The role of *Actinobacteria* in taste and odour episodes involving geosmin and 2-methylisoborneol in aquatic environments

Elise Anne Asquith, Craig Andrew Evans, Phillip Milton Geary, Richard Hugh Dunstan and Bruce Cole

ABSTRACT

The secondary metabolites geosmin and 2-methylisoborneol (2-MIB) provide soil with its characteristic earthy-musty odour, being notably produced by the abundant spore-forming filamentous bacterial genus *Streptomyces*, among other *Actinobacteria.* Taste and odour (T&O) problems attributed to these compounds affect drinking water supplies worldwide, often occurring sporadically and untraced to their biological origins. A number of prokaryotic and eukaryotic organisms are recognised geosmin and 2-MIB producers in aquatic environments. However, the focus of this paper is to assess the potential contribution of *Actinobacteria* to this water quality issue. To date, the aquatic ecology of these bacteria remains poorly understood and debate surrounds whether they exist solely as dormant spores of terrestrial origin or are capable of growing and biosynthesising these odourous compounds in aquatic environments. The *Actinobacteria* which are known to produce geosmin and 2-MIB are identified and a critical assessment of habitats within aquatic environments in which they may be metabolically active residents and thus potential sources of T&O is provided. Current understandings of the chemical ecology and biosynthetic pathways of geosmin and 2-MIB, as well as the conditions under which these secondary metabolites are produced by *Streptomyces,* are reviewed.

Key words | *Actinobacteria*, geosmin, odour, *Streptomyces*, taste, 2-methylisoborneol

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INTRODUCTION

Actinobacteria, notably the genus *Streptomyces*, have received significant research attention since the discovery of their superlative rich and diverse secondary metabolism in the 1940s, making them valuable providers of clinically important biomolecules. However, there are two secondary metabolites produced by *Streptomyces*, among other *Actinobacteria*, that are rather detested by the drinking water industry: the volatile tertiary alcohols geosmin and 2-methylisoborneol (2-MIB). Better recognised for providing soil with its pleasant earthy-musty odour, these compounds are also responsible for causing taste and odour (T&O) problems in drinking water supplies worldwide, leading to great consumer dissatisfaction and high treatment costs for water utilities. Numerous organisms, from prokaryotes to eukaryotes, have been implicated as the cause of this aesthetic water quality issue. Brief consideration will be paid to these, but it is the potential role that *Actinobacteria* play in generating unpleasant episodes of earthy-musty T&O that is the focus of this paper. An overview of the taxonomy, physiology and ecology of these bacteria is presented followed by a detailed review of the current evidence documented in the literature concerning geosmin and 2-MIB production by *Streptomyces* and other *Actinobacteria*. Importantly, the ability of these bacteria to be metabolically active in aquatic environments remains to

be elucidated, which ultimately leaves ambiguity concerning their role in T&O episodes.

WHAT ARE *ACTINOBACTERIA*?

Actinobacteria comprise a ubiquitous class of Gram-positive heterotrophic bacteria, characterised by DNA rich in guanine and cytosine which display diverse morphologies from simple bacterial shapes of cocci through to forms with differentiated branched mycelium (Vobis 1997; Ventura *et al.* 2007). In the literature relating to T&O in drinking water, the term 'actinomycetes' has been widely used to refer to taxa of this class, particularly the large filamentous genus *Streptomyces*. This was somewhat appropriate since the order *Actinomycetales*, from which this term is derived, previously contained T&O producing, filamentous genera. However, with the availability of new 16S rRNA gene sequence information, recent modifications in the phylogenetic relationships of the class *Actinobacteria* have been made, with the reconstruction of higher (taxonomic) ranks (Zhi *et al.* 2009; Ludwig *et al.* 2012). The order *Actinomycetales* now contains only one family (*Actinomycetaceae*), whereas the remaining families have been reclassified within new orders created by elevation of existing suborders to order status (e.g. *Streptomyces* now belongs to the new order *Streptomycetales*) (Ludwig *et al.* 2012). Hence the common term 'actinomycetes' will be avoided in this review. Rather these bacteria will be referred to more generally at their class level (*Actinobacteria*) or at genus level (mainly *Streptomyces*) where appropriate.

