Detection of aquatic streptomycetes by quantitative PCR for prediction of taste-and-odour episodes in water reservoirs

Jeanette E. Lylloff, Maria Hummelshøj Mogensen, Michele A. Burford, Louise Schlüter and Niels O. G. Jørgensen

ABSTRACT

Bacteria belonging to the *Streptomyces* genus are known to produce several taste-and-odour compounds (TOCs), but knowledge on the abundance of streptomycetes in drinking water reservoirs and other aquatic environments is scarce. In this study, quantitative real-time polymerase chain reaction (qPCR) was applied, for the first time, to determine densities of streptomycetes in a river, at a weir and in two reservoirs in subtropical Australia. The PCR approach was optimized with respect to (a) collection of streptomycetes in water, (b) extraction of DNA, and (c) a procedure to correct for inhibition of PCR amplification by natural substances in the water. Mean densities of *Streptomyces* cells at the study sites varied from 225 to 45,650 cells L^{-1}. The highest density occurred in bottom water (8.5 m deep) of one of the reservoirs, while densities in the Brisbane River varied between 260 and 7,950 cells L^{-1}. At the weir site, seasonal variation in abundance in winter and spring in surface water (mean densities of 430–13,550 cells L^{-1}) did not correlate with total bacterial abundance (0.9–3.5×10^9 cells L^{-1}). The qPCR approach shows that quantitation of streptomycetes in fresh water can be successfully achieved and may prove valuable in predicting TOC episodes in aquatic systems used for drinking water supplies.

Key words | drinking water reservoirs, geosmin, MIB, quantitative PCR, *Streptomyces*

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INTRODUCTION

Globally, taste-and-odour compounds (TOCs) produced by microorganisms reduce the availability and quality of surface water for drinking (Srinivasan & Sorial 2011). Commonly found TOCs in surface water are geosmin (earthy flavour) and 2-methylisoborneol (MIB; mildewed flavour) (Westerhoff *et al.* 2005). The TOCs are not removed by standard treatment for drinking water production but require supplementary treatment, e.g. activated carbon or biological filters (Ho *et al.* 2007). Geosmin and MIB are non-toxic, but their role in causing off-taste, even in low amounts, makes drinking water produced from surface waters non-palatable by many consumers.

TOCs are produced by several species of cyanobacteria, especially by filamentous forms, and by non-phototrophic bacteria belonging to the *Streptomyces* genus (Zaitlin & Watson 2006). Cyanobacteria are probably dominant TOC producers in fresh waters and reservoirs exposed to light, while streptomycete bacteria appear abundant in lakes in aerobic, organic-rich environments, such as the water–sediment interface (Klausen *et al.* 2004) and biofilms (Zaitlin & Watson 2006).

A major obstacle in studying the abundance of *Streptomyces* and their potential as TOC producers is the lack of knowledge on their abundance in aquatic environments. Traditional culture techniques are still widely used for enumeration of actinomycetes (a commonly used pseudonym for *Streptomyces* and other filamentous *Actinobacteria* (Stackebrandt *et al.* 1997)) in natural environments. For

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example, using agar plate cultures, the abundance of actinomycetes in running waters was determined to be 18×10^3 colony-forming units (CFU) L^{-1} in the Italian Arno River (Lanciotti *et al.* 2003) and 200×10^3 CFU L^{-1} in a Korean stream (Lee *et al.* 2011), while Zaitlin *et al.* (2003) found up to 60×10^3 CFU L^{-1} in water of Lake Ontario, Canada. Using a FISH technique (fluorescence *in situ* hybridization targeting the 16S ribosome), densities of filamentous *Actinobacteria* (assumed to mainly represent *Streptomyces* species) in water of aquaculture ponds and reservoirs were determined to be 10–$2{,}000 \times 10^6$ cells L^{-1} (Klausen *et al.* 2005; Nielsen *et al.* 2006). Although *Streptomyces* was not specifically identified in these studies, the high densities of filamentous *Actinobacteria* quantified by fluorescence *in-situ* hybridization (FISH) technique, relative to the culture approach, suggest that culture methods may underestimate the abundance of *Streptomyces* in aquatic environments.

Polymerase chain reaction (PCR)-based methods have not yet been applied for quantification of *Streptomyces* in aquatic environments, but the abundance of *Streptomyces* in soils has recently been determined by a quantitative PCR (qPCR) approach (Schlatter *et al.* 2010). Quantification of microorganisms in natural environments by PCR amplification may require adjustments of the experimental protocol, relative to their use in laboratory cultures. In particular, the DNA extraction efficiency must be optimized to obtain maximum template (sample) DNA, and the impact of natural substances that may inhibit the PCR amplification, such as humic and phenolic compounds and heavy metals (Wilson 1997; Schriewer *et al.* 2011), must be examined.

