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Abundance of actinobacteria and production of geosmin and 2-methylisoborneol in Danish streams and fish ponds

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Abstract

Occurrence of the odours geosmin and 2-methylisoborneol (MIB) in freshwater environments indicates that odour-producing organisms are commonly occurring. In the present study, we assumed actinomycetes to be a major source of the odours. Seasonal concentrations of odours and abundance of *Actinobacteria*, which includes actinomycetes and other G^+ and high GC bacteria, were determined in one oligotrophic and two eutrophic freshwater streams, as well as in aquacultures connected to these streams, in Denmark. Concentrations of geosmin and MIB ranged from 2 to 9 ng l^{-1} and were lowest in the winter. Passage of stream water in the aquacultures increased the amount of geosmin and MIB by up to 55% and 110%, respectively. Densities of actinobacteria were determined by fluorescence in situ hybridization with catalyzed reporter deposition (CARD-FISH) technique and were found to make up from 4 to 38×10^7 cells l^{-1}, corresponding to 3–9% of the total bacterial populations. The lowest densities of actinobacteria occurred in the winter. Filamentous bacteria targeted by the FISH probe made up about 2.7–38% (average was 22%) of the actinobacteria and were expected to be actinomycetes. Combined microautoradiography and CARD-FISH demonstrated that 10–38% (incorporation of ^{3}H-thymidine) and 41–65% (incorporation of ^{3}H-leucine) of the actinobacteria were metabolically active. The proportion of active actinobacteria increased up to 2-fold during passage of stream water in the aquacultures, and up to 98% of the cells became active. Sequencing of 16S rRNA genes in 8 bacterial isolates with typical actinomycete morphology from the streams and ponds demonstrated that most of them belonged to the genus *Streptomyces*. The isolated actinomycetes produced geosmin at rates from 0.1 to 35 ag geosmin bacterium^{-1} h^{-1}. MIB was produced at similar rates in 5 isolates, whereas no MIB was produced by three of the isolates. Addition of the odours to stream water demonstrated that indigenous stream bacteria were capable of reducing the odours, and that enrichment with LB medium stimulated the degradation. Our study shows that bacterial communities in freshwater include geosmin- and MIB-producing actinobacteria. However, the mechanisms controlling production as well as degradation of the odours in natural waters appear complex and require further research.
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Keywords: Actinomycetes; Geosmin; MIB; Freshwater streams; Aquacultures; CARD-FISH; Microautoradiography

1. Introduction

In natural environments, members of *Actinobacteria* are typically characterized as soil bacteria or plant pathogenic bacteria [1], but recent studies indicate that

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actinobacteria can be abundant planktonic microorganisms in freshwater, constituting in some cases >60% of the bacterial community [2,3]. Actinobacteria include morphologically diverse Gram-positive bacteria with a high GC content and with highly variable metabolic and physiological capacities (www.prokaryotes.com). A large number of genera within *Actinobacteria* have been isolated from terrestrial environments [1], but it is unknown if the same genera are abundant in aquatic ecosystems. Actinomycetes constitute a major group of rod-shaped and filamentous actinobacteria, including *Streptomyces* and *Actinomyces*.

Most research on aquatic actinomycetes has focused on production of antibiotics [4–6] and cytotoxical substances [7] rather than on their biological activity in the aquatic environment. Another feature of actinomycetes is their production of odours, such as geosmin and 2-methylisoborneol (MIB) [8]. These odours cause a reduced water quality in areas where surface water is used for drinking water [9]. Geosmin and MIB have also been found to reduce the quality of fish in freshwater aquacultures as the odours penetrate and accumulate in the fish, e.g. in rainbow trouts [10] and hereby lower the commercial value of the fish [11].

Occurrence of geosmin and MIB in freshwater is often supposed to be caused by cyanobacteria, and supporting this, enhanced odour concentrations have coincided with high densities of cyanobacteria [12,13]. However, presence of geosmin and MIB during periods of low algal biomass, e.g., during the winter [14,15], contradicts that cyanobacteria are the only source of odours in freshwaters. The observations by Lanciotti et al. [15] indicate that actinomycetes, possibly in association with microalgae, were the major odour producers in the winter in Arno river, Italy.

Preliminary observations on occurrence of geosmin and MIB in Danish streams and freshwater aquacultures during winter without planktonic primary production [16], inspired us to examine seasonal variations in abundance of actinomycetes and concentrations of geosmin and MIB in three streams and aquacultures receiving water from these streams. For detection of the actinomycetes, fluorescence in situ hybridization (FISH) technique was chosen. Unfortunately, the genetic variation within actinomycetes is so wide that available rRNA oligonucleotide probes targeting all actinomycetes also include other members of the *Actinobacteria* group [2]. Densities of *Actinobacteria* was determined by FISH with a horseradish peroxidase-labelled probe and catalyzed reporter deposition (CARD) to amplify the fluorescence signal [2,3]. In order to demonstrate that the actinobacteria were metabolically active in the water, we combined microautoradiography with CARD-FISH [17]. Pure culture isolates were obtained and analyzed for geosmin and MIB production. Finally the capacity of the freshwater bacteria for degradation of geosmin and MIB was examined in laboratory studies.

2. Material and methods

2.1. Sampling

Water samples were collected in 3 different freshwater streams, Funder, Holtum and Vorgod, and aquacultures receiving water from these streams. The streams are located in Jutland, western Denmark. Funder is an oligotrophic clearwater stream, while both Holtum and Vorgod are eutrophic streams, receiving nutrients from the surrounding agricultural areas. Before entering the aquacultures, the Funder and Vorgod stream water is treated with lime to maintain a pH of about 8, while no water treatment is applied in the Holtum aquaculture. In the aquacultures, water is distributed via an inlet channel to a number of fish ponds (typically about 50). After passage in the ponds, the water is converged into a collecting channel and subsequently treated by mechanical and biological procedures to reduce the content of nutrients and organic matter. Finally, the water is returned to the stream.

Sampling in streams and aquacultures was performed at about 2-month intervals from summer 2002 to summer 2003 (August, September, October and December in 2002; March, May, June and July in 2003). Samples were collected in the streams and in 7 locations within the aquacultures (inlet channel, four selected ponds, collecting channel and outlet). Immediately after collection, [^{3}H]-thymidine was added to subsamples for measurement of bacterial production (see below). Additional subsamples were preserved with formaldehyde (2% final conc.) for total bacterial counts or ethanol (50% final conc.) for CARD-FISH, or were 0.2 μm membrane filtered for chemical analysis (see below). Samples for cultivation of bacteria on different media were brought to the laboratory in coolers (temperature of 3–6 °C) and processed the following day.

2.2. Bacterial production and abundance

Bacterial production was determined by incorporation of [^{3}H]-thymidine according to Fuhrman et al. [18], but applying the centrifugation procedure for bacterial incorporation of [^{3}H]-leucine by Smith and Azam [19]. Triplicate 1 ml water samples and a control with 2% formaldehyde (final conc.) were incubated in 2 ml test tubes for 30 min (120 min in March 2003). The incubations were terminated by addition of 100 μl 50% trichloroacetic acid (TCA), followed by addition of 20 μl 5% bovine serum albumin to visualize the pellet formation during the subsequent centrifugation at 14,000*g* for

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5 min. The pellet was washed with 1 ml 5% TCA and centrifuged. After discarding TCA, 1 ml of a commercial scintillation cocktail was added and the samples were finally radioassayed by liquid scintillation counting. Incorporated thymidine was converted to bacterial cell production using a conversion factor of 2.15×10^{18} cells per mole thymidine [20].