Streptomyces, the most representative genus, among other *Actinobacteria*, exhibit a filamentous growth habit and produce spores, morphological traits which confused their discoverers in the 1870s, growing like fungi, but with tiny dimensions like bacteria. They are recognised by the tough, chalky or leathery and often pigmented colonies they develop on solid media (Figure 1). Their resilient spores disperse in the environment and germinate to form a dense branching hydrophilic vegetative mycelium on solid substrates, which forages for nutrients and assembles them into cellular structures. Upon depletion of nutrients, this biomass is utilised to build a vertically growing hydrophobic aerial (secondary) mycelium. As these specialised hyphae grow and mature, synchronised cell septations develop between nuclear areas, resulting in the formation of spores (Figure 2) (Goodfellow & Williams 1983; Williams *et al.* 1984).

THE ECOLOGICAL SIGNIFICANCE OF *ACTINOBACTERIA*

Actinobacteria are a successful class of bacteria, occurring in a multiplicity of terrestrial, aquatic and man-made environments. Soil is their greatest natural reservoir, where they constitute a significant proportion of the bacterial community (Goodfellow & Williams 1983; Williams
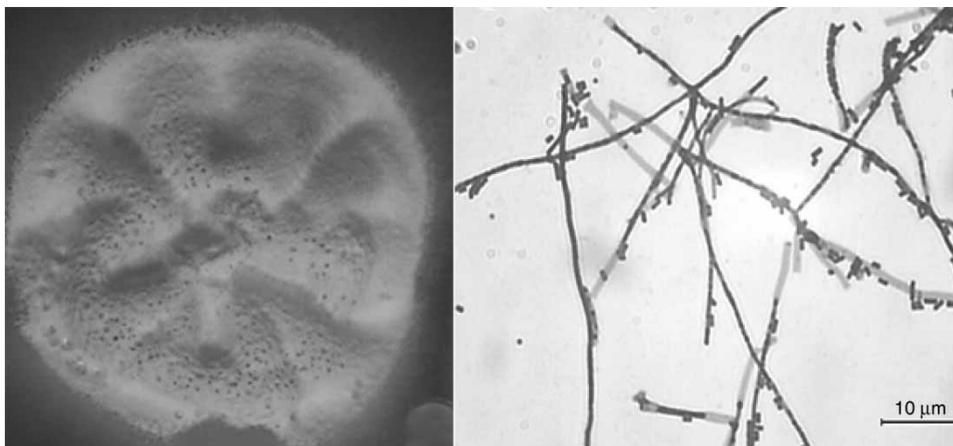
Figure 1 | Sporulating *Streptomyces* sp. colony growing on starch-casein agar (left). Photomicrograph of stained isolate detailing the presence of hyphae and spores (right).
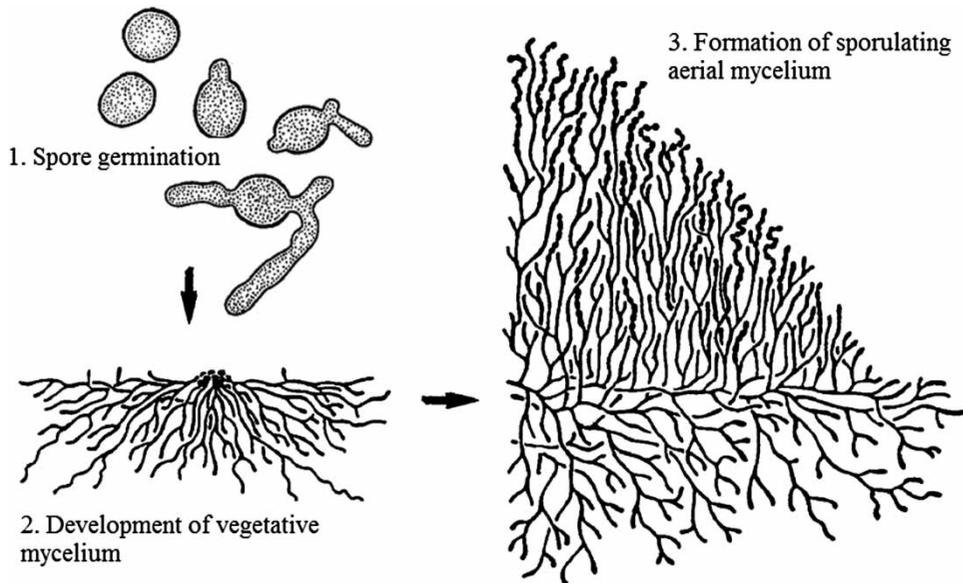
Figure 2 | Development of mycelium in *Streptomyces*. Modified from Vobis (1997).

