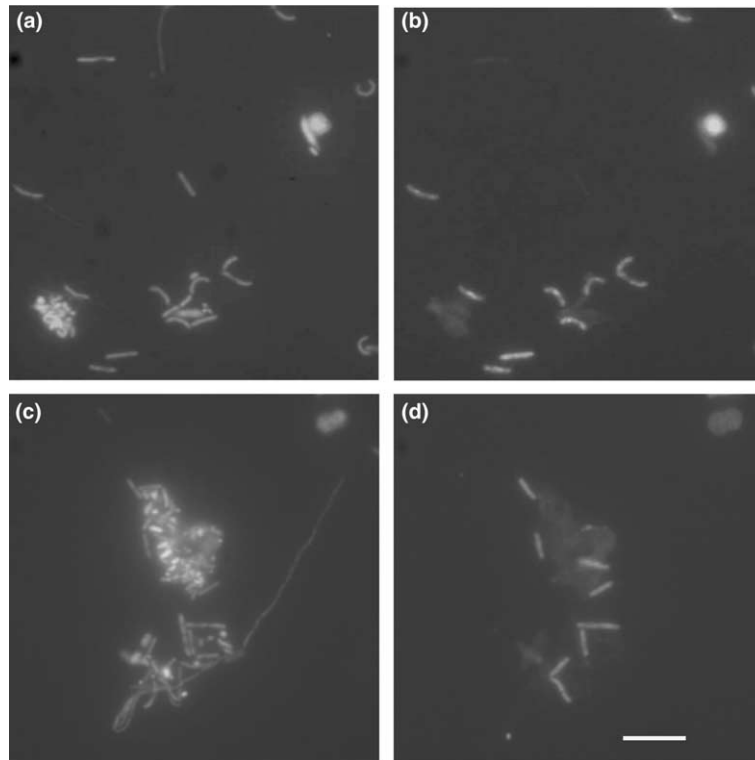


Fig. 2. In situ detection of “morphotype R” in the chemocline of Lake Cadagno after hybridization with probes DsmA455 (a and b) and DsmB455 (c and d). The left panel shows all organisms in the same sample after DAPI-staining (a and c). Bar represents 10  $\mu\text{m}$ .

quantitative analyses in the anoxic water column (i.e., the chemocline and monimolimnion) and in the anoxic sediment of Lake Cadagno were performed with a mixture of both probes DsmA455 and DsmB455.

### 3.2. Microbial analyses

Physico-chemical conditions in the chemocline and monimolimnion resembled patterns typically found in

Lake Cadagno [3,5]. Sulfate concentrations were high, but relatively stable with values ranging from 1.25 to 1.67 mM (data not shown). Oxygen was rapidly depleted at the upper border of the chemocline (Fig. 3), and sulfide concentrations increased with depth to maximum values of about 0.5 mM close to the sediment at a depth of 20 m (Fig. 3). Corresponding to the turbidity values, numbers of DAPI-stained cells increased in the chemocline with numbers of up to  $7 \pm 2 \times 10^6$  cells  $\text{ml}^{-1}$ . Num-

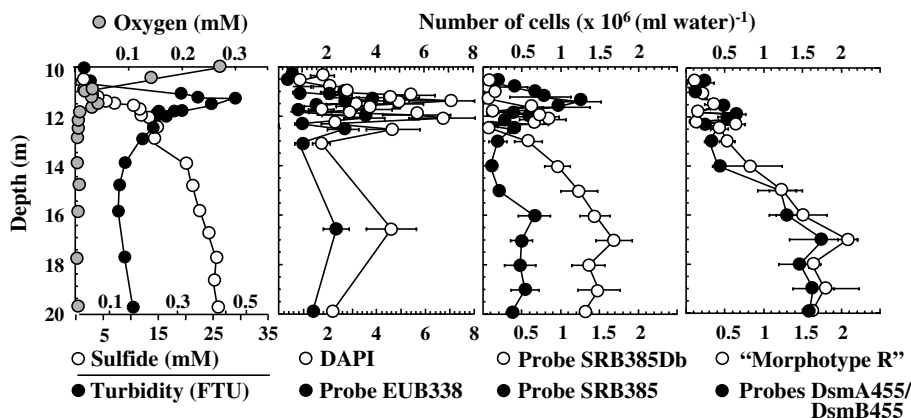


Fig. 3. Basic physico-chemical conditions (left panel), and distribution profiles of sulfate-reducing bacteria of the families Desulfovibrionaceae (probe SRB385) and Desulfobacteriaceae (SRB385Db) (middle panel), and of “morphotype R” detected with probes DsmA455 and DsmB455 or based on morphological criteria (right panel) in the water column of Lake Cadagno between 10 and 20 m depth. Circles represent means of 40 counts (means  $\pm$  SE).