For determination of bacterial abundance, the formaldehyde-preserved samples were treated in a sonication bath for 20 s. to release attached bacteria. Bacterial densities were measured by flow cytometry, using a Becton Dickinson Calibur flow cytometer after staining with SYBR Green1 (Molecular Probes, USA). Briefly, 5 μl of 1:60 diluted fluorochrome and 100,000 fluorescent microspheres with a diameter of 2 μm were added to 500 μl sample. The number of particles within a characteristic and well-defined size region (fluorescence intensity vs. side scatter) was counted until 10,000 bacteria were acquired. The simultaneous counting of fluorescent microspheres was used to calculate the analyzed volume and determine of the bacterial density in the water samples. SD of replicate flow cytometer cell counts was determined to <5%.

2.3. Fluorescence in situ hybridization with catalyzed reporter deposition

Detection of actinobacteria was performed by CARD-FISH according to protocol by Sekar et al. [3]:

(1) Bacteria in the 5 ml ethanol-preserved samples were filtered onto 0.2 μm pore size 25 mm diameter polycarbonate filters (Poretics; www.osmolabstore.com), followed by washing with Milli-Q water (www.millipore.com) and air drying.
(2) A wedge corresponding to 1/8 of the filter area was cut from each filter. The filter pieces were embedded in low gelling point agarose (Metaphor, 0.2% conc.; FMC Bioproducts, Rockland, ME, USA) and air dried for 10 min.
(3) For permeabilization of the bacteria, the filter pieces were treated with lysozyme and achromopeptidase. The filter pieces were incubated in a fresh, buffered lysozyme solution (10 mg ml^{-1}, dissolved in 0.05 M EDTA at pH 8.0 and 0.1 M Tris–HCl at pH 7.4; Sigma–Aldrich, St. Louis, USA) for 60 min at 37 °C and were subsequently washed in Milli-Q water. The filter pieces were transferred to a fresh achromopeptidase solution (60 units ml^{-1}, dissolved in 0.01 M NaCl and 0.01 M Tris–HCl; Sigma–Aldrich) and incubated for 30 min at 37 °C. The filter pieces were finally washed in Milli-Q water, incubated in 0.01 M HCl to destroy bacterial enzymes, washed in Milli-Q water and 96% ethanol, and air dried.
(4) The permeabilized cells were hybridized with the 5′-HRP (horse radish peroxidase)-labelled oligonucleotide probe HG1-654 [2] targeting 16S rRNA of *Actinobacteria* (ThermoHybaid, Interactiva Division, Ulm, Germany). Hybridization buffer for the probe was 0.9 M NaCl, 20 mM Tris–HCl (pH 7.4), 10% dextran sulfate (wt/vol), 30% vol/vol formamide, 1% blocking reagent (Boehringer, Mannheim, Germany), and 0.05% Triton X-100 (vol/vol). The blocking reagent was prepared by dissolving in a maleic acid buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5) to a final concentration of 10% (wt/vol). The filter pieces were immersed in a mixture of 400 μl hybridization buffer and 4 μl of probe working solution (50 ng/μl) and incubated in a 1.5-ml test tube at 35 °C with a rotation shaker over night. Next day the filter pieces were transferred to 50 ml prewarmed washing buffer and incubated at 37 °C for 10 min. The washing buffer was 112 mM NaCl, 5 mM EDTA (pH 8.0), 20 mM Tris–HCl (pH 7.4), and 0.02% (wt/vol) sodium dodecyl sulfate.
(5) In preparation for the tyramide signal amplification step, the filter pieces were shortly dried on a blotting paper before incubation in PBS with 0.05% Triton X-100 for 15 min at low agitation. The filter sections were shortly dried and transferred to the tyramide substrate mix (2 μl Cy3- or fluorescein-labelled tyramide stock solution (1 μg μl^{-1}); NEN Life Science Products, Boston, MA, USA) and 200 μl diluted amplification buffer (PBS containing 0.0015% H_2O_2 and 0.1% blocking reagent) for 10 min at 37 °C. Finally, the filter sections were washed in the PBS–Triton solution (15 min), Milli-Q water (1 min) and 96% ethanol (1 min).
(6) Filter sections with hybridized cells were mounted on slides using low-quenching immersion oil and counted at 1000× magnification. Eight to 10 microscope fields, each with an area of 75×105 μm, and representing between 80 and 220 cells, were counted. In some of the samples the abundance of individual, hybridized cells inside filaments was determined and related to the number of unicellular, hybridized bacteria.

2.4. FISH without CARD technique

Numbers of *Actinobacteria* in the streams from September and December 2002 and March 2003 were determined by traditional FISH technique in February 2004. These samples were originally collected for enumeration of total cells densities only and were preserved with formaldehyde (see above). Attempts to process these samples with the CARD-FISH approach were not

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successful, but a modified, traditional FISH procedure, involving an enzyme pretreatment to enhance permeabilization of the cells, proved successful. Based on a series of laboratory tests, the following procedure was applied to the formaldehyde-preserved samples. Three millilitres of water sample was mixed with 3 ml 97% ethanol and filtered onto a 0.2 μm polycarbonate membrane filter. The bacteria were embedded in 0.2% agarose solution as above. For permeabilization of the cells, the filters were incubated in a fresh proteinase K solution (2.5 mg ml^{-1}, dissolved in 0.05 M EDTA at pH 8.0 and 0.1 M Tris–HCl at pH 7.4; Sigma–Aldrich) for 30 min at 37 °C. The filters were washed in Milli-Q water and incubated in 96% ethanol and air dried. The hybridization step was performed as described above in (4), except that a fluorescein-labelled HG1-654 probe was used (no HRP enzyme attached), the incubation temperature was 46 °C for 90 min, and temperature of the washing buffer was 48 °C. The filters were finally washed in Milli-Q water and ethanol before mounting on microscope slides.

When compared to fresh, formaldehyde-preserved bacteria in stream water, the hybridized bacteria in the stored samples were smaller and had lower fluorescence intensity. This probably reflects a degradation of rRNA and implies that the observed number of FISH-positive cells in these samples might underestimate the actual abundance of actinobacteria. Laboratory tests with cultivated actinomycetes indicated that short-term preservation (1 week) with formaldehyde and EtOH (as above) produced identical cell densities with the modified FISH technique and with CARD-FISH technique, respectively. The effect of long-term storage of actinobacteria in formaldehyde was not determined.

2.5. Isolation of actinomycetes

Bacteria in the streams and aquacultures were cultivated on agar plates (1/10 tryptic soy agar, TSA) after serial dilutions. Characteristic actinomycete colonies (grey-brownish colour and leathery surface) were picked after 8–10 days and transferred to new 1/10 TSA. Purity of the isolates were controlled by regrowth in liquid media (1/10 tryptic soy broth, TSB), followed by plating on 1/10 TSA. The isolates were used for measuring production of geosmin and MIB.