et al. 1984). Their impressive saprophytic abilities are perhaps their greatest ecological significance in soil, delivering an immense contribution to organic matter turnover. Hydrolytic extracellular enzyme secretion combined with the penetrating abilities of their hyphae, make filamentous *Actinobacteria* highly adapted to extensively colonising complex organic substrates (e.g. lignocellulose) which other microbes cannot exploit (McCarthy & Williams 1992). In the rhizosphere, they protect plant roots by antagonising phytopathogens and exhibit traits of plant growth promoting rhizobacteria. The genus *Frankia* has long been recognised for its occurrence in the root nodules of certain shrubs and trees where it fixes atmospheric nitrogen (Goodfellow & Williams 1983; Williams *et al.* 1984). *Actinobacteria*, particularly *Streptomyces*, are of great medical significance, being a major source of enzymes, vitamins and secondary metabolites which have a broad spectrum of biological activities (e.g. antibacterial, antifungal and enzyme inhibitory) (Ventura *et al.* 2007).

Actinobacteria have provided mankind and the environment with many beneficial microorganisms but also some of its greatest foes, namely pathogenic forms which cause some widespread and intensely studied animal and plant diseases (e.g. the genus *Corynebacterium* which causes diphtheria and *Mycobacterium* which causes leprosy and tuberculosis) (Ventura *et al.* 2007).

Actinobacteria are also problematic by causing immensely thick scumming on the surface of secondary sewage treatment aeration tanks and their overgrowth can create problems with sludge bulking, impairing sludge settling and reducing effluent quality (Kämpfer & Wagner 2002). The impartment of T&O into drinking water supplies is another significant challenge these bacteria present to the water industry. A review is hereafter presented regarding the problem of biologically produced T&O involving geosmin and 2-MIB in aquatic environments, with an emphasis on the role of *Streptomyces* and other *Actinobacteria*.

GEOSMIN AND 2-MIB IN DRINKING WATER SUPPLIES

Surface waters, including reservoirs, natural lakes and rivers, are important sources of potable water throughout the world, in which water utilities often encounter sporadic episodes of earthy-musty T&O, rendering it unpalatable for human consumption. Despite numerous microorganisms (e.g. cyanobacteria, *Actinobacteria* and fungi) being implicated as sources, the majority of T&O outbreaks are neither anticipated nor traced to their biological origins (Juttner & Watson 2007). The status of the problem in

Australia was captured in a 2005 survey involving 37 drinking water providers, representing five million consumers. Approximately 78% of the suppliers reported problems regarding earthy-musty T&O and only five providers had identified a definite link between potential causative microorganisms (namely cyanobacteria) and T&O causing compounds (Hobson *et al.* 2010). The ambiguity of the cause of this serious aesthetic water quality problem is certainly not restricted to Australia; it affects drinking water utilities worldwide.

