

## **Actinomycetes may also produce taste and odour**

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## Occurrence of odour-producing actinomycetes and other bacteria in the North Pine River Dam, Brisbane, Australia

By

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### Abstract

In the summer period, North Pine Dam in southeast Queensland, Australia, is used as a supplementary source of drinking water for the city of Brisbane. Relatively high concentrations of geosmin and methylisoborneol (MIB) are frequently detected in the water in summer. The abundance of cyanobacteria (= blue-green algae) that are known to produce these compounds, is generally low, which suggests that the odours are produced by other organisms. In this short study, we examined the abundance of geosmin- and MIB-producing actinomycetes in the water and sediment of the reservoir. The results show that actinobacteria (actinomycetes are included in the taxonomic group of *Actinobacteria*) made up 18 to 24% of all bacteria in the dam water during a period of relatively high geosmin and MIB concentrations. The high density of actinobacteria in the dam suggests that these bacteria may be more dominant in production of geosmin and MIB than previously expected, and that they should be included in future studies of odour problems in freshwater reservoirs.

### Introduction

Biological production of the taste and odour components (TOCs) geosmin and methylisoborneol (MIB) reduce the application of surface water for drinking (Cook et al., 2001; Smith et al., 2002; Lanciotti et al., 2003). The origin of TOCs is generally believed to be cyanobacteria, but certain bacteria may also contribute TOC in water reservoirs. Among geosmin- and MIB-producing

bacteria are actinomycetes, which according to recent research are common microorganisms in most aquatic environments (Sekar et al., 2003; Klausen et al., 2003; Burkert et al., 2003). Actinomycetes are Gram-positive, filamentous (most species), spore-forming bacteria, which produce colonies with a leathery surface and greyish colours on agar media.

The significance of TOC production by actinomycetes in Australian water reservoirs has not been studied, as well as the occurrence of these bacteria. The purpose of this research was to quantify the abundance of actinomycetes in water and sediment of one of these reservoirs, North Pine Dam, in Brisbane.

## **Locations and methods**

### *Sampling locations*

Water was collected from North Pine Dam, a drinking water reservoir 30 km north of Brisbane, Australia. Four sites were sampled on 10<sup>th</sup> and 18<sup>th</sup> December 2003: station 10001, 100 m from the dam wall, 20 m deep (surface and bottom); 10003, mid lake, 11 m deep (surface and bottom); 10006, Clear Mountain resort intake, 2 m deep (surface); and 10010, Kobble Ck arm, 9 m deep (surface and bottom). For the surface samples, a 3 m depth integrated sample was taken with a pipe, and for the bottom sample, a Niskin bottle was used. Water samples were kept in the dark until returned to the laboratory within 2 h. In the case of samples for geosmin/MIB analysis, subsamples were placed in dark glass bottles and kept on ice until returned to the laboratory.

Sediment material (from the upper 0.5 cm sediment) was also collected from stations 10001, 10010, 10003 and 10006 on 10<sup>th</sup> December 2003. Samples were kept in the dark until returned to the laboratory within 2 h. In the case of samples for geosmin/MIB analysis, subsamples were placed in dark glass bottles, filled with water from the site and kept on ice until returned to the laboratory.

### *Total bacterial counts*

All samples were preserved with formaldehyde (2% final conc.) about 2 h after collection. Before analysis, the samples were vigorously shaken by whirl-mixing to release attached bacteria. Bacterial densities in the water samples were measured by flow cytometry, using a Becton Dickinson Calibur flow cytometer after staining with SYBR Green1 (Molecular Probes, USA) (Jochem, 2001).

### *Bacterial growth*

The bacterial production in the water column was determined by incorporation of <sup>3</sup>H-thymidine (Fuhrman and Azam, 1982). <sup>3</sup>H-thymidine to a concentration of 10 nM was added to 1 ml water samples. Samples were incubated for 30 to 45 min. The incubations were terminated by addition of

trichloroacetic acid (TCA) to a final concentration of 5%. Finally, the samples were centrifuged and washed twice in 5% TCA. Scintillation liquid was added and radioactivity was measured with a scintillation counter (Packard). For conversion of incorporation of radioactivity into cell production, a factor of  $2.15 \times 10^{18}$  cells per mol thymidine was applied (Smits and Riemann, 1988).

#### *Occurrence of actinomycetes by FISH technique*

Enumeration of actinomycetes was performed using fluorescent in-situ hybridization (FISH) with signal amplification (catalyzed reporter deposition, CARD) according to Sekar et al. (2003). The oligonucleotide probe for the hybridization was HG654, which is specific for *Actinobacteria* (Glöckner et al., 2000). *Actinobacteria* include actinomycetes and a few other naturally occurring bacteria, mainly *Arthrobacter* and *Corynebacteria*. The probe was combined with a horseradish peroxidase enzyme (HRP) (ThermoHybaid, Ulm, Germany).