2.6. Sequencing of isolated actinomycetes

The isolates were grown in 25 ml Luria–Bertani (LB) media until visible turbidity. Cells were pelleted by centrifugation, and DNA was extracted using a commercial kit (FastDNA kit BIO101; www.qbiogene.com). The 16S rRNA gene was amplified using the universal primers 8F (5′-AGA GTT TGA TCM TGG CTC AG [21] and 1492R (5′-GGT TAC CTT GTT ACG ACT T [22]. PCR was carried out in 50 μl reactions with 2 mM MgCl, 0.2 mM of each primer, 2.5 U Taq polymerase (Sigma–Aldrich) and 0.125 mM dNTP each. Cycling conditions were: 1 min at 93 °C followed by 35 cycles of 30 s at 92 °C, 1 min at 57 °C and 45 s at 72 °C, with a final elongation step at 72 °C for 5 min.

Two PCR reactions were pooled and purified before sequencing using a commercial kit (Qiagen CR purification kit; www.qiagen.com) according to the manufacturer's instructions. Sequencing was performed in both directions (GATC Biotech, Germany) using the primers 341F (5′-CCT ACG GGA GGC AGC AG [21] or 8F sequencing in one direction, and 907R (5′-CCG TCA ATT CCT TTG AGT TT [23] for sequencing in the opposite direction. Obtained sequences were subject to a BLAST search [24] to affiliate the sequences to genus level.

2.7. MAR-FISH

Activity of actinobacteria in streams and collection channels was measured in May 2003 by combined microautoradiography (MAR) and CARD-FISH technique, using the MAR protocol by Nielsen et al. [25] and Lee et al. [26] with some modifications. Briefly, 4 ml water samples were incubated in 9 ml serum bottles with 20 μCi [^{3}H]-thymidine and unlabelled thymidine (or 20 μCi [^{3}H]-leucine and unlabelled leucine) to a final concentration of 50 μM for 5 h under aerobic conditions. The samples were fixed by addition of freshly prepared paraformaldehyde (PFA) in PBS to a final concentration of 4% and allowed to stand for 2 h at 4 °C. Excess radioactivity was removed by two centrifugation steps (10 min at 10,000*g*) and resuspension in sterile-filtered sample water. The samples were gently homogenized using a glass tissue grinder (Thomas Scientific®, USA) before being transferred to gelatine-coated cover glasses (24 × 60 mm) and allowed to dry at 50 °C. Hybridization with catalyzed reporter deposition (CARD-FISH) was carried out according to protocol by Pernthaler et al. [27] except that all steps were performed directly on the cover glass instead of filters. The applied probe was HG1-654 (see above). After hybridization, the cover glasses were carefully dipped in prewarmed (43 °C) LM-1 emulsion (www.amershambiosciences.com) and exposed at 4 °C for 7 days, followed by development (3 min) in Kodak developer L-19 as described by Lee et al. The MAR-CARD-FISH preparations were examined in a LSM 510 scanning confocal microscope (Carl Zeiss, Germany). The percentages of FISH-positive actinobacteria that assimilated thymidine and leucine were estimated by enumerating MAR-positive microcolonies within 200 FISH-positive cells on 2 cover glasses.

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2.8. Analysis of geosmin and MIB

Geosmin (*trans*-1,10,-dimethyl-*trans*-(9)-decalol) and MIB (2-methylisoborneol (exo-1,2,7,7-tetramethyl-[2.2.1]heptan-2-ol) were extracted from water samples by solid phase micro-extraction (SPME), using a 2 cm 50/30 μm StableFlex fibre (part number 57348-U) in a manual fibre holder (part number 57330-U), both from Supelco (Sigma–Aldrich). Water samples of 50 ml were gently decanted into 100 ml serum flasks in the field, followed by addition of 12 g NaCl (to increase the volatility of the odours) and a magnetic stir bar. Finally the flasks were tightly closed with silicone-PTFE caps. In the laboratory, the SPME fibre was injected into headspace of the sample flask, which was lowered into a 60 °C water bath for 15 min to release the odours. The magnetic stir bar was vigorously rotated to enhance the vaporisation. After the absorption, the fibre was desorbed for 3 min at 260 °C in the splitless injector of a gas chromatograph–mass spectrometer (GC–MS) (TRACE GC with Polaris GCQ, ThermoFinnigan, Austin, TX, USA). The GC column was 30 m × 0.25 mm id, 0.25 μm film thickness Rtx–5MS (Restek, Bellefonte, PA, USA) with He carrier gas at constant flow of 1.2 ml min^{-1}. The temperature program was 45 °C (3 min), 30 °C min^{-1} to 250 °C, 100 °C min^{-1} to 300 °C (2 min). Temperature of the transfer line and the ion source was 275 and 200 °C, respectively, and the scan range was 30 to 200 *m*/*z*. Quantification of geosmin and MIB was made by selective ion monitoring of the 112 and 95 *m*/*z* fragments, respectively.

Linearity and detection limit of the GC–MS procedure was tested using a standard series of MIB and geosmin of 10–100 ng l^{-1}. A mixture including both compounds was obtained from Supelco, product no. 47525-U (100 μg ml^{-1} in MeOH). Purity of the compounds was 98.2% (geosmin) and 99.1% (MIB). For the geosmin production experiments, standards up to 100,000 ng l^{-1} were applied. An acceptable linearity were obtained in all cases, with R^2 values >0.99. The analytical detection limit was estimated to 0.2 ng l^{-1}, and the precision for concentrations <30 ng l^{-1} was 6–9%.

2.9. Production of geosmin and MIB by isolated actinomycetes

Five ml of an actively growing culture (in liquid 1/10 TSB medium) was transferred to 4 × 20 ml liquid 1/10 TSB media in 100 ml sterile serum bottles containing a stir bar. The cultures were incubated at 28 °C on a shaking table. After 1 h, two of the bottles were transferred to an ice bath and were used to determine initial values of geosmin concentrations and bacterial density. The remaining two cultures were incubated for additionally 20 h. Geosmin and MIB concentrations were measured as above, except that no NaCl was added, as this would require opening of the serum bottles. To ensure that elimination of salt did not affect the concentration measurements, geosmin and MIB standards were prepared without salt. Following the geosmin/MIB analysis, the actinomycete cells were transferred to 20 ml centrifuge tubes and centrifuged for 5 min at 5000*g* to concentrate the cells.

In order to measure the cell-specific odour production by the bacteria, it was attempted to determine the number of actinomycete cells in each of the cultures. The cells were centrifuged, resuspended in 2 ml water and homogenized in a micro tissue grinder with about 10 μm distance between pestle and grinder tube (Kontes Glass Company, Vineland, NJ, USA). A microscopic analysis of cells stained with SYBR Green1 (as above) demonstrated, however, that it was extremely difficult to enumerate individual cells in the entangled filaments. An exception was culture 4M in which individual cells inside the filaments were identifiable and countable. To enumerate the cell density in the remaining actinomycete cultures, a relationship between cell density and fluorescence intensity of culture 4M, after labelling with SYBR Green1, was applied. Subsamples of homogenized actinomycete filaments were stained with SYBR Green1 and analyzed in a fluorescence spectrophotometer at excitation and emission wavelengths of 495 and 537 nm, respectively, according to specifications by Molecular Probes. The cell-specific fluorescence of the 4M culture was used for calculation of a fluorescence-to-cell conversion factor and used for estimating the cell number in the other actinomycete cultures. For calculation of the cell-specific geosmin production, a mean cell number between start ($t = 1$ h) and end of the incubation ($t = 21$ h) for each of the isolates was determined.