In this study, a qPCR protocol was applied to determine the abundance of *Streptomyces* in water reservoirs in subtropical Queensland, Australia. Streptomycetes were detected by the TaqMan PCR method (Rintala & Nevalainen 2006). Briefly, the TaqMan reaction combines primer and probe technology to form a fluorescent product in proportion to the target DNA (23S rRNA gene). To test for possible interference on the PCR amplification by substances in DNA extracted from the water samples, a plasmid-based assay was developed and validated. The abundance of *Streptomyces* was related to concentrations of geosmin and MIB in selected samples to test for a possible relationship between these potential TOC-producing streptomycetes and concentrations of TOCs in the water, e.g. for future monitoring of TOC episodes in water reservoirs.

MATERIAL AND METHODS

Sampling

Water samples were collected in the mid-Brisbane River and eight adjoining creeks, Mt Crosby Weir on the mid-Brisbane River, and Little Nerang and Hinze reservoirs in southeastern Queensland, Australia in the austral autumn (April) and from winter to spring at one site (June–September) (Table 1). All samples were taken 1.0–1.5 m below the surface, unless otherwise indicated, with a clean, stainless steel water sampler. The samples were brought to the laboratory within 2 h of collection and kept at +3 °C in the dark for up to 24 h. Subsamples were used for analysis of the microbial communities, total bacterial counts, and geosmin and MIB concentrations (some samples only).

Separation of microorganisms and extraction of DNA

Microorganisms in the water samples were initially collected by filtration onto glass-fibre filters (Whatman GF/F), but the glass-fibre material produced a thick porridge-like matrix that interfered with the DNA extraction and severely reduced the extraction efficiency. Instead, microorganisms were separated from the water by centrifugation at high gravity. A total volume of 123 mL water was centrifuged in three subsequent centrifugations (15 min at 14,990 rpm, corresponding to 26,800 g, at 4 °C) of 41 mL each. The supernatant were discarded after each centrifugation. Pellets in the tubes were suspended in 0.5 mL 96% ethanol (EtOH) simultaneous with ultrasonic treatment for about 10 s. The pelleted material was transferred to DNA-free vials. The EtOH treatment was repeated twice to ensure removal of all pelleted material. All suspended pelleted material was collected in one vial and kept at room temperature until further treatment.

The efficiency of centrifugation for separation of *Streptomyces* cells from the water was examined by addition of *S. coelicolor* cells to bacteria-free lake water. Briefly, a culture of *S. coelicolor* (DSM 40233) was gently homogenized in a micro-tissue grinder with 10 μm distance between pestle and grinder tube (Kontes Glass Company, Vineland, NJ, USA). Homogenization was needed to separate the ball-like

Table 1 | Sampling stations in mid-Brisbane River and adjacent creeks, and in water reservoirs in southeast Queensland in 2010

Site name and number	Sampling date
BR 1: Unnamed creek flowing into mid Brisbane River	8 April
BR 2: Unnamed creek flowing into mid Brisbane River	14 April
BR 3: Mid Brisbane River	15 April
BR 4: Unnamed creek with sediment deposition, flowing into mid Brisbane River	8, 15 April
BR 5: Far end of backwater Holcim, flowing into mid Brisbane River	8 April
BR 6: Downstream Coal Creek, flowing into mid Brisbane River	15 April
BR 7: 19 Inside Coal Creek, flowing into mid Brisbane River	8 April
BR 8: Kholo Crossing (mid Brisbane River)	15 April
MC 1: Mount Crosby Weir (1 m below surface)	15 April and June–Sept.
MC 2: Mount Crosby Weir at 8.5 m (bottom)	15 April
HR 1: Hinze reservoir 1.5 m below surface	15 April
HR 2: Hinze reservoir at 40 m depth (bottom)	15 April
LN 1: Little Nerang reservoir at 1.6 m depth	14 April
LN 2: Little Nerang reservoir at 35 m depth (bottom)	14 April

cell structures that typically are formed during growth in cultures. Identical portions of the cell suspension were added to tubes with 0.2 μm filtered lake water. To test if centrifugation at 26,800 g removed all streptomycete cells from the water phase, half of the tubes were centrifuged as above, while the remaining tubes were not. Samples of 1 mL were taken from all tubes (centrifuged or non-centrifuged) and stained with SYBR-Green 1 (10 μL of 1:100-fold diluted stock; www.invitrogen.com). The stained samples were used for microscopic inspection of cell densities after filtration onto 0.2 μm polycarbonate filters and for analysis of relative cell abundance by fluorescence spectrophotometry at an excitation wavelength of 497 nm and emission wavelength of 520 nm.

Before extraction of DNA in the pelleted material from the water samples, the procedure for DNA extraction was optimized. Cells of *S. coelicolor* and *Streptomyces* isolate 40006 (Klausen *et al.* 2005) from laboratory cultures were harvested by centrifugation as described above. DNA of the pellets was extracted with two commercial kits: Rapid Water® kit by Mobio (www.mobio.com); and FastDNA® kit by Qbiogene (www.qbiogene.com) according to instructions by the manufacturers. The classical phenol-chloroform extraction procedure was also used (Bickley & Owen 1995). Before phenol-chloroform extraction, the cells were treated with an in-house procedure using lysozyme treatment at 37 °C for 1 h (10 mg mL^{-1} in STET buffer (www.sigma-aldrich.com), followed by the addition of 10% SDS at 37 °C (30 min) and 65 °C (30 min)).