Before hybridization, bacteria in the water and sediment samples were filtered onto 0.2  $\mu\text{m}$  polycarbonate filters (Millipore, GTTP 00025). The sediment material was initially sonicated (Branson probe, max. power and 50% duty for 1 min) before a 100- fold dilution in distilled water. Bacteria on the polycarbonate filters were embedded in 5% agarose prior to hybridization.

The hybridized cells were dipped in a solution of tyramine, which was converted into tyramide by peroxidase. Tyramide is a strong protein-absorbing compound, which readily combine with proteins in the bacterial cells. The fluorochrome fluorescein (linked to tyramide) produced brightly green fluorescent cells that were visualized by epifluorescence microscopy.

#### *Bacterial plate counts (isolation of actinomycetes)*

Bacteria in water and sediment from the different stations were isolated by plating on 5% R2A agar (Merck, Darmstadt, Germany). The agar medium was enriched with chitin to a final concentration of 0.1%. To obtain a full-strength agar concentration, the 5% R2A medium was further added 5 g agar per l before autoclaving and decanting into Petri dishes. The media was dispersed into 50 agar plates (9 cm diameter). Water samples were diluted 1000- and 10000-fold and spread on the agar plates. Samples from the sediment were diluted 50-fold (0.5 gram sediment in 25 ml Milli-Q water) and were further diluted 100-fold and 1000-fold and spread on agar plates. Selected colonies with a typical actinomycete morphology (solid, leathery surface and a greyish-brownish colour) were further isolated after 5 days on the same media.

#### *Content of geosmin and MIB*

Sediment and water samples collected on 10<sup>th</sup> December were analysed for geosmin and MIB concentrations using a purge-and-trap GC MS technique (<http://www.chem.agilent.com>). In the

case of sediment samples, 1 g ww of sediment was added to 40 ml deionised water, shaken for 1 h, then 25 ml of suspension was injected into the GC-MS. All analyses were performed as single measurements. The detection limit was 4 ng l<sup>-1</sup> and the analytical precision was determined to ± 3%. Analysis of duplicate samples from the same locations typically demonstrate a variation <10%.

## Results and Discussion

### *Bacterial density and production*

The bacterial density in the reservoir water varied from 6 to 10 x 10<sup>9</sup> cells l<sup>-1</sup> (**Fig. 1**). The lowest number was found in surface and bottom water of the 20 m deep station 10001, near the dam wall. Maximum density occurred in the surface of the 9 m deep Kobble Ck arm (station 10010). The bacterial density was 5 to 18% lower in the bottom water than in the surface water at stations 10001 and 10010, but no difference was seen at station 10003 (mid lake). The measured bacterial numbers in North Pine Dam are typical of eutrophic freshwater environments during spring and summer periods (Simon, 1994; Cole and Pace, 1995).

The bacterial production in the reservoir water on December 10<sup>th</sup> and 18<sup>th</sup> varied between 70 and 150 x 10<sup>6</sup> cells l<sup>-1</sup> h<sup>-1</sup>, except at station 10001, where < 38 x 10<sup>6</sup> cells l<sup>-1</sup> h<sup>-1</sup> were found at surface and bottom (indicated by an asterisk) (**Fig. 2**). We expect these two results to be incorrect, possibly due to a contamination. When ignoring these two samplings, the bacterial production was rather similar at both sampling dates, except at station 10010, where a low bacterial growth was measured on December 18<sup>th</sup> (45% below the rate on December 10<sup>th</sup>). The bacterial growth in the bottom water was reduced relative to the surface (16% lower at station 10001 on Dec. 18<sup>th</sup> and 28% lower at station 10010 on Dec. 10<sup>th</sup>), except at station 10010 where a two-fold higher production was found on Dec. 18<sup>th</sup>.

The variable bacterial production in the surface water, relative to the bottom, may reflect that a changeable portion of the organic matter was degraded in the photic zone. In addition, anoxic conditions in the bottom strata may also have influenced the bacterial growth. The bacterial doubling times were about 70 hours (bacterial density divided by the cell production) as compared to doubling times of 10 to 24 hours typically observed in eutrophic lakes (Simon, 1994). This means that the bacterial growth rate in North Pine River was lower than that often measured in eutrophic freshwater. However, the present rates compare well with the bacterial production of 4 g C m<sup>-2</sup> d<sup>-1</sup> previously measured in Australian, subtropical rivers (Pollard, 2004). Assuming a water depth of 4 m and a bacterial C content of 10<sup>-13</sup> g (Kroer, 1994), the present rates correspond to 8 g C m<sup>-2</sup> d<sup>-1</sup>. The estimated 2-fold higher bacterial production measured here may reflect a higher production of organic matter in North Pine River than in the rivers studied by Pollard.

### *Occurrence of actinobacteria in water and sediment*

Bacterial samples collected on December 18<sup>th</sup> were analyzed for presence of bacteria belonging to the *Actinobacteria* group by the CARD-FISH technique. A photomicrograph of FISH-positive cells is shown in **Fig. 3**.