2.10. Degradation of geosmin and MIB by indigenous stream bacteria

Degradation of geosmin and MIB, or geosmin alone, was studied in two laboratory experiments. In both experiments, 50 ml volumes of Holtum Stream water in 100 ml serum bottles with a stir bar were enriched with the odours and incubated for 8 days (geosmin and MIB at 50 ng l^{-1}) or 35 days (geosmin at 1000 ng l^{-1}) on a shaking table at 150 rpm. Additional serum bottles were further enriched with 50 μl pure ethanol or 75 μl LB medium to stimulate the bacterial alcohol dehydrogenase activity [28] and the overall bacterial activity. At the end of the incubations, geosmin and/or MIB was measured by SPME as above. In the short-term experiment (8-day duration), no NaCl was added to the bottles before absorption to the fibre, while NaCl quickly was added before absorption of the 35-day experiment.

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2.11. Other chemical analyses

Total dissolved nitrogen (TDN) was measured in 0.2 μm membrane filtered water from the sampled streams and fish ponds by chemiluminescence after conversion to NO_x [29]. Concentrations of TDN were used as indicators of trophic level in the water.

3. Results and discussion

3.1. Total dissolved nitrogen and abundance and production of bacteria in the streams

The trophic level in the three streams differed significantly, as exemplified by the concentration of total dissolved nitrogen (TDN) (Fig. 1, upper panel). In Funder, TDN concentrations were 50–70 μM N, while 230–480 μM N were measured in the other two streams. In Funder, similar amounts of ammonia and nitrate occurred and made up about 80% of TDN, while nitrate was the dominant N compound in the other two streams and made up about 75–85% of TDN (own unpublished data).

Bacterial density was lowest in Funder (0.8 to 3.6×10^9 l^{-1}) as compared to Holtum (2 to 10×10^9 l^{-1}) and Vorgod (1 to 8×10^9 l^{-1}) (Fig. 1, middle panel). The highest densities occurred in spring and summer. The bacterial production to some extent reflected the bacterial densities, with the lowest rates in the winter, but large fluctuations were observed. Annual variations in the production rates were 2.3 to 35×10^6 cells l^{-1} h^{-1} (Funder), 3 to 64×10^6 cells l^{-1} h^{-1} (Holtum) and 3 to 113×10^6 cells l^{-1} h^{-1} (Vorgod) (Fig. 1, lower panel).

3.2. Seasonal abundance of actinobacteria in the three streams

Occurrence of actinobacteria in the streams varied from 3.9×10^7 l^{-1} (Vorgod Stream, December 2002) to 37.4×10^7 l^{-1} (Funder Stream, July 2003) (Fig. 2). Actinobacteria were most abundant in the Funder Stream, both with respect to average density (17.3×10^7 l^{-1}) and proportion of the total bacterial populations (average of 9.2%), as compared to Holtum Stream (16.6×10^7 l^{-1} and 3.1%) and Vorgod Stream (15.8×10^7 l^{-1} and 3.1%).

The seasonal variations indicate a lower abundance of actinobacteria in the winter season (September to May) as compared to June and July. In contrast, the relative abundance of actinobacteria to all bacteria was

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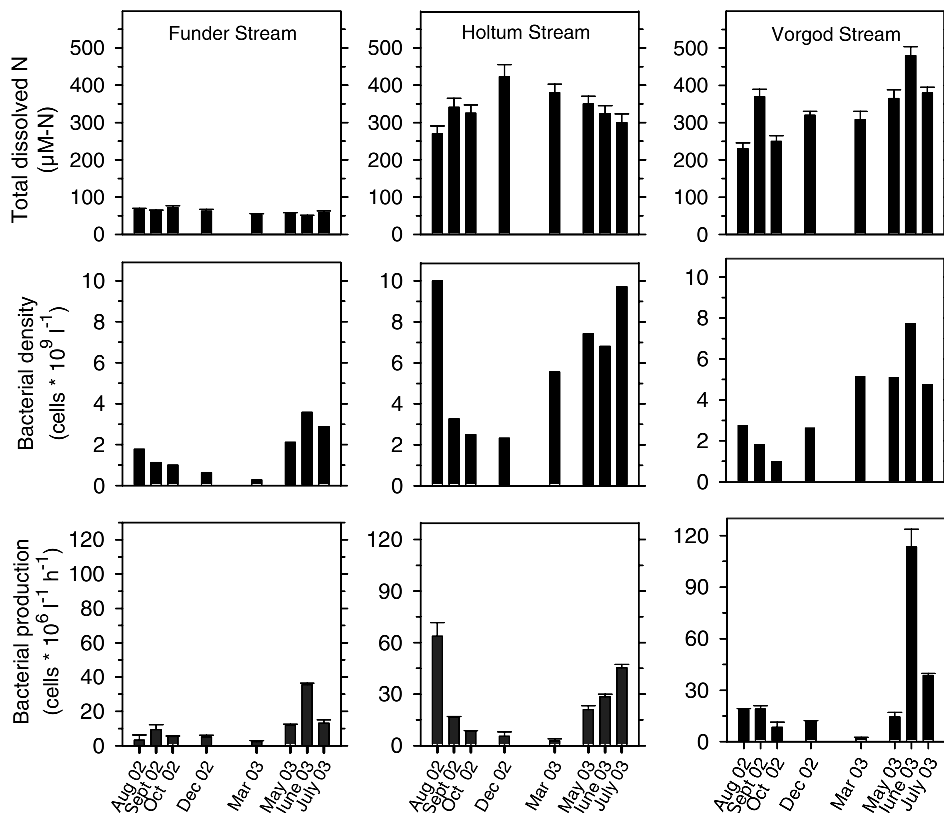

Fig. 1. Background information on the three streams. Concentrations and seasonal changes in total dissolved nitrogen (upper panel), bacterial density (middle panel) and bacterial production (lower panel; thymidine incorporation). Means ± 1 SD of three replicates shown, except for the bacterial numbers (SD was <5%; not shown).

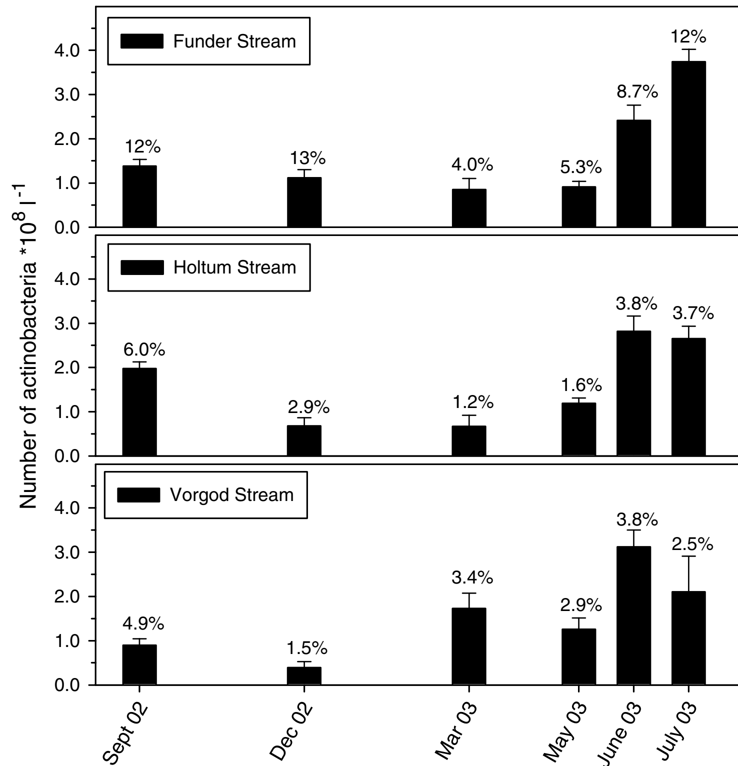

Fig. 2. Number of actinobacteria (CARD-FISH-positive cells) in the three streams. Abundance of actinobacteria relative to the total bacterial densities is shown above the columns. Error bars indicate SE of the cell numbers.

highest in September (Holtum Stream (4.9%) and Vorgod Stream (6%)), or in December (Funder Stream (13%)). It should be mentioned that this seasonal difference may be biased by the two procedures used for enumeration of the actinobacteria. In September and December 2002 and March 2003, actinobacteria were hybridized by traditional FISH technique, while CARD-FISH technique was applied in May, June and July 2003 (see Section 2).