Although a number of microbial secondary metabolites give rise to unpleasant T&O in water, attention is mainly focused on earthy geosmin 2β,6α-dimethylbicyclo[4.4.0]decan-1β-ol and musty 2-MIB ((1R-exo)-1,2,7,7-tetramethylbicyclo[2.2.1]heptan-2-ol) (Figure 3). While these semi-volatile terpenoids pose no risk to human health, T&O is perceived by the general public as a primary indicator of the safety and acceptability of drinking water and encourages them to switch to alternative supplies such as bottled water (Juttner & Watson 2007; Srinivasan & Sorial 2011). A major problem concerning these compounds in drinking water is that they can be detected by human olfactory sense at exceptionally low concentrations, the odour threshold concentrations being as low as 4 and 9 ng L^{-1} for geosmin and 2-MIB, respectively. Owing to their low concentration and chemical stability, their removal from drinking water has proven to be a difficult task. Some treatment technologies have been employed with variable success, including adsorption by activated carbon, advanced oxidation processes and biological/physical filtration technologies (Srinivasan & Sorial 2011; Ho *et al.* 2012).
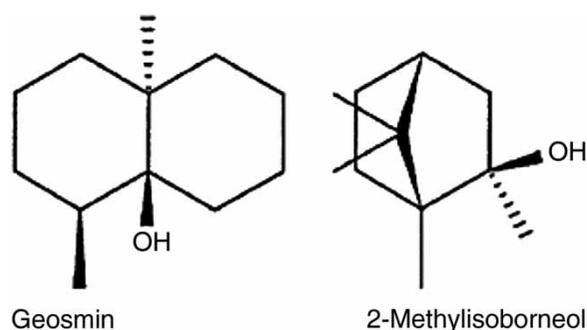
Figure 3 | Molecular structures of geosmin and 2-MIB. Modified from Juttner & Watson (2007).

NON-ACTINOBACTERIAL PRODUCERS OF GEOSMIN AND 2-MIB

Predicting and preventing geosmin and 2-MIB accumulation in drinking water supplies is vital for long term delivery of high quality water to consumers. The literature demonstrates the multiplicity of microorganisms that could produce these compounds and the range of habitats that could foster their production. The phenomenon is highly complex and as such, a large number of T&O cases remain unsolved. Photoautotrophic cyanobacteria, being widely distributed in freshwater systems, are thought to be the main producers of T&O. The regular monitoring of cyanobacterial seasonal dynamics in drinking water storages has provided ample opportunity to analyse relationships between these bacteria and T&O (Juttner & Watson 2007). Often, strong correlations between planktonic cyanobacteria and T&O have been documented (Izaguirre *et al.* 1999; Park *et al.* 2001; Zhang *et al.* 2010). Geosmin and 2-MIB occurrences have been well correlated with abundance of planktonic *Anabaena* (Bowmen *et al.* 1992; Jones & Korth 1995; Izaguirre *et al.* 1999; Uwins *et al.* 2007; Hobson *et al.* 2010). Some reports indicate that benthic cyanobacteria such as *Aphanizomenon*, *Oscillatoria*, *Phormidium* and *Pseudanabaena* are also associated with earthy-musty odour (Vilalta *et al.* 2004; Izaguirre & Taylor 2007).

The presence of geosmin and 2-MIB during periods of low cyanobacterial biomass indicates that these bacteria are not the only source (Klausen *et al.* 2004; Dzialowski *et al.* 2009). Indeed, other microbes, notably *Actinobacteria*, may also be responsible for their production and in some cases, both have been implicated in T&O episodes (Sugiura & Nakano 2000; Lanciotti *et al.* 2003). *Myxobacteria*, ameoba and liverworts have also been confirmed as geosmin producers (Hayes *et al.* 1991; Sporle *et al.* 1991; Yamamoto *et al.* 1994; Schulz *et al.* 2004; Dickschat *et al.* 2005). In particular, some fungi produce geosmin and as these organisms are readily detectable in aquatic environments, they may be an underestimated source of T&O which are yet to be systematically investigated (Kikuchi *et al.* 1981; Borjesson *et al.* 1993; Larsen & Frisvad 1995).