Concentrations and purity of the extracted DNA were measured in a NanoDrop spectrophotometer (www.nanodrop.com) and were further visualized by gel electrophoresis in agarose stained with ethidium bromide. The phenol-chloroform method produced five to 11-fold higher concentrations of DNA and had a higher purity than the two commercial kits. Based on these results, the phenol-chloroform method was chosen for extraction of DNA in this study. Before the extraction of DNA in pellet material from the water samples, EtOH was removed using vacuum centrifugation and the pellets were subsequently dissolved in TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR protocols and amplification efficiency

For detection and quantification of streptomycetes, the TaqMan procedure targeting the 23S rRNA gene for qPCR (Rintala & Nevalainen 2006) was applied with minor adjustments. The PCR reactions were performed in a Mx3000P system (Stratagene, La Jolla, USA) using 10 μL 2× TaqMan Universal PCR Master Mix (including PCR buffer, AmpliTaq Gold DNA polymerase, AmpErase UNG, deoxynucleoside triphosphates (dNTPs) and passive reference dye;

www.appliedbiosystems.com); 0.4 μM (each) primer SMfw8 (5′-GCCGATTGTGGTGAAGTGGA-3′) and SMrev9 (5′-GTACGGGCCGCCATGAAA-3′); 0.2 μM of the probe SMp6 (5′-FAM-ATCCTATGCTGTCGAGAAAAGCCTCTAGCG-TAMRA-3′); 0.2 mg mL^{-1} of bovine serum albumin (New England Biolabs, Ipswich, MA, USA); 2 μL of template DNA; and DNase/RNase-free water (Sigma-Aldrich) for a final volume of 20 μL. The qPCR thermal cycling program consisted of the following steps: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, and 1 min at 58 °C for primer and probe annealing and extension.

The amplification products were separated by gel electrophoresis in 2.5% agarose, stained with ethidium bromide and visualized by UV light.

Specificity of the PCR reaction for *Streptomyces* species was previously tested by Rintala & Nevalainen (2006) for indoor house dust. Application of the method for microorganisms in rivers and water reservoirs therefore needed further specificity testing. Several taxonomically different prokaryotes were cultivated for the test and DNA was subsequently extracted by the phenol-chloroform procedure. The following microorganisms were examined: *Sphingomonas* sp. (Gram-negative; G^-), *E. coli* (G^-), *Shewanella* sp. (G^-), *Pseudomonas* sp. (G^-), *Bacillus* sp. (G^+), *Rhodococcus* sp. (G^+), *Arthrobacter* sp. (G^+) (all non-phototrophic organisms); *Synechocystis sp*. PCC 6803, *Synechococcus sp*. PCC 7002, *Nodularia spumigena* (two isolates originating from brackish water in the Baltic Sea and Australia, respectively), *Microcystis* sp. 7820, *Oscillatoria* sp., *Cylindrospermopsis* sp., *Aphanizonemon* sp., *Nostoc* punctiforme ATTC 29133, *Anabaena lemmermani* and *A. circinalis* (all cyanobacteria).

Control plasmid construct pJET-*Sc23*

In order to determine if substances in the pellet material were inhibitory to the qPCR reactions, the amplification efficiency of a plasmid construct (pJET-*Sc23*) was tested with and without addition of DNA extracted from the water samples. Control plasmid pJET-*Sc23* was constructed by cloning of target 23S rRNA PCR fragment (from PCR amplification of *S. coelicolor* DNA with primer SMfw8 and SMrev9 as described previously) into pJET1.2/blunt Cloning VectorCloneJET (PCR Cloning Kit; www.fermentas.com).

For detection and quantification of pJET-*Sc23*, the PCR assay described above was applied, except that the vector-specific primers pJET 1.2 F 5′-d(CGACTCACTATAGGGAGAGCGGC)-3′ and pJET 1.2 R 5′-d(AAGAACATCGATTTTCCATGGCAG)-3′ were used to amplify the inserted 23S rRNA region. Amplification efficiency of the primers was tested with 10-fold serial dilution of pJET-*Sc23*. Amplification of DNA from water samples with pJET 1.2 F and pJET 1.2 R was not detected.

Template DNA, i.e. DNA extracted from the centrifugation pellets, was added to a known concentration of pJET-*Sc23*. The 23S region was amplified using the thermal cycling program as described above. After amplification, the qPCR cycle threshold (C_t) was compared with C_t value of the reaction with pJET-*Sc23* alone. All water samples were tested individually. The PCR reaction contained 10 μL 2× TaqMan Universal PCR Master Mix; 0.4 μM (each) primer pJET 1.2 F and pJET 1.2R; 0.2 μM of the probe SMp6; 0.2 mg mL^{-1} of bovine serum albumin (New England Biolabs, Ipswich, MA, USA); 2 μL of water sample template DNA and 2 μL pJET-*Sc23* DNA; and DNase/RNase-free water (Sigma-Aldrich) to a final volume of 20 μL.