3.3. Abundance of unicellular and filamentous actinobacteria in streams and aquacultures

The proportion of unicellular and filamentous CARD-FISH positive cells was analyzed in the three streams and connecting aquacultures (collecting channels). Microscope photographs of the two morphological cell types at one of the localities, Holtum Stream, are shown in Fig. 3 (upper panel).

The number of the two cell types at the 3 locations together ranged from 0.18 (Funder Stream) to 0.22×10^9 cells l^{-1} (Funder collecting channel), and constituted from 3.6% (Holtum Stream) to 8.1% (Funder collecting channel) of all bacteria in the water (Fig. 3). Filamentous forms among the actinobacteria ranged from 11% to 20% in the eutrophic Vorgod and Holtum streams and aquacultures, but in the oligotrophic Funder Stream and aquaculture, the filamentous forms made up 38%. The average proportion of filamentous actinobacteria at the 3 locations was 22%. Relative to the total bacterial densities in the streams, filamentous actinobacteria constituted from 0.5% (Holtum Stream) to 3.1% (Funder Stream).

During passage of stream water in the Holtum fish ponds, the number of actinobacteria increased by 16% ($p < 0.05$, t-test). Similarly, abundance of filamentous forms increased from 14% (stream) to 20% (collecting channel) ($p < 0.05$, t-test) in this aquaculture. No statistically significant changes in morphological forms were found in the other two aquacultures.

The proportion of actinobacteria in the streams and aquacultures (0.2–9.1% of the bacteria were actinobacteria) was lower than the proportion of 15–60% found in two alpine lakes [2], but it supports that actinobacteria can be abundant aquatic microorganisms. Actinobacteria have typically been considered as terrestrial microorganisms [30], except for some foam-forming species in activated sludge [31]. The applied FISH probe targeted all actinobacteria and did not provide specific information on abundance of actinomycetes in the water. Separation of CARD-FISH positive cells into unicellular and filamentous morphological types indicated that on the average 22% of the actinobacteria were filamentous forms. Since morphology of the filamentous types closely resembled that of filamentous actinomycetes (short, twisted filaments with irregular bends), we assume

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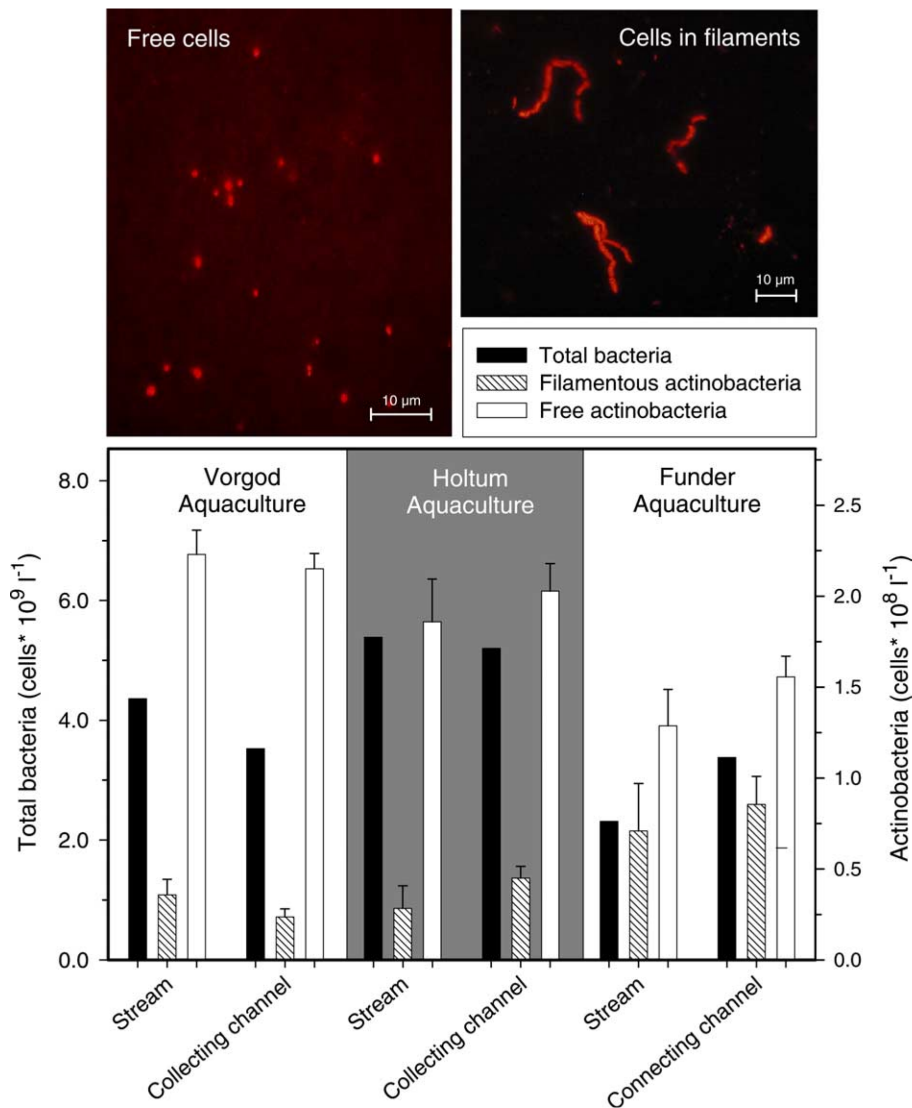

Fig. 3. Microscope photographs of free and filamentous actinobacteria (CARD-FISH positive cells) in Holtum Stream (upper panel). Total bacterial densities and abundance of free and filamentous actinobacteria in the three streams and collection channels of aquacultures connected to the streams (lower panel). Sampling date was May 5th 2003. Error bars indicate SE of the CARD-FISH positive cells. SD of the total bacterial counts were <5% (not shown).

CARD-FISH positive filaments in our samples to be actinomycetes. Filamentous actinobacteria made up from 0.5% (eutrophic streams) to 3.1% (oligotrophic stream) of bacteria in the streams and aquacultures. The actual abundance of actinomycetes might have been higher, if unicellular actinomycetes also were common. It should be mentioned that Glöckner et al. [2] suspect that the applied FISH probe (HG1-654) can make cross reactions with some members of the *Cytophaga*-*Flavobacter* group, but the extent of such cross hybridizations and their implication to aquatic environments have not been studied (F.O. Glöckner, pers. com.).