bers declined in the monimolimnion to values between 2 and  $5 \times 10^6$  cells  $\text{ml}^{-1}$  (Fig. 3). In situ hybridization with probe EUB338 targeting members of the Bacteria on the Domain level, detected about 50% of all DAPI-stained organisms in both chemocline and monimolimnion (Fig. 3). Highest values were obtained with 61% in the monimolimnion close to the sediment at a depth of 20 m, which was comparable to percentages obtained in previous studies [46]. In the chemocline, however, previous studies detected higher numbers of DAPI-stained organisms (up to  $11 \times 10^6$  cells  $\text{ml}^{-1}$ ) and cells hybridizing with probe EUB338 (up to 90%) [7,18,46]. These differences most likely reflect seasonal changes [16,18].

In the chemocline where sulfide concentrations were still low though increasing with depth to up to 0.2 mM, sulfate-reducing bacteria were predominantly represented by bacteria detected with probe SRB385, i.e. members of the Desulfovibrionaceae (Fig. 3). These most likely resembled bacteria related to *Desulfocapsa thiozymogenes* that had been found to be the numerically most important sulfate-reducing bacterium in the chemocline accounting for up to 99% of the Desulfovibrionaceae in previous studies [7,15–18]. In these studies, population patterns were similar to those of cells detected with probe SRB385Db, i.e. members of the Desulfobacteriaceae, however, numbers of Desulfobacteriaceae were found to be more important in spring and summer rather than in fall as obtained in this study. In the monimolimnion, cells detected with probe SRB385Db became more prominent with numbers of up to  $1.5 \times 10^6$   $\text{ml}^{-1}$  close to the sediment being three times as high as those of the Desulfovibrionaceae (Fig. 3). Numbers increased with depth and profiles were positively related to increasing sulfide concentrations (Fig. 3). In the monimolimnion, Desulfobacteriaceae represented up to 29% of the DAPI-stained cells, and about 75% of the cells detected with probe EUB338. The numerical dominance of the Desulfobacteriaceae over Desulfovibrionaceae in the monimolimnion of Lake Cadagno suggests ecophysiological adaptations to the environmental conditions.

Profiles and numbers of Desulfobacteriaceae determined after in situ hybridization with probe SRB385Db resembled that of “morphotype R” analyzed by using morphological criteria (Fig. 3). These numbers were similar to those of earlier studies in 1993 in which “morphotype R” was found to represent 30% of all organisms in the monimolimnion of Lake Cadagno with numbers of up to  $1.5 \times 10^6$  cells  $\text{ml}^{-1}$  [19]. This percentage reflects average numbers encountered throughout the season though values between 17% and 44% have been reported with maximum numbers of up to  $44 \times 10^6$  cells  $\text{ml}^{-1}$  in early summer in the monimolimnion [46]. Comparable cell numbers as for “morphotype R” in Lake Cadagno have been obtained for the proposed relative “morphotype T5” with up to  $1.5 \times 10^6$  cells  $\text{ml}^{-1}$  in Lake Plußsee,

Germany [47]. The importance of this organism in the anaerobic water column was even more pronounced in Lakes Wintergreen and Burke (Michigan, USA) where “morphotype T5” could represent up to 60% of all Bacteria [20].

The profile of “morphotype R” perfectly matched with that retrieved after hybridization with probes DsmA455 and DsmB455 (Fig. 3) indicating that the sequences in our clones resemble the numerically most important members of the Desulfobacteriaceae in the monimolimnion of Lake Cadagno, and that our probes enabled us to detect them quantitatively. Our analysis showed that the Desulfobacteriaceae in the monimolimnion were entirely represented by “morphotype R” and consisted of at least two major populations with the closest cultured relatives belonging to the genus *Desulfomonile*.