All qPCR reactions were performed in triplicate and at least three negative controls containing water instead of DNA, as well as two negative samples containing non-target DNA were included in each run. Serial dilutions for calibration curves were also carried out in triplicate. All reactions were performed in 96-well PCR plates (Thermo-Fast® 96 Non-Skirted) with optical caps (www.thermofisher.com).

Calibration curves

S. coelicolor and *Streptomyces* isolate 40006 (Klausen *et al*. 2005) were grown on solid oatmeal agar medium (60 g oatmeal and 12.5 g agar L^{-1}) for production of spores (conidia). When a dense formation of spores was observed on the agar plates, spores were collected after gentle washing of the agar surface with sterile water. DNA was extracted from spores using the phenol-chloroform extraction protocol and densities of spores were determined by fluorescence microscopy after staining with SYBR Green 1 (www.invitrogen.com).

Densities of streptomycete spores were related to the formed PCR products using C_t values of the PCR reactions.

Calibrations curves were determined by relating a certain spore number to a specific C_t value. Copy number of the 23S rDNA gene in *Streptomyces* was obtained from the *rrn*DB website (http://rrndb.mmg.msu.edu).

Bacterial densities in the water samples

Total bacterial number in the water samples was determined by epifluorescence microscopy. Briefly, water samples of 1 mL were preserved with glutaraldehyde (2% final conc.), stained with SYBR Green 1 and filtered onto 0.2 μm black polycarbonate filters. The filters were mounted in immersion oil on a glass slide and counted at 1,000× magnification under a microscope. Average number of bacteria was determined from counting of cells in 10–15 ocular fields.

RESULTS AND DISCUSSION

Initially, the protocol for detection of aquatic *Streptomyces* was optimized with respect to: (1) collection of streptomycetes from water by centrifugation; (2) DNA extraction procedure; (3) potential inhibition of the PCR reaction by natural substances in the water; (4) primer specificity; and finally (5) calibration curves for conversion of PCR products to cell number.

Efficiency of centrifugation for concentration of microorganisms

Tests of centrifugation efficiency for collection of suspended *Streptomyces* cells into pellets could not be performed by microscopic counting of *Streptomyces* cells in the water, before and after centrifugation, because the filaments hampered identification of individual cells. Instead, the abundance of streptomycetes was measured by fluorescence spectrophotometry of SYBR Green 1 stained cells in suspensions in untreated and centrifuged samples. Measurements of fluorescence showed that 44% ± 5 ($n = 4$ centrifugation tubes) of the cell biomass remained in suspension after centrifugation. The cell abundance in suspension before and after centrifugation is visualized in Figure 1. Filamentous and vegetative cells of *Streptomyces* were difficult to concentrate by centrifugation, even at 26,000 g for 15 min. This is in contrast to spores (conidia) of *Streptomyces* that can be concentrated by centrifugation at about 10,000 g (N. Jørgensen, unpublished observation).

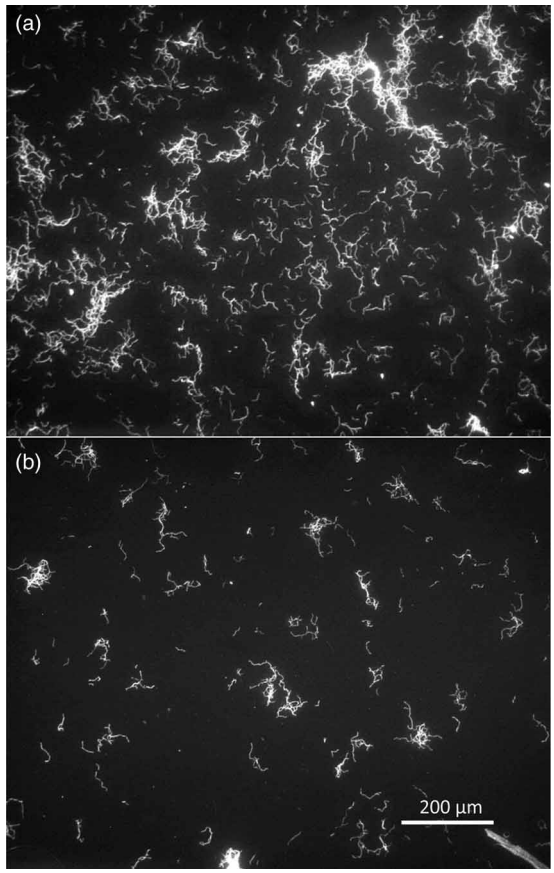

Figure 1 | Effect of centrifugation of water sample enriched with *Streptomyces coelicolor*. Abundance of *S. coelicolor* filaments before (a) and after (b) centrifugation at 26,000 g for 15 min. One mL water samples were stained with SYBR Green 1 and filtered onto 0.2 μm pore size polycarbonate filters. The filters were examined at 20× magnification in a fluorescence microscope.