The density of actinobacteria in the dam water ranged from about  $1.4 \times 10^9$  cells  $l^{-1}$  in surface and bottom water at stations 10001 and 10010, to about  $1.8 \times 10^6$  cells  $ml^{-1}$  at station 10003 and 10006 (surface) (**Fig. 4**). The actinobacteria typically made up about 20% of all bacteria in the water (range 17.9 to 23.6%).

The number of FISH-positive cells in surface sediment from 4 stations in the dam varied from  $20 \times 10^6$  cells  $g^{-1}$  wt at stations 10001 and 10010, to 50 to  $60 \times 10^6$  cells  $g^{-1}$  wt at stations 10003 and 10006. The proportion of actinobacteria relative to all bacteria in the sediment could unfortunately not be determined, as the fluorescent stain for microscopic quantification of the total bacterial densities faded before quantification of the cells was possible. Flow cytometer counting of sediment bacteria is not possible due to presence of large particles that will damage the inlet quartz tube of the flow cytometer.

The observed amounts of actinobacteria in the dam water is comparable with recent studies of these bacteria in lakes and streams in Europe (Glöckner et al., 2000; Klausen et al., 2004). In these studies, actinobacteria were found to constitute from a few percent and up to 63% of the bacterial populations. The proportion of actinomycetes among the actinobacteria has not been determined, but the fraction of filamentous bacteria in similar FISH analyses of Danish streams was found to be 24-54% (Klausen et al., 2003). The only filament-forming actinobacteria are actinomycetes. Using the actinobacteria/actinomycete proportion in Danish streams and considering that some actinomycete species are non-filamentous and unicellular, the density of actinomycetes in North Pine River may be 5 to 10% of the bacterial populations.

The ecological function of actinomycetes in freshwater is unknown. Actinomycetes produce a wide range of hydrolytic enzymes (Madigan et al., 2003) and may be expected to be efficient in degrading various types of organic matter. Recent observations from manipulation of the content or dissolved organic matter in humic lake water suggest that actinomycetes can be outcompeted by faster-growing species (Sekar et al., 2003). Speculatively, this may indicate that the function of actinomycetes in freshwater is degradation of relatively recalcitrant organic species.

### *Isolation of Actinomycetes*

Inoculation of dam water on Petri dishes with 5% R2A media demonstrated single actinomycete colonies, but other and faster-growing bacteria in the dam water tended to overgrow the

actinomycete colonies. In contrast, several actinomycete colonies were observed in all the sediment samples when plating suspended sediment material on the R2A media. The actinomycete colonies were identified by morphology only (**Fig. 5a**). Filaments of the bacteria were hybridized with the CARD-FISH technique to demonstrate that the applied HG654 probe targets actinomycetes (**Fig. 5b**). These isolates had a characteristic earthy odour, typical of geosmin.

#### *Content of geosmin and MIB in surface water and sediment*

Geosmin and MIB were present in the water at detectable levels at all sites (**Table 1**). The concentration of MIB was below  $9 \text{ ng l}^{-1}$ , except at the 2 m deep station 10006, where concentrations were  $15 \text{ ng l}^{-1}$ . Geosmin concentrations ranged from 10 to  $15 \text{ ng l}^{-1}$ , but near the dam wall (St. 10001), less than  $5 \text{ ng l}^{-1}$  were measured. Sediment data was only available for sites 10006 and 10010. At these stations, the concentrations of both odours were 500 to  $650 \text{ ng kg}^{-1}$ . The amount of geosmin was about 15% above the amount of MIB at the two sediment stations.

Off-take water from NPD was analysed for geosmin and MIB during the period that this study was undertaken. MIB was present on all sampling days in December (10 to  $23 \text{ ng l}^{-1}$ ), while geosmin was present just above the  $4 \text{ ng l}^{-1}$  detection limit on over half the days of sampling (**Fig. 6**). On Dec. 18<sup>th</sup>, when the density of actinobacteria was determined, the MIB and geosmin concentrations were 13 and  $10 \text{ ng l}^{-1}$ , respectively. The continuous abundance of the TOCs in the raw water demonstrates that there was an ongoing production of these compounds. Therefore, although actinobacterial densities were only determined for December 18<sup>th</sup>, while analysis for TOCs occurred on December 10<sup>th</sup>, it is evident that the presence of TOCs in the water was not an isolated event.

The concentrations of geosmin and MIB in North Pine River correspond to the levels measured in other freshwaters in Australia (Jones and Korth, 1995) and in eutrophic lakes and rivers with cyanobacterial populations, e.g., Lake Zurich (only geosmin was measured; Durrer (1999)) and Arno River, Italy (Lanciotti et al., 2003). Cyanobacteria are generally believed to be the main source of the odours. Supporting this, removal of a cyanobacterial-rich biofilm on canal walls in Phoenix, Arizona, was found to significantly reduce the odour level in the water (Hu et al., 2003). Also, during a high zooplankton grazing on cyanobacteria in the Lake Zurich, the geosmin level rose from 3 to  $21 \text{ ng l}^{-1}$  (Durrer et al., 1999). However, during periods with a low cyanobacterial biomass, the source of geosmin and MIB has been suggested to be actinomycetes rather than cyanobacteria (Lanciotti et al., 2003). This is supported by our own analysis of streams in Denmark, where up to  $12 \text{ ng geosmin and MIB l}^{-1}$  were detected, although no cyanobacteria were present (Klausen et al., 2004).