Environmental conditions in the anoxic sediment of Lake Cadagno were characterized by increasing sulfide and decreasing sulfate concentrations with depth (Fig. 4) indicating active sulfate reduction. DAPI staining detected between 2 and  $6 \times 10^{10}$  cells (g sediment dry weight) $^{-1}$  in the upper 10 cm, and less than  $1 \times 10^{10}$  cells (g sediment dry weight) $^{-1}$  below (Fig. 4). Similar to the monimolimnion, in situ hybridization with probe EUB338 detected about 50% of these cells. The profile generated with EUB338 was matched by profiles obtained after hybridization with probes SRB385 and SRB385Db. Each probe detected about the same number of cells that added up to the number detected with probe EUB338 (Fig. 4) indicating the dominance of sulfate-reducing bacteria in the anoxic sediment of Lake Cadagno. In contrast to the monimolimnion, however, members of the Desulfobacteriaceae and the Desulfovibrionaceae were equally important numerically. “Morphotype R” represented the Desulfobacteriaceae with about  $5 \times 10^9$  cells (g sediment dry weight) $^{-1}$  entirely in the upper cm only. Below the upper cm, number and relevance of “morphotype R” declined significantly with depth. In the second and fourth cm, for example, cell numbers of “morphotype R” that were still entirely retrieved by our probes DsmA445 and DsmB455 (Fig. 4) declined from 3 to  $2 \times 10^9$  cells (g sediment dry weight) $^{-1}$ , and represented only about 25%, and 20% of the Desulfobacteriaceae, respectively. Although these data indicate effects of environmental conditions on the fate of “morphotype R”, a causal relationship between population development of “morphotype R” and specific environmental conditions could not be established.

“Morphotype R” resembles *D. tiedjei* and *D. limimaris* morphologically, and with respect to habitat occurrence. *D. tiedjei* and *D. limimaris* have been isolated and described recently [21,22]. Both are large, Gram-negative rods with a collar girdling each cell and have the ability to degrade halogenated organic compounds (e.g., 3-chlorobenzoate) by halorespiration or reductive

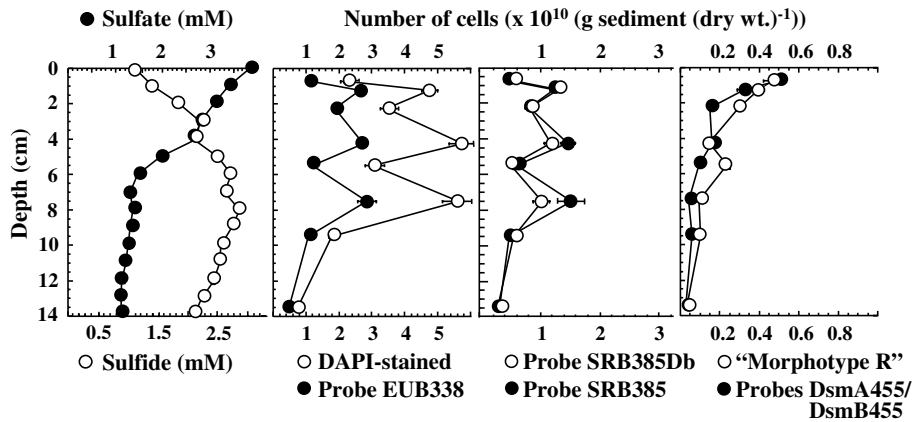


Fig. 4. Pore water concentrations of sulfide and sulfate in the upper 14 cm of sediment of Lake Cadagno (left panel), and distribution profiles of sulfate-reducing bacteria of the families Desulfovibrionaceae (probe SRB385) and Desulfobacteriaceae (SRB385Db) (middle panel), and of “morphotype R” detected with probes DsmA455 and DsmB455 or based on morphological criteria (right panel). Circles represent means of 40 counts (means  $\pm$  SE).

halogenation in which the halogenated compounds are used as terminal electron acceptors instead of  $\text{SO}_4^{2-}$ . Although phylogenetic relationships between *D. tiedjei* and *D. limimaris* and “morphotype R” from Lake Cadagno are not that close with homology values of 95% and 93%, respectively, and do not necessarily reflect physiological relationships [48–50], it is tempting to use specific traits and isolation conditions of these *Desulfomonile* sp. as basis for enrichment cultures of the individual populations of “morphotype R” from Lake Cadagno. The molecular analysis presented in this study provides the baseline data and tools to monitor the enrichment of the specific populations in order to obtain pure cultures that are necessary to study the physiological properties of “morphotype R” and its subpopulations. This strategy has been successful for other members of the microbial community in Lake Cadagno such as purple sulfur bacteria and sulfate-reducing bacteria [15], as well as in other studies [29,51,52].

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