Isolation of actinomycetes has often been performed by embedding in water agar [32]. In initial studies, we cultivated bacteria from streams and aquacultures in water agar (5% agar in water, enriched with mineral salts) to determine the density of actinomycetes. The maximum number of actinomycetes colonies (identified by morphology after 3–4 weeks) obtained by this technique was 2200 CFU ml^{-1} (unpublished results). Relative to the presently estimated density of actinomycetes by FISH technique ($10–60 \times 10^6$ l^{-1}, assuming that 22% of the actinobacteria were actinomycetes (see above)), the traditional water agar cultivation seriously underestimated the abundance of actinomycetes in the streams.

3.4. Activity of actinobacteria in streams and aquacultures

MAR-CARD-FISH analysis of bacteria in streams and collecting channels of the aquacultures showed that the fraction of metabolically active actinobacteria

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increased after passage through the aquacultures. Depending on whether DNA or protein synthesis was the target of the applied radioisotope, the proportion of active actinobacteria in the streams varied from 10% to 65% (Table 1). Actinobacteria in the oligotrophic Funder stream had the lowest proportion of active cells with both isotopes, as compared to the two eutrophic streams.

In the aquaculture collecting channels, the proportion of active actinobacteria was significantly higher than in the streams. Incorporation of [^{3}H]-thymidine showed that the number of active actinobacteria had increased 2- to 3.7-fold, and incorporation of [^{3}H]-leucine indicated a 0.2- to 2-fold higher proportion of active cells (Table 1). Most bacteria (76–98%) in the two aquacultures receiving eutrophic stream water were active, while only 37–71% were active in the oligotrophic Funder ponds. Microscope photographs of a MAR-CARD-FISH preparation are shown in Fig. 4.

The fish (rainbow trouts) are fed dry pellets consisting of lipids, proteins, carbohydrates and cellulose fibres (www.biomar-fishfeed.com). Uneaten food pellets, faecal matter and fish debris enrich the pond water with organic matter and stimulate the bacterial activity. As an illustration of the higher nutrient content in the ponds, the amount of dissolved nitrogen typically increased by 20–70% during passage (own unpublished data). The substances stimulating the DNA and protein synthesis by actinobacteria in the aquacultures are unknown. Within *Actinobacteria*, actinomycetes are known to produce a large variety of hydrolytic enzymes [33,34] and possibly, degradation of complex organic matter caused the observed stimulation of the bacterial activity.

Despite their high capability for producing hydrolytic enzymes, actinomycetes and other actinobacteria appear not to belong to the fastest growing microorganisms in aquatic environments. When manipulating the content or dissolved organic matter in humic lake water, Burkert et al. [35] observed that actinobacteria were outcompeted by faster growing species. Our observations of an increasing activity of actinobacteria in the aquacultures contradict that actinobacteria are slow-growing, but specific actinobacteria-stimulating nutrient conditions may have prevailed in the fish ponds. An additional effect of actinomycetes in the studied freshwater systems may be production of antibiotics as observed by marine actinomycetes [5,6]. Speculatively, antimicrobial compounds produced by actinomycetes may give these bacteria a competitive advantage in the streams and aquacultures.

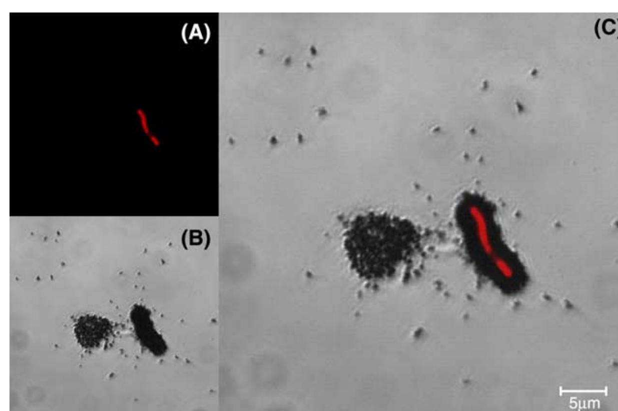

Fig. 4. Micrographs showing combination of MAR- and CARD-FISH-positive cells of water sample from the oligotrophic Funder Stream. (A) CARD-FISH image showing a bacterium hybridized with the HG1-654 probe (red colour) and (B) the same field with a bright-field image of MAR (incubated with [^{3}H]-thymidine). (C) shows the overlay of A and B.

3.5. Occurrence of geosmin and MIB in streams and aquacultures

The lowest geosmin concentrations were measured in the oligotrophic Funder stream (1.0–2.4 ng l^{-1}), while higher concentrations occurred in the eutrophic Holtum and Vorgod streams (2–6 ng l^{-1}, except for 12 ng l^{-1} in June 2003) (Fig. 5). In Funder Stream, maximum concentrations occurred in December and March, but in the other two streams the lowest concentrations occurred in these months. This suggests that no general seasonal trends in occurrence of geosmin can be expected. Concentrations of MIB varied between 2 ng l^{-1} (Vorgod Stream, December 2002) to 9.6 ng l^{-1} (Holtum Stream, July 2003). Despite the relative few measurements (no samples for MIB analysis were taken in August and September 2002), the results suggest a seasonal variation with lower concentrations in winter than summer, except for a low concentration (2.5 ng l^{-1}) in Vorgod Stream in May.

Table 1
Proportion of MAR-CARD-FISH-positive actinobacteria in streams and aquacultures after incubation with [^{3}H]-thymidine or [^{3}H]-leucine

Location	Vorgod		Funder		Holtum	
	Stream (%)	Collecting channel (%)	Stream (%)	Collection channel (%)	Stream (%)	Collecting channel (%)
Substrate						
[^{3}H]-thymidine	34	96	10	37	38	79
[^{3}H]-leucine	45	98	41	71	65	76

A total of 200 CARD-FISH-positive cells were counted from each sample. Samples were collected May 5th 2003.

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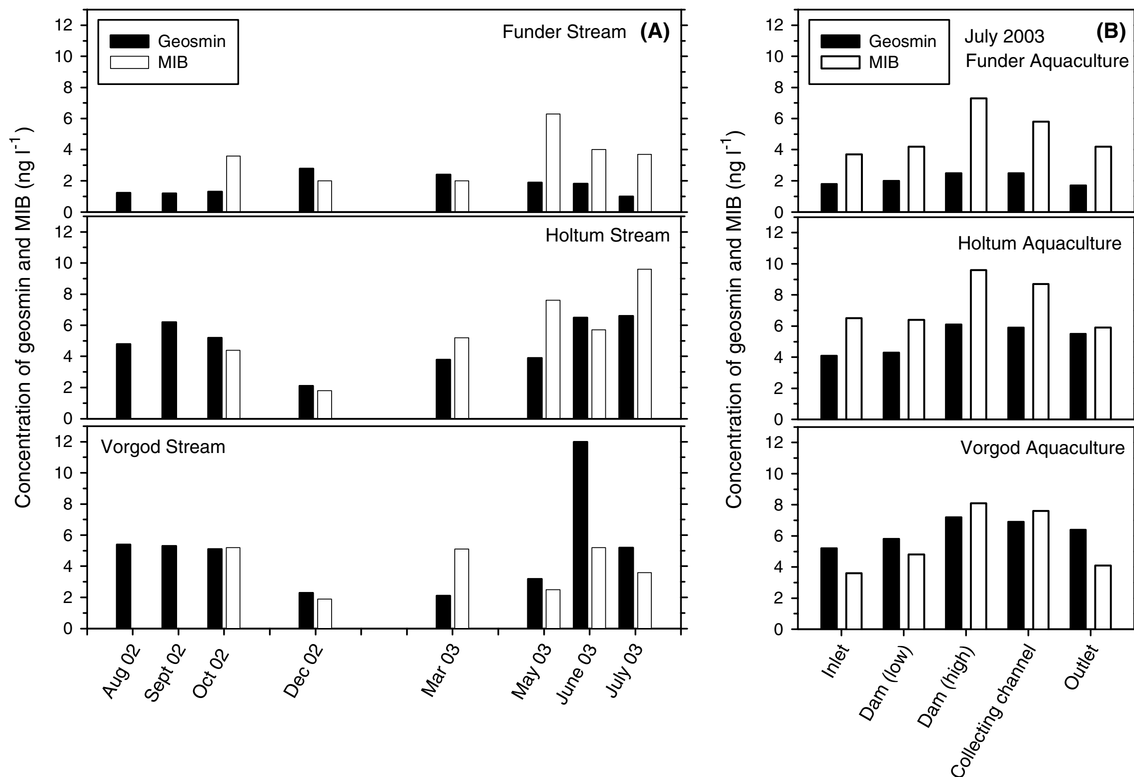