Concentration of aquatic bacteria by centrifugation is not widely reported in the literature but in a recent study, centrifugation at 5,500–20,000 g was tested for concentration of non-filamentous, aquatic bacteria (Duhamel *et al.* 2008). The authors found that a maximum of 93% of the cells could be removed from the supernatant by application of 20,000 g for 30 min or more. Centrifugation is an attractive method for concentration of microorganisms in dilute environments, such as natural water samples, since interference from filter material in the DNA extraction procedure is eliminated. However, more work is needed to optimize the efficiency of centrifugation.

Extraction of DNA

Relative to the two commercial kits for extraction of DNA (FastDNA® by Qbiogene and RapidWater® by Mobio), the classical phenol-chloroform procedure significantly improved the extraction efficiency and purity of DNA from both cell cultures and the environmental samples. Concentrations of DNA obtained with the phenol-chloroform extraction were five to 11-fold higher and the purity was markedly higher than when using the kits (quantification of DNA by NanoDrop spectrophotometry), and reproducibility among the samples was improved. Extraction of DNA from the centrifugation pellets was performed by the phenol-chloroform method, and concentrations of 4–93 ng μL^{-1} were obtained.

The commercial kits are intended for universal DNA extraction (FastDNA®) or optimized for extraction of DNA in material collected on filters (RapidWater®). The extraction method of both kits includes bead beating, lysing solution and binding of DNA to a silica matrix. Extraction efficiency of microbial DNA in particulate matter in lakes and rivers has not previously been studied, but application of a commercial kit (QIAamp® by Qiagen) for extraction of DNA from the bacterium *Ralstonia solanacearum*, released into irrigation water, demonstrated a successful detection of the bacteria (Poussier *et al.* 2002). In a test of six commercial DNA extraction kits for isolation of DNA in *Bacillus* endospores added to soil, an extraction efficiency of up to 35% was found for sandy soils using the FastDNA® kit, but below 1% was obtained for most of the soil samples (Dineen *et al.* 2010). The authors concluded that binding of cells to soil particles, physical contact during bead beating, and inhibitory substances such as humic substances all may have influenced the efficiency of the DNA extraction. Also, in environmental samples, extracted DNA may adsorb to substances and surfaces, and introduce a qualitative and quantitative bias in PCR reactions (Levy-Booth *et al.* 2007).

The specific reason for the higher yield by the phenol-chloroform approach in our study was not examined but the sample matrix (mixed organic and inorganic particulate matter in freshwater) and the thick cell walls of *Streptomyces* may reduce the effectiveness of the kits. A difference between the procedure recommended for the kits and the phenol-chloroform extraction was pre-treatment of the samples with lysozyme and SDS at 37 and 65 °C before the phenol-chloroform treatment. Supporting a positive effect of the enzyme pretreatment, Ferrera *et al.* (2010) found that lysozyme as well as proteinase K treatment significantly increased the yield of DNA extracted from lake water biofilms.

PCR efficiencies and primer specificity

Testing of possible interference from substances in the extracted DNA on the qPCR reaction was performed by comparing qPCR C_t values of the pJET-*Sc23* control plasmid with and without addition of DNA extracted from the individual water samples. Inhibition of the pJET-*Sc23* PCR amplification was also assumed to reflect inhibition of the TaqMan-based amplification of the 23S rRNA gene in *Streptomyces*.

The plasmid approach showed that substances in the DNA extracts inhibited the PCR reaction in 16 of 23 samples, inhibiting the reaction by 33% (range 14–70%). Triplicate runs of the plasmid approach for each water sample further indicated up to 56% variation in C_t value per sample. The calculated densities of *Streptomyces* cells in the water samples were corrected for the inhibition and the observed per-sample variation was applied (see below).

Inhibition of PCR amplification by DNA extracted from environmental samples has been addressed in several studies. Dominant inhibitors appear to be naturally occurring organic compounds, e.g. humic acids and phenolic substances, but some heavy metals may also obstruct the amplification (De Boer *et al.* 1995; Wilson 1997; Schriewer *et al.* 2011). Various approaches have been applied to purify DNA isolated from environmental samples, including addition of proteins (bovine serum albumin and skimmed milk), precipitation with polyethylene glycol and other polymers, and adsorption of the inhibiting substances to various polymer materials (Arbeli 2007; Schriewer *et al.* 2011), but with variable results. Alternatively, the extracted DNA can be diluted to minimize the presence of inhibitory substances, but this may also reduce the concentration of the target gene to below detection limits (Lindberg *et al.* 2007). The specific reason for inhibition of the PCR reaction in our study was not examined, and dilution techniques were not applied due to the low density of *Streptomyces* in the water.