GEOSMIN AND 2-MIB PRODUCTION BY *ACTINOBACTERIA*

Actinobacteria, particularly the genus *Streptomyces*, have gained significant notoriety throughout the drinking water industry as a major source of geosmin and 2-MIB since these secondary metabolites were first extracted from *Streptomyces* cultures (Gerber & Lecheval 1965; Gerber, 1969). They were initially dealt with in the literature as a potential source of T&O at the end of the 1920s followed by a surge of studies from the 1950s ascribing almost all earthy odours in natural waters to *Actinobacteria* (Silvey *et al.* 1950; Silvey & Roach 1953, 1975; Silvey 1963; Higgins & Silvey 1966). Silvey & Roach (1975) stated that 'there is a direct relationship between the actinomycete [*Actinobacteria*] population that occurs in raw water and the T&O compounds that are produced by these organisms' (p. 270). These reports stimulated microbiologists to count 'aquatic' *Actinobacteria* in water supplies. Such monitoring efforts regularly failed to produce clear relationships. Cross (1981) provided a simple explanation for this: spores, being the principal propagules found in water, are not metabolically active and therefore cannot produce odourous secondary metabolites. Rather these compounds arise subsequent to secondary mycelial development. As spores and vegetative forms are indistinguishable using traditional culture techniques, it remains uncertain if *Streptomyces* or other *Actinobacteria* can be metabolically active in freshwater environments and contribute to earthy-musty odour T&O.

Conditions of production

In order to elucidate the conditions under which *Streptomyces* produce geosmin and 2-MIB and therefore better predict T&O outbreaks, the effect of physical and chemical parameters on production rates have been investigated. The prevalence of earthy-musty T&O during warmer seasons is well documented. Undoubtedly, temperature is an important parameter affecting the metabolic activity of *Streptomyces*, which are predominately mesophilic, exhibiting optimum growth between 25 and 30 °C (Goodfellow & Williams 1983). Wood *et al.* (1985) determined that the minimum temperature for geosmin production by *S. albidoflavus*

in nutrient amended reservoir water was 15 °C and that all documented cases of earthy odour problems occurred when the water temperature exceeded this. However, more recently, Zuo *et al.* (2010) found that some sediment isolates of *Streptomyces* could grow slowly and produce relatively low concentrations of geosmin even at 4 and 10 °C. Generally, *Streptomyces* cultures increase their production yields with increasing incubation temperature (≤ 40 °C) (Dionigi & Ingram 1994; Blevins *et al.* 1995).

Being secondary metabolites, geosmin and 2-MIB are produced by *Streptomyces* during secondary mycelial growth coinciding with sporulation. This has been demonstrated by the inhibition of geosmin production by *Streptomyces* mutants incapable of aerial mycelium development as well as by non-mutants growing on media not conductive to sporulation (Bentley & Meganathan 1981; Dionigi *et al.* 1992). As the secondary mycelium is obligately aerobic, *Streptomyces* require the presence of oxygen for geosmin and 2-MIB production. Supporting this, oxygen enrichment (30% O_2 atmosphere) of *S. tendae* cultures was found to increase geosmin production (48 ng mg^{-1}) compared to ambient atmosphere-incubated controls (42 ng mg^{-1}). Exposure to low oxygen concentrations (5 and 10%) further reduced geosmin synthesis (Dionigi & Ingram 1994). Similar findings were reported by Schrader & Blevins (1999). Though these baceria are neutrophiles, they have been detected in both moderately acidic (pH 5) and alkaline (pH 9) aquatic environments (Jiang & Xu 1996). Blevins *et al.* (1995) showed that *S. halsetdii* grew optimally in a neutral pH range (6–7) but interestingly, the highest geosmin production occurred at pH 9 and in the extensive range of 6–11.