Fig. 5. Concentrations of geosmin (August 2002 to July 2003) and MIB (October 2002 to July 2003) (A) and changes in geosmin and MIB in stream water during passage in the aquacultures (B). Four dams were analyzed for content of geosmin and MIB, but only concentrations in dams with the lowest and the highest amounts of odours are shown. Concentrations of geosmin and MIB were determined from single injections.

In the aquacultures receiving water from the three streams, a production of both geosmin and MIB was measured during passage of the water in ponds and collection channels. The concentration of the two odours increased up to 55% (geosmin) and 110% (MIB) (Fig. 5). Before discharge of pond water back into the streams, treatment of the water (combined aeration and biological degradation) reduced the geosmin and MIB concentrations.

The observed concentrations of geosmin and MIB correspond to levels measured in eutrophic lake water, e.g., in Lake Zurich (only geosmin was studied) [14] and in the Arno River, Italy [15]. In freshwater with a high primary production, cyanobacteria may be more important than actinomycetes in producing odours [13]. Supporting this, removal of a cyanobacterial-rich biofilm on water canal walls in Arizona significantly reduced the odours in the water [12], and during a high zooplankton grazing on cyanobacteria in Lake Zurich, geosmin increased from 3 to 21 ng l^{-1} [14]. But in periods with a low occurrence of cyanobacteria in the Arno River, geosmin and MIB were assumed to originate from actinomycetes [15]. In the Danish streams, pigment analysis of phytoplankton >0.7 μm showed that cyanobacteria only were present at low densities in the two eutrophic streams in late August 2002 (zeaxanthin/chlorophyll *b* ratio; W. Martinsen, personal communication). Thus, except for short summer periods, we assume actinomycetes to be the dominant producers of geosmin and MIB in the streams.

The higher concentration of geosmin and MIB in the aquaculture ponds than in the streams shows that odours were produced in the ponds. The increased metabolic activity of actinobacteria in the ponds relative to the streams, lends support to actinomycetes being responsible for the odour production. The lower odour concentration in the outlet channels (Fig. 5) probably does not indicate a degradation of geosmin, as this is a slow process (see below). Rather, sprinkling of pond water in the cleaning procedure before discharge back into the stream, reduced the odour content (geosmin and MIB are both volatile substances). The level of geosmin and MIB in the aquacultures does not exceed that observed in surface water used for drinking water [9], yet the odours penetrate and accumulate in the rainbow trouts and reduce their commercial value [11].

3.6. Identification of isolated actinomycetes

Based on sequencing of the 16S rRNA gene, 8 actinomycetes isolated from the streams and aquacultures were identified to the genera *Streptomyces* (4 isolates;

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no species identity was obvious), *Streptomyces intermedius* (one isolate), *Streptomyces rubrogriseus* (one isolate) and *Rothia* sp. (one isolate) (Table 2). The BLAST analysis showed that three of the *Streptomyces* sp. isolates had a similar 16S rRNA gene sequence. One of the isolates, 1NV, did for unknown reasons not produce a positive PCR product with the universal primers 8F and 1492R.

3.7. Production of geosmin and MIB by isolated actinomycetes

Accumulation of geosmin and MIB in liquid cultures of the 8 isolated actinomycetes showed a variable production of odours. Geosmin was produced by all actinomycetes, but three of the isolates had no production of MIB (2B, 2M and 1EJ). The calculated cell-specific production of geosmin varied from 0.13 to 35 ag bacterium^{-1} h^{-1} (Table 3). Comparable rates were found for MIB, but the maximum rate was 15 ag MIB bacterium^{-1} h^{-1}. The highest production of the two odours occurred by the same species, isolate 2R. Three of the 5 MIB-producing actinomycetes had a higher production of MIB than of geosmin.

Sequencing of 16S rRNA genes indicated a complete similarity between isolate 2M, 4M and 2R, but these isolates varied significantly with respect to odour production. Isolate 2M only produced geosmin, and isolates 2M and 4M had a >7-fold (MIB) and >70-fold (geosmin) difference in odour production, despite the isolates grew in the same media.

We are not aware of previous results on cell-specific geosmin or MIB production by actinomycetes. Instead we estimated the geosmin production rate by two streptomycetes from published data. Assuming a cell dry weight of 10^{-12} g, the geosmin production by *Streptomyces citreus* was estimated to 0.1 ag bacterium^{-1} h^{-1} (data in Fig. 1 by Pollak and Berger [36]), while *Streptomyces tendae* had a rate of 0.3–0.6 ag bacterium^{-1} h^{-1} (data in Table 2; Dionigi et al. [37]). Despite differences in growth media and cultivation types (*S. citreus* was grown in liquid media and *S. tendae* was grown on agar plates), these calculated rates are within the range observed in our study.

Table 3
Cell-specific production of geosmin and MIB by 8 isolates during a 21-h incubation period

Isolate	Geosmin (ag bacterium^{-1} h^{-1})	MIB (ag bacterium^{-1} h^{-1})
1B	0.83	0.45
2B	0.14	nd
1NV	0.17	1.24
2M	2.38	nd
3M	0.12	0.16
4M	0.47	2.03
2R	34.85	15.34
1EJ	6.34	nd

Geosmin and MIB concentrations were determined from single injections.
ag, atto gram; nd, no production of geosmin or MIB detected.

It is unknown to which extent geosmin production is influenced by differences in cultivation conditions, e.g. type and concentration of media, as well as temperature. Different growth media significantly changed the geosmin production by *S. tendae* [37]. In contrast, Schrader and Blevins [38] observed that substances which stimulated the growth of *Streptomyces halstedii*, such as mannitol and galactose, only in some cases lead to a higher geosmin synthesis. When *S. tendae* was grown on media that did not introduce sporulation, no geosmin was produced [37]. In support of a relation between sporulation and geosmin synthesis, Gust et al. [39] found that mutations in sesquiterpene synthase (involved in the initial steps in geosmin synthesis) in *Streptomyces* also reduced the sporulation. Since sporulation is expected to occur at insufficient nutrient conditions [1], a higher geosmin synthesis may also be expected when nutrients are low. Therefore, the dilute nutrient media used in our cultures (0.4 g TSB l^{-1}) as compared to the medium for *S. citreus* (mixture of glucose, malt, yeast extract and asparagine to a total of 25 g l^{-1}; [36]) may speculatively explain the significantly higher geosmin production by our isolates than by *S. citreus*.