Tests of specificity of the TaqMan PCR reaction for *Streptomyces* species showed that DNA from a range of

taxonomically diverse prokaryotes did not form PCR products with the primer and probe combination (data not shown). This result confirms the findings of Rintala & Nevalainen (2006).

Calibration curves

Calibration curves (C_t value vs. cell number) were produced from analysis of spores of *S. coelicolor* and *Streptomyces sp.* isolate 40006 and showed a high correlation within the examined cell densities ($10–4 \times 10^6$ spores; $R^2 = 0.994$) (Figure 2). If assuming a methodological threshold at a C_t value of 40 (e.g. Rintala and Nevalainen (2006)), the theoretical detection limit of the present qPCR reaction is 0.5 cells or approximately one cell.

The copy number of the 23S RNA gene in *Streptomyces* species (including also *S. coelicolor*) has been determined to six copies per genome (*rrn*DB website). Copy number of *Streptomyces* isolate 40006 is unknown, but the positive agreement between calibration curves of the two *Streptomyces* species in our study suggests a similar 23S RNA copy number in both species. In order to calculate cell densities in the water samples (see below), it was assumed that *Streptomyces* in the water also had six copies per genome.

Quantification and abundance of streptomycetes in water

The optimized qPCR protocol for *Streptomyces* was applied to DNA in particulate matter from water samples

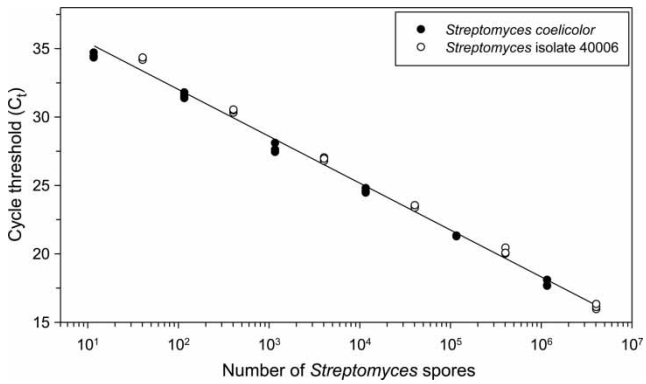

Figure 2 | Standard curve for quantitative PCR assay of *Streptomyces*, based on spores from cultures of *S. coelicolor* and *Streptomyces* isolate 40006. Linear regression of spore number vs. C_t value was: $Y = -3.433 \times \log x + 37.04$; $R^2 = 0.994$; $n = 3$ (at each dilution).

from the Brisbane River, weir and water reservoirs. Densities of *Streptomyces* were corrected for centrifugation and amplification efficiencies and were found to vary from 225 to 45,650 cells L^{-1} (mean values) in the surface water of Hinze reservoir (site HR 1) and bottom water (8.5 m deep) at the Mt Crosby Weir (site MC 2), respectively (Table 2). In the mid-Brisbane River (site BR 3 and BR 8) and the adjacent shallow streams (BR 1 and BR 4–7) there was a large variation in abundance of streptomycetes, as indicated by mean densities from 260 and up to 7,948 cells L^{-1}.

Seasonal variation in *Streptomyces* abundance was studied in Mt Crosby Weir surface water from June to September. The densities varied significantly, with mean numbers from 433 to 13,549 cells L^{-1} (Figure 3). The highest densities (>10,000 cells L^{-1}) coincided with the highest total bacterial abundances ($>3 \times 10^9$ cells L^{-1}; Figure 3) but there was no statistically significant correlation between the abundance of all bacteria and streptomycetes over the 10 week period (Spearman rank correlation, $R > 0.05$).

The high density of *Streptomyces* in the bottom water at Mt Crosby Weir (site MC 2) (45,650 cells L^{-1}) may indicate that streptomycetes are most abundant at the water-sediment interface. Filamentous *Actinobacteria* (assumed to be streptomycetes) have previously been found to be abundant in surface sediment of a southeast Queensland water reservoir and coincided with high concentrations of geosmin (Klausen *et al.* 2004). However, here we did not detect *Streptomyces* in bottom water of Hinze (40 m depth) and Little Nerang reservoirs (35 m depth). The reason for these differences is not clear but one cause might be that the anoxic bottom water in these two reservoirs inhibited growth of streptomycetes. Largely all streptomycetes are obligate aerobic organisms. In contrast, in the Klausen *et al.* (2004) study, the bottom waters were artificially mixed with a destratification unit.