Although low nutrient concentration was found by Wood *et al.* (1985) to be a major constraint on the growth and geosmin production by a *Streptomyces* isolate in the water mass of oligotrophic reservoirs, nutrient poor conditions have also supported the production of T&O compounds by these bacteria (Zaitlin *et al.* 2003a, b). Schrader & Blevins (2001) found that easily assimilated carbon sources (e.g. glucose and maltose) promoted biomass production whilst providing carbon sources such as mannitol and glycerol, promoted maximal geosmin production by *S. halstedii*. Similarly, Saadoun (2005) found that glycerol promoted 2-MIB production compared to glucose which was

more favourable for biomass development in cultures of *S. violaceusniger*. Carbon sources which readily serve as growth substrates often repress the need for secondary metabolism and therefore T&O production by *Streptomyces*, being normally triggered in nutrient limiting conditions. Similarly, repression of cyanobacterial growth appears to promote geosmin biosynthesis due to the increased availability of precursor compounds (e.g. pyruvate and acetyl-CoA) (Blevins *et al.* 1995; Saadoun *et al.* 2001).

Blevins *et al.* (1995) observed that limiting concentrations of nitrate and ammonia (16.5 and 13.1 µg L^{-1} respectively) induced geosmin production (45.8 and 62.5 ng mg^{-1} respectively) and suppressed growth by *S. halstedii*. However, increasing the phosphorus concentration (up to 36.2 µM) increased geosmin and biomass production, with no geosmin detected at the lowest concentrations tested (0.7–7.3 µM) (Schrader & Blevins 2001). The effect of micronutrients on geosmin production by *S. halstedii* were also investigated, with low concentrations of K, Fe, Co, Ca, Cu, Mn and Zn stimulating geosmin synthesis while higher concentrations inhibited or repressed production. Significant geosmin production was still observed in the absence of some of these trace elements (Fe, Co, Ca and Mn) (Schrader & Blevins 2001). Salinity has also been observed to affect geosmin production, with Rezanka & Votruba (1998) reporting that an increase in sodium chloride concentration (0–12%) in *S. avermitilis* cultures decreased geosmin biosynthesis.

Pathways of biosynthesis

Despite the identification of geosmin and 2-MIB in the late 1960s, their puzzling biosynthesis remained unsolved for several decades. By feeding radioactively labelled precursors to cultures of *S. antibioticus*, Bentley & Meganathan (1981) demonstrated that these compounds were terpenes, geosmin a degraded sesquiterpene and 2-MIB a methylated monoterpene. Two distinct pathways of terpenoid biosynthesis exist: the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP) or the mevalonic acid (MVA) pathway. Both pathways produce isopentenyl diphosphate (IPP), the 5-carbon building block of terpene synthesis which forms geranyl diphosphate (GPP), the universal 10-carbon precursor of monoterpenes (e.g. 2-MIB) and farnesyl diphosphate (FPP), the 15-carbon precursor

of sesquiterpenes (e.g. geosmin) (Lange *et al.* 2000). The MEP pathway is believed to be the major biosynthetic pathway to produce these precursors in *Streptomyces* (Spiteller *et al.* 2002; Singh *et al.* 2009). Of recent years, a significant focus has been the elucidation of the genes and enzymes responsible for the final steps by which geosmin and 2-MIB are produced from these precursors.

Geosmin synthase has been identified as the enzyme responsible for the two-step production of geosmin. This bifunctional sesquiterpene synthase, first isolated from *S. coelicolor* A3(2) consists of 726 amino acids, encoded by the 2181-bp SCO6073 (cyc2) gene (Cane & Watt 2003; Gust *et al.* 2003; Jiang *et al.* 2007). The enzyme's two active sites include an N-terminal domain which catalyses the cyclisation of FPP into the sesquiterpene alcohol (1(10)*E*,5*E*)-germacradien-11-ol (germacradienol) and germacrene D (bicyclic hydrocarbon) and the highly homologous C-terminal active site which diffusively rebinds the released germacradienol product and catalyses proton initiated cyclisation-fragmentation of this molecule via the intermediate 8,10-dimethyl-1-octalin to give geosmin. Both sites have an absolute dependence on the cofactor Mg^{2+}, with each possessing two strictly conserved motifs for binding this cation (Cane *et al.* 2006; Jiang *et al.* 2007). Homologous genes encoding geosmin synthases have been also identified in the genomes of *S. avermitilis* (SAV2163 or geoA, 725 aa) (Cane *et al.* 2006) and *S. peucetius* (Spterp13, 2,199 bp, 732 aa) (Ghimire *et al.* 2008; Singh *et al.* 2009). A geosmin synthase encoding gene has also been identified from the cyanobacteria *Nostoc punctiforme* (Giglio *et al.* 2008). Citron *et al.* (2012) screened bacterial genomes for amino acid sequences with high similarity to the *S. coelicolor* geosmin synthase gene and identified putative geosmin synthases in the genomes of 49 strains of bacteria, including all *Actinobacteria*.