### *Tests for stimulation and inhibition of actinomycete growth in water*

In pilot experiments, we tested if growth of actinomycetes can be stimulated or inhibited. Presence of actinomycetes might be detected from their odours alone, but this requires a sufficiently high density. Therefore, if the abundance is low, a stimulated growth would be useful. Using FISH technique, we found that addition of the polymer substance, chitin, significantly promoted the growth of the actinomycete in dam water within two weeks (data not shown). Chitin is efficiently degraded by actinomycetes and has been used a selective medium for these bacteria (Hsu and Lockwood, 1975).

Inhibition of actinomycete growth in dam water was tested by addition of the antibiotic Bacitracin, which inhibits cell divisions of Gram-positive bacteria such as actinobacteria. The number FISH-positive cells in the Bacitracin-treated samples was identical to that in the controls, despite chitin was added (data not shown). This confirmed that the actinomycete growth was prevented. Obviously the antibiotic did not have an effect on existing Gram-positive cells present in the sample.

These pilot experiments show that the growth of actinomycetes can be rather easily controlled under experimental conditions. Manipulation of the actinomycete density in natural water samples will be useful when examining their potential as producers of TOCs.

### **Conclusions**

The abundance of actinobacteria (includes actinomycetes and a few other bacterial groups) in both water and sediment of the North Pine Dam suggests that these microorganisms can be potentially important producers of geosmin and MIB. Production of TOCs by actinomycetes is well-documented, but their biology, natural growth rates and actual significance as producers of odours in natural waters must be examined.

Future studies on actinomycetes in water reservoirs should include a more intensive spatial and temporal sampling program to test for covariation in occurrence of actinomycetes and TOCs. The field samplings should be accompanied by laboratory incubations to test for increase in TOCs in relation to occurrence and growth of actinomycetes. Also, in an attempt to obtain more specific information on actinomycetes in water and sediment, the presently applied FISH probe should be redesigned to target only actinomycetes.

The abundance of TOC-producing actinomycetes in North Pine River emphasizes that cyanobacteria may not be the only producers of odours in the dam. The high concentrations of geosmin (up to 15 ng l<sup>-1</sup>) and MIB (up to 23 ng l<sup>-1</sup>) measured in December 2003 in the dam, despite a low abundance of potential TOC-producing cyanobacteria such as *Anabaena* sp. (own unpublished observations) support that focus should be placed on other TOC producers than cyanobacteria. Yet,



the present data are too sparse to draw conclusions on actinomycetes as a major source of odours in the dam and further studies are needed.

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Table 1. Concentrations of geosmin and MIB in surface water (0-3 m integrated samples) and in the upper 2 cm sediment in North Pine Dam. na = not analyzed

Station	Water (ng l <sup>-1</sup> )		Sediment (ng kg <sup>-1</sup> )	
	MIB	Geosmin	MIB	Geosmin
10001	8.8	<5	na	Na
10003	7.2	13	na	Na
10006	15	10	556	651
10010	6.2	15	501	578

## Figure legends

Fig. 1. Number of bacteria in surface and bottom water of selected stations in North Pine River reservoir. Variations in cell number in duplicate flowcytometer counts were <3% (not shown).

Fig. 2. Bacterial production in the North Pine River reservoir, determined by incorporation of <sup>3</sup>H-thymidine into bacterial DNA. The low production measured at station 10001 on Dec. 10<sup>th</sup> probably reflects an analytical error. Mean values ± 1 SD of triplicate measurements are shown.

Fig. 3. Actinobacteria in North Pine River water, detected by CARD-FISH technique. See textbox for more details on the method.

Fig. 4. Abundance of *Actinobacteria* in North Pine River water (left axis) and their proportion of the total bacterial density (right axis). Mean values of 10 to 15 microscope fields of the CARD-FISH preparations (each with a size of 110 x 110 μm) ± 1 SD are shown.

Fig. 5. Unidentified actinomycete species isolated from sediment material of North Pine River dam (grown on R2A agar; left) and a microphotograph of a CARD-FISH preparation of filaments from the isolate (right).

Fig. 6. Concentrations of geosmin and MIB in raw water off-take pipe on the dam wall in December 2003. No geosmin or MIB were detected on December 17<sup>th</sup>. The detection limit of geosmin and MIB was 4 ng l<sup>-1</sup>.