Natural small-scale variations in the abundance of *Streptomyces* cells in the water column might impact reproducibility of the present method. To test for such variability, triplicate 123 mL samples were taken from each of three river water samples. The mean variation in cell number per 123 mL sample was 61%. Due to the limited extent of this test, variation in cell densities in Table 2 is

Table 2 | Sampling sites, concentrations of geosmin, MIB, densities of *Streptomyces* sp. (mean densities ± range observed by the plasmid-based assay) and densities of all bacteria (determined by epifluorescence microscopy). Empty fields = no data available. NP = No PCR product

Site	Date	Geosmin ng L^{-1}	MIB ng L^{-1}	*Streptomyces* sp. cells L^{-1}	Bacterial abundance $\times 10^9$ cells L^{-1}
BR 1	Apr 8	3.5	10	4,740 ± 2,667	2.6
BR 2	Apr 14	3.6	6.1	NP	2.8
BR 3	Apr 15	2.0	8.5	1,500 ± 840	3.4
BR 4	Apr 8	2.1	59	1,205 ± 725	4.0
BR 4	Apr 14			1,096 ± 620	2.5
BR 4	Apr 15	2.4	11	5,705 ± 3,225	3.6
BR 5	Apr 8	2.4	24	7,948 ± 4,463	4.0
BR 6	Apr 15	2.8	6.5	260 ± 150	4.2
BR 7	Apr 8	2.0	2.0	1,801 ± 1,019	2.7
BR 8	Apr 15	3.5[a]	30[a]	660 ± 360	2.8
MC 1	Apr 15		25	3,715 ± 2,117	3.7
MC 2	Apr 15			45,650 ± 25,765	3.8
HR 1	Apr 15			225 ± 135	2.4
HR 2	Apr 15			NP	2.2
LN 1	Apr 14			2,230 ± 1,250	2.3
LN 2	Apr 14			NP	1.7
MC 1	Jun 2			5,974 ± 3,488	3.4
MC 1	Jun 16			12,908 ± 7,299	3.6
MC 1	Jun 23			433 ± 238	1.9
MC 1	Jul 13			7,656 ± 4,331	0.9
MC 1	Jul 21			912 ± 504	0.9
MC 1	Aug 4			750 ± 426	1.4
MC 1	Aug 18			822 ± 462	3.5
MC 1	Aug 25			13,549 ± 7,644	2.7
MC 1	Sep 1			7,655 ± 4,307	2.6
MC 1	Sep 10			829 ± 466	2.1

[a]Concentrations in samples collected April 21.

only indicated from the range observed by the plasmid control assay.

The abundance of streptomycetes in aquatic environments has not previously been quantified by PCR. Culture assays (growth on selected agar media) suggest that streptomycetes in various freshwater environments make up from 10^2 to 10^5 CFU L^{-1} (Lanciotti *et al.* 2003; Zaitlin *et al.* 2003; Lee *et al.* 2011). Significantly higher densities of filamentous *Actinobacteria* (assumed to include a large fraction of *Streptomyces*) were determined in fresh waters by FISH technique (densities of 10–2,000 $\times 10^6$ cells L^{-1}) (Klausen *et al.* 2005; Nielsen *et al.* 2006). Relative to these abundances, the present qPCR-based densities of *Streptomyces* appear to align with cell densities determined by culture-based approaches for actinomycetes in freshwater but were below densities of filamentous *Actinobacteria* determined by FISH. However, comparison of abundances of *Streptomyces* by qPCR and culture assays should be conducted on identical water samples before general conclusions on the reliability of methods are drawn.

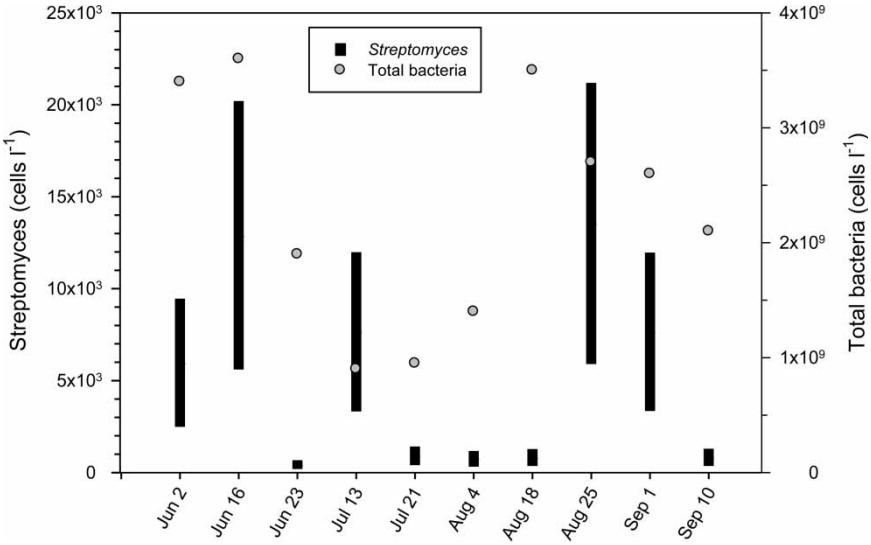

Figure 3 | Abundance of streptomycetes and total bacteria in surface water at Mt Crosby Weir. Ranges (minimum–maximum) of *Streptomyces* abundance were determined by the plasmid assay (see Material and methods). For abundance of total bacteria, means of 10 microscope fields (representing a minimum of 250 cells) are shown. Standard variations were <5% (not shown).