The pathway of 2-MIB biosynthesis in *Nannocystis exedens* was unravelled in feeding experiments by Dickschat *et al.* (2007) and the same enzymatic mechanism has been confirmed in *Streptomyces* and cyanobacteria (Wang & Cane 2008; Giglio *et al.* 2011; Wang *et al.* 2011). The pathway proceeds through the *S*-adenosylmethionine-dependent methylation of GPP to (*E*)-2-methylgeranyl diphosphate (2-methyl-GPP) catalysed by a GPP-2-methyltransferase (2-methyl-GPP synthase) and its subsequent cyclisation to 2-MIB, catalysed by a C$_{11}$ homo-monoterpene synthase

(2-MIB synthase). In *S. coelicolor* A3(2), these enzymes are encoded by two adjacent genes including SCO7701 and SCO7700 together with an upstream regulatory cyclic nucleotide binding protein (cnb) gene in a three-gene operon (Wang & Cane 2008). These genes share highly conserved motifs for Mg^{2+} binding and recently, the X-ray crystal structures of both enzymes have been identified (Koksal *et al.* 2012a, b). Komatsu *et al.* (2008), using bioinformatic analysis, identified homologous genes constituting the 2-MIB synthase operon in the genome of other *Streptomyces* capable of producing this compound. Homologous genes for the same biosynthetic machinery have been also identified in cyanobacteria, suggesting that 2-MIB biosynthesis and the associated genes in these bacteria have a common origin (Komatsu *et al.* 2008; Giglio *et al.* 2011; Wang *et al.* 2011). Citron *et al.* (2012) conducted a BLAST search against the amino acid sequence of 2-MIB synthase from *S. coelicolor* (SCO7701), revealing the presence of homologues in 23 *Actinobacteria*.

Purpose of biosynthesis

Secondary metabolite production in *Streptomyces* is generally regarded as a defensive strategy in times of adversity to ensure survivability of the next generation of germinating spores. Thus it seems reasonable to assume that geosmin and 2-MIB may act as allelogens. *In vitro* assays have demonstrated that these compounds can inhibit the growth of *Salmonella typhimurium*, reduce cell viability and damage DNA in rainbow trout hepatocytes and are toxic to sea urchin embryos using concentrations in parts per million (0.45–45 mg L^{-1}) (Dionigi *et al.* 1993; Nakajima *et al.* 1996; Gagne *et al.* 1999). These excessively high concentrations far exceed those levels normally encountered in freshwater environments (<500 ng L^{-1}) which are unlikely to exhibit allelopathic activity. However, Zaitlin & Watson (2006) maintain that the concentrations used in the above studies may be encountered by organisms in close proximity to source microorganisms or at the microscale level in sediments, soil and biofilms. The increased biomass of a non-axenic green algal culture (*Selenastrum*) in the presence of geosmin (480 ng L^{-1}) suggests that it may function as an antibiotic, reducing the populations of parasitic bacteria or fungi that antagonise algae. Indeed, several studies have

provided evidence that *Streptomyces* reduced the activity of other bacteria and fungi in aquatic environments (Aumen 1980; Wohl & McArthur 2001). Recently, the antifungal activity of geosmin and 2-methylisoborneol, among other volatile organic compounds produced by *S. alboflavus* was reported (Wang *et al.* 2013).