Temperature effect on geosmin synthesis in *Streptomyces* was studied by Aoyama et al. [40] who found that the geosmin production by three *Streptomyces* species was temperature dependent and had a lower optimum temperature than cell growth. Aoyama et al. observed

Table 2
Results from BLAST search on 16S rRNA gene sequences of 8 isolates

Isolate	Number of nucleotides	Affiliation	Closest hit in GenBank (Accession No.)	Similarity (%)
2M	1280	*Actinomycetales*	*Streptomyces* sp. 40006 (AY295794)	100
3M	1279	*Actinomycetales*	*Streptomyces intermedius* (Z76686)	99
4M	1280	*Actinomycetales*	*Streptomyces* sp. 40006 (AY295794)	100
2R	1122	*Actinomycetales*	*Streptomyces* sp. 40006 (AY295794)	100
1EJ	1105	*Actinomycetales*	*Streptomyces* sp. VK-A60 (AY079156)	100
1B	1309	*Actinomycetales*	*Rothia*-like sp. CCUG 35957 (AJ131121)	98
2B	439	*Actinomycetales*	*Streptomyces rubrogriseus* (AF503501)	99

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a similar temperature relation for production of antibiotics by *Streptomyces*.

The ratio between production of geosmin and MIB by the five actinomycetes in our study was 0.14–2.3. This is more variable than geosmin/MIB production rates of 1.0–1.4 measured by 40 actinomycetes isolated from a lake sediment by Sugiura and Nakano [41]. It is doubtful if biochemical pathways for synthesis of geosmin and MIB are related, as some actinomycetes only produce one of the odours in this study. Production of only one odour was also found in a Canadian River, in which 47% of the isolated actinomycetes produced both odours, whereas only geosmin or MIB was produced by 35% and 6%, respectively, and 12% did not produce any of the odours [42].

Three of the actinomycete isolates (2M, 4M and 2R) in our study had similar 16S rRNA gene sequences, yet their production of geosmin and MIB varied considerably. Whether this divergence in odour production reflects geno- or phenotypic differences among the isolates is unknown. The synthesis of geosmin and other sesquiterpene derivatives by *Streptomyces* require several enzymatic reactions, but knowledge on genes encoding these enzymes and their control is limited [39,43].

3.8. Degradation of geosmin and MIB in streams and aquacultures

Capacity by indigenous stream bacteria for degradation of geosmin was tested by addition of 1000 ng geosmin l^{-1} to Holtum Stream water. After 35 days, geosmin was reduced to 110 ng l^{-1}in the untreated stream water, while 179 and 16 ng l^{-1} remained in the samples enriched with ethanol and LB medium, respectively (Table 4). In the natural, untreated stream water, geosmin was reduced from 1.3 to 0.6 ng l^{-1} during the period. The geosmin content in the control (Milli-Q water with 1000 ng geosmin l^{-1}) declined to 891 ng l^{-1} during the incubation. This reduction may reflect leakage during the 35-day period as well as loss during opening of the bottles for addition of NaCl before the SPME absorption. A similar loss may be expected to have occurred in the other bottles.

To address more realistic concentrations of the odours, geosmin and MIB were added to the Holtum Stream water at 50 ng l^{-1} and incubated for a shorter period (8 days). A reduction of geosmin was only observed in stream water enriched with LB medium (Table 4). In contrast, MIB was reduced to 18–23 ng l^{-1} in all stream samples enriched with MIB. No changes in geosmin and MIB were found in the untreated stream water during the incubation.

In laboratory cultures, bacteria belonging to *Actinobacteria*, among these the actinomycete *Rhodococcus*, have been found to degrade geosmin [44]. In another study, MIB was degraded by *Pseudomonas* sp. and *Enterobacter* sp. [45]. Since both *Actinobacteria* and *Pseudomonas* are abundant microorganisms in nature, geosmin and MIB might be expected to be degraded in natural waters. Supporting this, a slow geosmin degradation was measured in a biological filter containing lake bacteria, and the degradation was further stimulated by enrichment with ethanol [28]. The effect of ethanol could be activation of alcohol dehydrogenase. This enzyme might also promote degradation of the geosmin and MIB as both compounds are alcohols.

Our observations support that aquatic bacteria can degrade geosmin and MIB, but a stimulatory effect was only observed for LB media, not for ethanol. The effect of LB enrichment suggests that the most important factor in stimulating degradation of geosmin is a high bacterial activity. The slow degradation of geosmin and MIB by indigenous bacteria in non-enriched stream water indicates that microbial removal of the odours from water bodies with high odour content and a short residence time, e.g., reservoirs for drinking water and freshwater aquacultures, most likely will be insignificant. Stimulation of the bacterial activity by nutrient enrichment may increase the odour degradation, but it may also increase the odour production as observed in the present aquacultures. The most efficient process for removal of geosmin and MIB probably is by mechanical procedures (aeration) or combined physical and

Table 4
Degradation of geosmin and MIB by Holtum Stream bacteria during 35 days (geosmin only; added concentration was 1000 ag l^{-1}) and 8 days (geosmin and MIB; added concentrations were 50 ag l^{-1})

	Concentration after 35 days	Concentration after 8 days	
	Geosmin (ng l^{-1})	Geosmin (ng l^{-1})	MIB (ng l^{-1})
Stream water (no additions)	0.6 ± 0.3	3.0 ± 0.4	6.3 ± 0.7
Stream water + geosmin/MIB	110 ± 9	44.2 ± 7.1	21.8 ± 5.1
Stream water + geosmin/MIB + EtOH	179 ± 29	43.6 ± 6.9	18.0 ± 5.4
Stream water + geosmin/MIB + LB	16.8 ± 4.3	30.4 ± 6.2	23.0 ± 4.8
Milli-Q water + geosmin/MIB	891 ± 18	41.5 ± 0.9	43.4 ± 7.8

In the 35-day experiment, the initial, natural concentration of geosmin was 1.3 ± 0.3 ng l^{-1}. In the 8-day incubations, no changes in the natural geosmin and MIB content was found. Error bars represent deviation from duplicate measurements (one injection from each of two bottles).

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chemical approaches such as the recently developed TiO_2 catalyzer with UV-irradiation [11].

3.9. Conclusions

Until recently, actinobacteria have not been considered abundant organisms in aquatic environments, but our study support that they can be commonly occurring bacteria in freshwater. Filamentous actinobacteria in the streams constituted about 3% of the bacterial populations and were assumed to be actinomycetes. Actinomycetes isolated from the streams and aquacultures were all found to produce geosmin and some also produced MIB. Despite the low number of isolates studied, actinomycetes seem to have a major impact on the quality of stream water for aquaculture purposes due to their odour production. The extremely variable odour production by the isolated actinomycetes, even in isolates with identical 16S rRNA sequences, demonstrated that these bacteria possess a wide diversity in cellular functions. The stimulation of the metabolic activity in actinomycetes during passage in the nutrient-enriched aquacultures indicates that aquatic actinomycetes may promote degradation of organic matter and hereby help improving the water quality. In our study, the application of a gene probe targeting all actinobacteria did not allow a quantification of the actual densities of actinomycetes in the water. In future studies of aquatic actinomycetes, gene probes with a narrower taxonomic specificity should be used, if occurrence, biology and taxonomy within this interesting group of bacteria should be characterized.

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