It is possible that the present qPCR method did not target all *Streptomyces* species in the water with similar efficiency. In a study of house dust, Rintala & Nevalainen (2006) observed that the TaqMan approach produced a weak product for *S. albus* and a thermophilic strain, suggesting that abundance of single *Streptomyces* strains may be underestimated by the qPCR assay. Another factor that may influence the quantification is the presence of S*treptomyces* species not yet isolated and characterized with respect to rRNA gene sequence. Such species might not be detected by the applied primers and probe. Speculatively, the application of conidia (spore) DNA for quantification of vegetative streptomycete cells in the water might have biased the C_t-value-to-cell conversion, e.g. due to different copy numbers of genes encoding ribosomes in vegetative cells and in *Streptomyces* conidia. However, chromosome sets of spores and their vegetative cells have been assumed to be identical (Errington 2003).

The main advantages of the qPCR approach, relative to culture methods, include detection of very low cell numbers, unique identification of *Streptomyces*, and a short analytical turnaround time that can be applied for rapid monitoring of taste-and-odour episodes.

Occurrence and production of geosmin and MIB relative to cell densities

In the Brisbane River and connecting creeks, concentrations of geosmin were 2–3.6 ng L^{-1} while higher, but more variable concentrations of MIB (2–59 ng L^{-1}) were measured in April 2010. The significance of *Streptomyces* species as potential producers of these TOCs may tentatively be estimated from known production rates measured in laboratory cultures. Cell-specific production of geosmin and MIB by selected *Streptomyces* strains, isolated from aquatic environments, was estimated to $0.1–30 \times 10^{-18}$ g $cell^{-1}\,h^{-1}$ for both geosmin and MIB by Klausen *et al.* (2005). Assuming that streptomycetes in water of the mid-Brisbane River had similar production rates, from 10^3 to 10^6 days were needed to produce a concentration of geosmin or MIB of 5 ng L^{-1} (representative level in mid-Brisbane River, Table 2). In bottom waters of Mt Crosby Weir (site MC 2) the higher densities of *Streptomyces* means that at least 84 days were required to produce geosmin or MIB to this level. In these estimates no degradation of geosmin or MIB is considered but microbial degradation of the off-flavours has been demonstrated in stream water and by bacteria isolated from water treatment plants (Klausen *et al.* 2005; Ho *et al.* 2007).

Although actual production and degradation rates of geosmin and MIB in the water were unknown, the calculations of potential production rates suggest that streptomycetes may contribute to the TOC pool in the water, but they appear not to be major sources of TOCs in water of southeast Queensland reservoirs. However, more information on *in situ* production of geosmin and MIB by streptomycetes in water of the river, weir and reservoirs are needed, and further tests should be performed to ensure that all streptomycetes are included in the TaqMan qPCR approach.

During the seasonal sampling at Mt Crosby Weir (MC 1) from the austral winter, June through to spring (September), concentrations of geosmin and MIB in periodic samples were <3.6 and <7.1 ng L^{-1}, respectively. Unfortunately, the exact dates for analysis of TOC and bacteria did not coincide. This discrepancy and the low number of observations of TOCs do not allow analysis of possible correlations between the occurrence of *Streptomyces*, geosmin and MIB. The low level of geosmin and MIB in the Brisbane River in the austral winter (concentrations close to the human taste threshold) contrasts with episodes of high geosmin concentrations of >25 ng L^{-1} at Mt Crosby Weir (receives water from Brisbane River) in December 2010 (Seqwater, unpublished data), and MIB concentrations of 30 ng L^{-1} in the mid-Brisbane River (Table 2). In future studies, sampling for TOC-producing organisms should be carried out in periods with high and variable geosmin concentrations, e.g. during the austral summer.

It should be mentioned that streptomycetes are probably not the only non-phototrophic, geosmin-producing microorganisms in freshwater environments. Recent genome analyses show that the geosmin synthase gene may also occur in microbes that have not previously been known to produce geosmin, e.g. *Frankia* (symbiotic N_2 fixing bacterium), *Saccharopolyspora* (related to *Streptomyces*) and the common slime bacterium *Myxobacteria* (Giglio *et al.* 2008). If these microorganisms also contribute geosmin to the water, the total TOC production by non-photosynthetic organisms may be larger than previously assumed.

CONCLUSIONS

We applied, for the first time, real-time (quantitative) PCR to detect and quantify the Gram-positive bacterium *Streptomyces* in river, weir and reservoir water in southeast Queensland, Australia. The method was optimized with respect to collection of bacterial cells from the water and interference by naturally occurring substances on the PCR amplification. The measured abundance of *Streptomyces* cells in the water was a relatively small fraction of the total bacterial populations, yet they may be important producers of off-flavours in some habitats, e.g. in bottom water of reservoirs. In future studies, specificity of the qPCR method should be optimized to ensure inclusion of all streptomycetes, and actual production rates of TOCs by aquatic streptomycetes should be determined.

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