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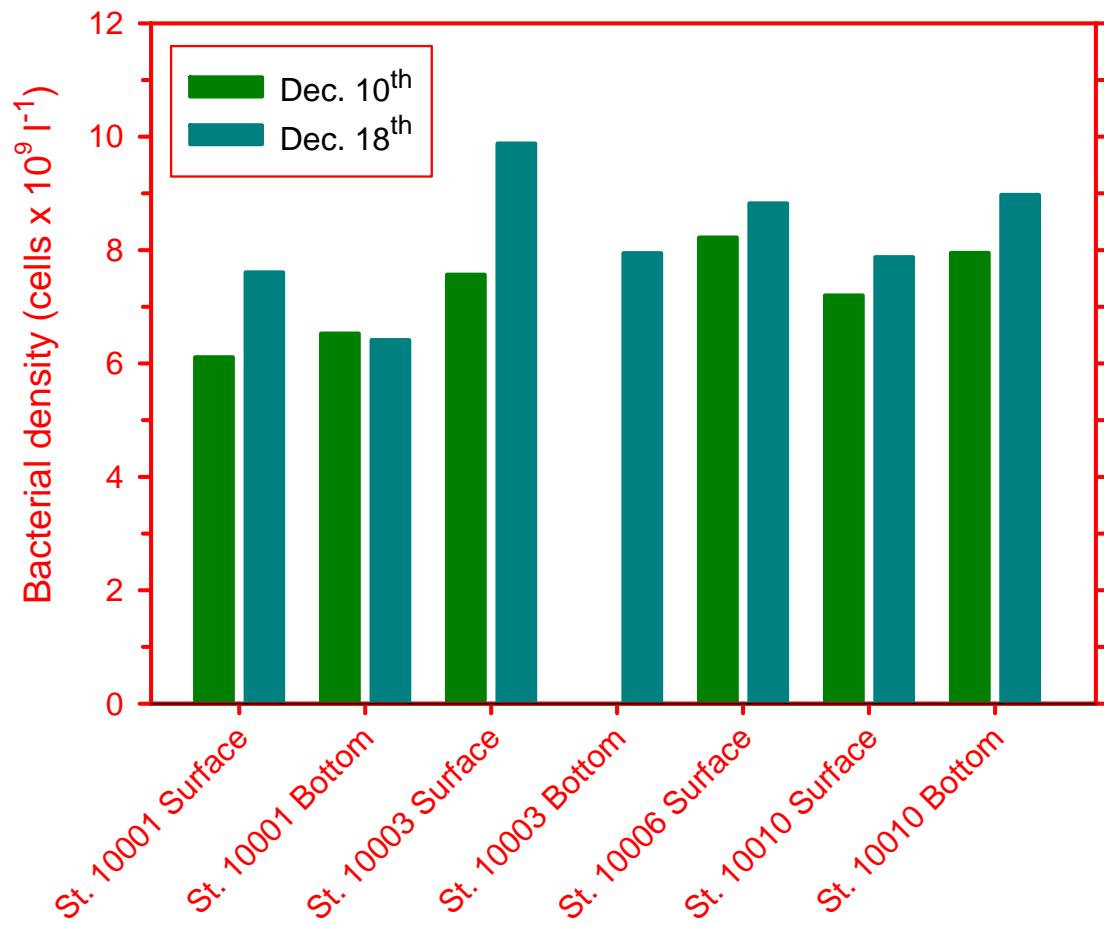


Fig. 1

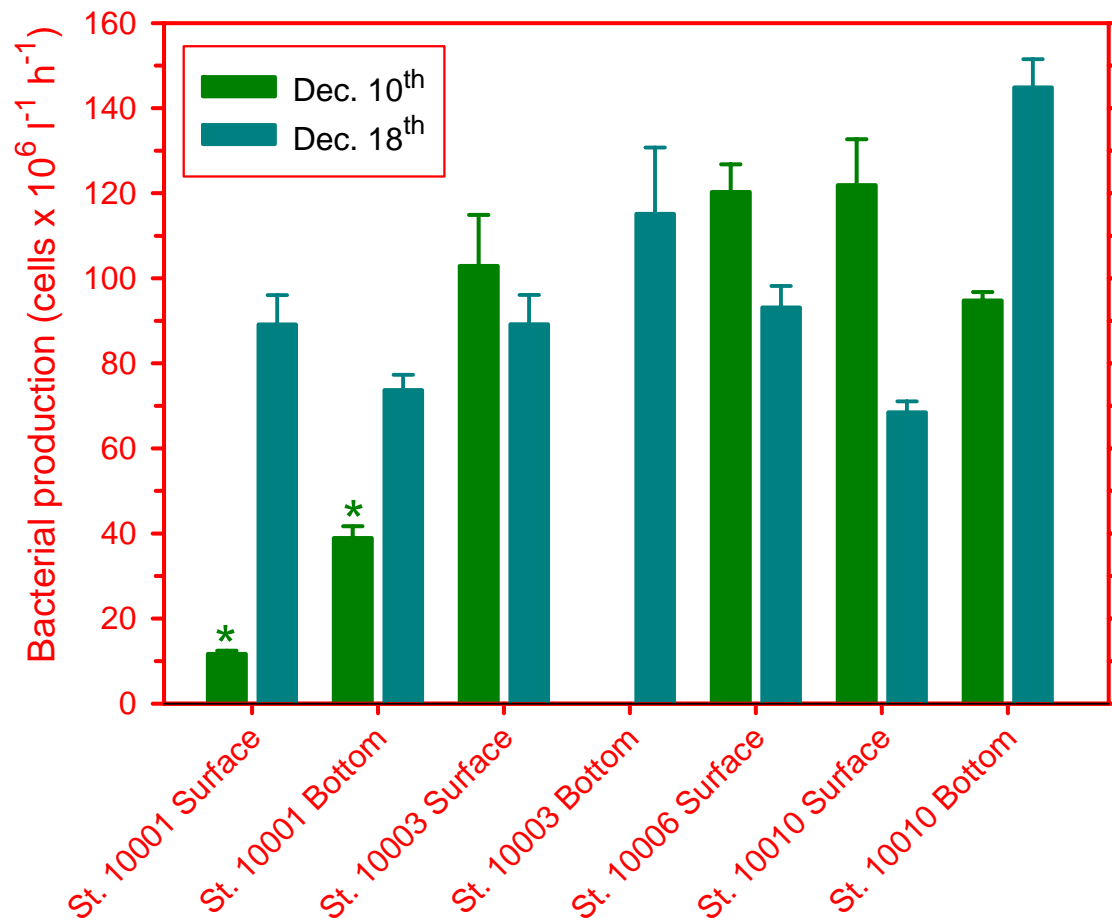


Fig. 2

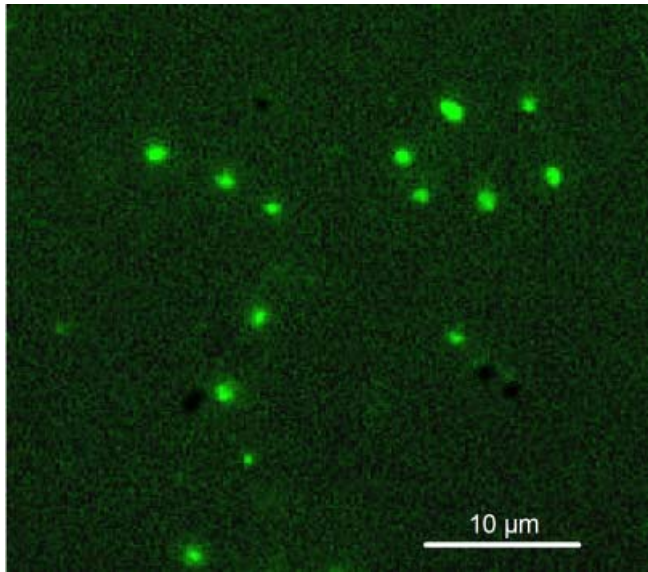


Fig. 3



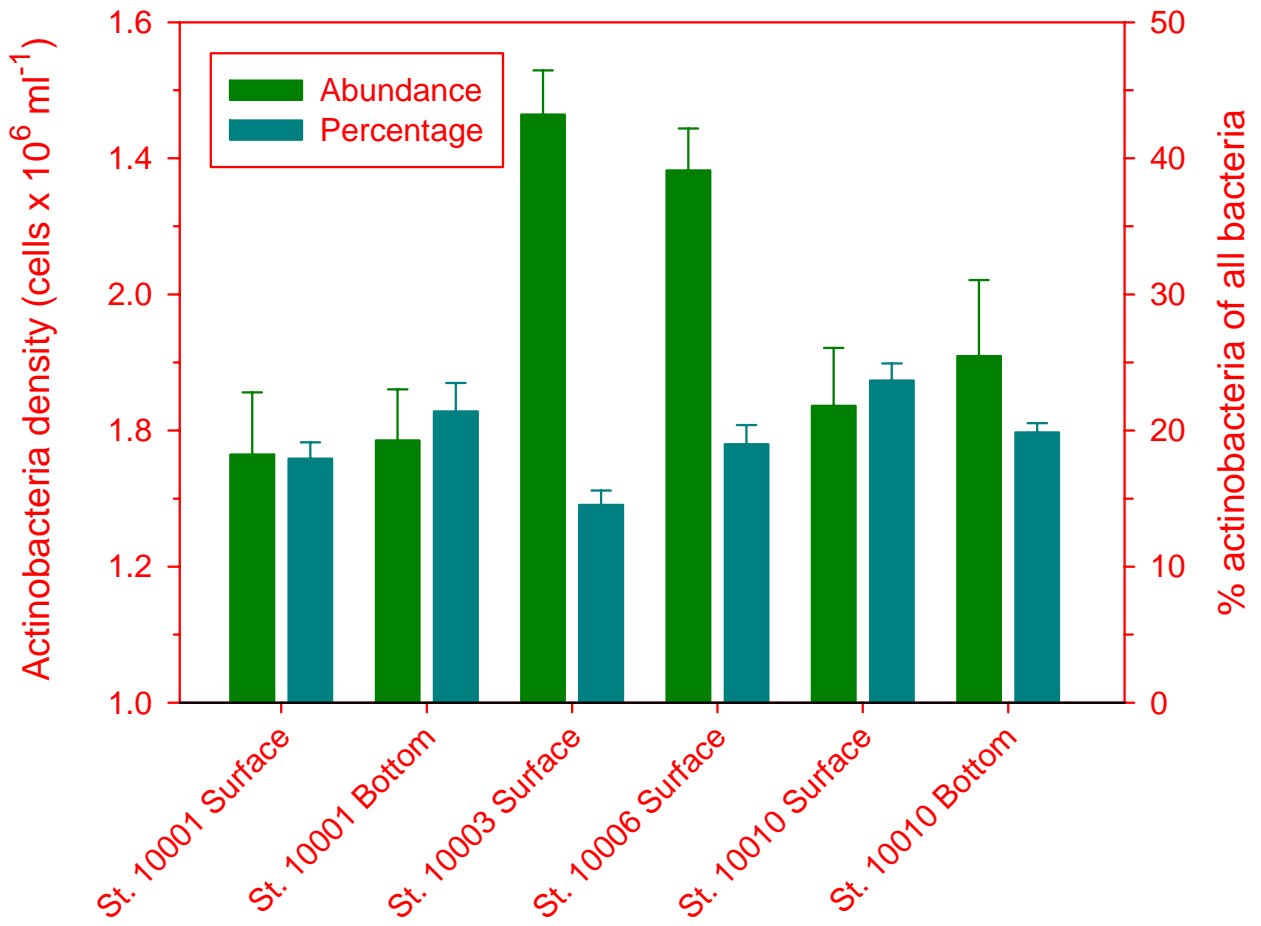


Fig. 4

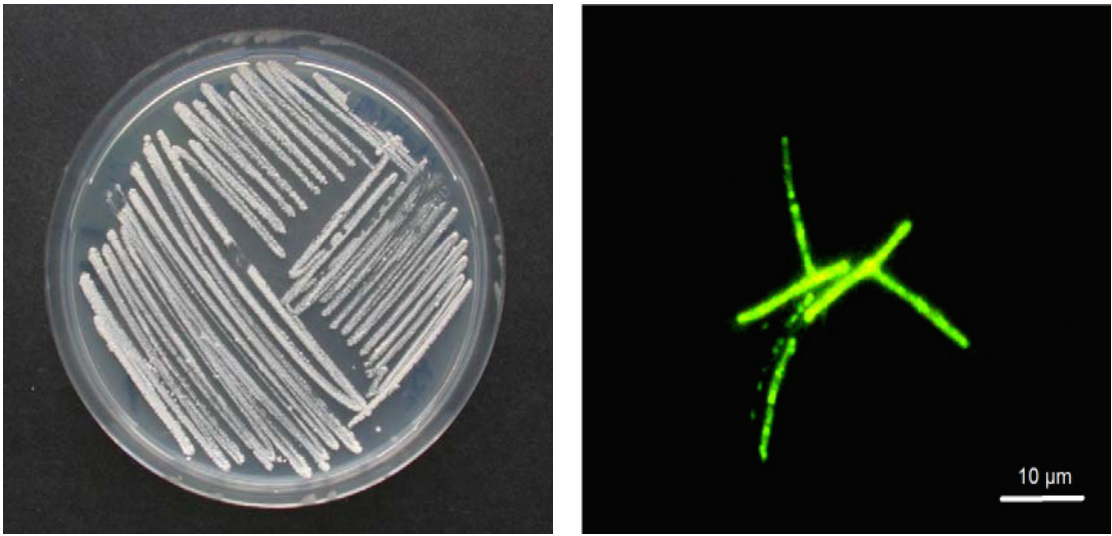


Fig. 5

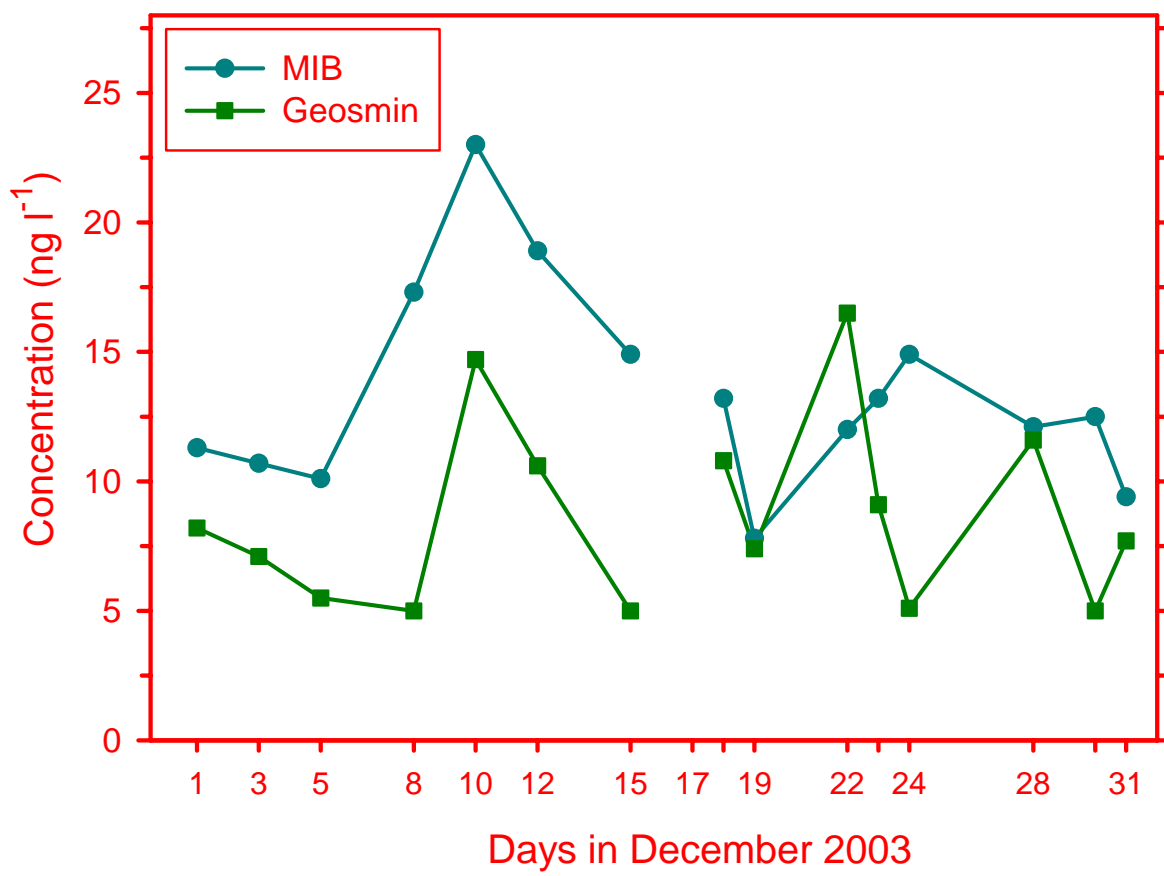


Fig. 6

## Text box : FISH technique

Only a small fraction of naturally occurring prokaryotes (bacteria within the domains *Bacteria* and *Archaea*) are identifiable by traditional culture methods, but the development of molecular tracers have made it possible to detect and identify microorganisms that cannot be cultivated in the laboratory. Among the successfully applied molecular tracers is the genetic information of the prokaryote ribosomes, especially the 16S ribosome. Analysis of the nucleotide sequence of 16S rRNA has shown that 16S rRNA possesses both highly conserved and highly variable regions. Conserved regions, for example, show the same sequence for all prokaryotes, while highly variable regions can help to identify specific groups of prokaryotes. Based on these differences, it is possible to design RNA probes with different selectivity and specificity, e.g. targeting major bacterial domains or single genera. RNA probes contain approx. 20 nucleotides that are complementary to the selected target region on the RNA.

When RNA probes are used to detect bacteria in environmental samples, cell walls of the bacteria are made permeable to allow the probe to diffuse into the cells. The probe will bind to (hybridize with) its complementary target on the rRNA in the bacterial cell without destroying its structure. If a fluorescent tag is added to the probe, the targeted bacteria can be detected by fluorescence microscopy (see figure). The method is commonly named *fluorescence in situ hybridization* (FISH) as the probe is applied to RNA in whole cells. FISH has been used to detect several specific bacterial groups in natural environments, e.g., subdivisions of *Proteobacteria*, the *Cytophaga-Flavobacterium* group and *Actinobacteria*, as well as single genera, e.g. *Nitrosomonas*. Different probes can be used in each sample if the probes are tagged with different fluorochromes.

If bacteria in natural environments are slowly growing and only contain few ribosomes, the fluorescent signal obtained by the FISH method may too low for microscopic observations. To compensate for this, a modified procedure, named *signal amplification*, has been developed. An example of signal amplification is the application of the compound tyramine, often labelled with Cy3 as a fluorescent tag. Instead of a fluorescent label, the RNA probe is combined with an enzyme (in our case a horse radish peroxidase). Peroxidase converts tyramine to tyramide, which combines with proteins in the bacterial cells. The deposition of fluorescent stain the cells leads to brightly fluorescent cells. The signal amplification has been named *catalyzed reported deposition* or CARD. The CARD-FISH approach was used in the present study of *Actinobacteria* in North Pine River dam.