A TERRESTRIAL ORIGIN OF *ACTINOBACTERIA* AND T&O?

As soil is a rich reservoir of *Actinobacteria*, records of their occurrence in water samples must be considered critically with regards to their origin, specifically whether or not they represent indigenous aquatic inhabitants or terrestrial wash-in forms (Cross 1981). Periods of high runoff can introduce the soils of drainage basins, entrained with *Actinobacteria* and their associated odourous secondary metabolites into surface waters causing T&O episodes. The source of annual spring earthy-musty odour in the Saskatchewan River (Canada) was traced to high numbers of *Streptomyces* (up to 4.5×10^4 cfu mL^{-1}), which, along with their odourous metabolites, were thought to be introduced into these surface waters during snowmelt and subsequent runoff (Jensen *et al.* 1994). The majority of isolates (58%) were able to produce geosmin and/or 2-MIB. Futhermore, in the Elbow River Basin and Lake Ontario (Canada), Actinobacterial abundance, geosmin and 2-MIB were associated with parameters indicative of terrestrial runoff (*E. coli*, turbidity and suspended sediment) (Zaitlin *et al.* 2003a, b).

ACTINOBACTERIA AND T&O PRODUCTION IN AQUATIC ENVIRONMENTS

The capacity of *Actinobacteria* to be metabolically active in aquatic environments has not yet been elucidated and many researchers argue that those isolated from such environments are not truly aquatic, but rather an artefact of runoff and spore survival (Johnston & Cross 1976a; Cross 1981). Indeed Cross (1981) referred to aquatic *Actinobacteria* as 'mythical' organisms. Wood *et al.* (1983) argued that whilst it is plausible to consider the terrestrial wash-in hypothesis of *Actinobacteria*, there seems no reason to assume that

they cannot grow in aquatic environments, given suitable environmental conditions for their growth and activity. These bacteria readily grow and produce earthy-musty odour in liquid media and there are numerous reports of *Streptomyces* differentiation and sporulation occurring under such conditions (van Keulen *et al.* 2003; Manteca *et al.* 2008). Furthermore, gas vesicle protein gene clusters with close homology to those found in cyanobacteria have been detected in the genome of *Streptomyces*, implying that they may also possess gas-filled organelles that function as flotation devices. This suggests that these bacteria, primarily regarded as soil saprophytes, may also be physiologically adapted to the water environment (van Keulen *et al.* 2005).

Actinobacteria have been isolated from samples taken many kilometres from land and at great oceanic depths and research using molecular techniques is providing more evidence supporting that they may be autochthonous and metabolically active inhabitants of marine communities (Jensen *et al.* 1991, 2005; Bredholt *et al.* 2008). Moran *et al.* (1995) extracted *Streptomyces* rRNA from coastal salt marsh sediment at concentrations representing 2–5% of the sediment RNA. As dormant spores cannot produce rRNA, hybridisation analyses using *Streptomyces* specific probes indicated they were actively growing. Isolates of the genus *Salinispora* were found to be present as vegetative forms in marine sediments at great depths (1,100 m) by using a DNA extraction technique which excludes spores (Mincer *et al.* 2005).

What evidence do we currently have for their growth within freshwater environments? Actinobacterial genera such as *Streptomyces, Micromonospora, Actinoplanes, Rhodococcus* and *Thermoactinomyces* have been frequently isolated from freshwater habitats including the waters, sediments and vegetation of lakes, reservoirs and rivers. However, the extent to which they represent a physiologically active and ecologically significant component of freshwater microbial communities remains unresolved. Such knowledge is critical to understand their capacity to contribute to *in situ* T&O production (Wood *et al.* 1983; Zaitlin & Watson 2006). *Streptomyces*, the main genus known to produce odourous metabolites, were described by Cross (1981) as 'terrigenous species which occasionally find themselves in water' (p. 407). However, the isolation of several phages specific to *Streptomyces* from the surface

of lake mud provided early evidence that they may be active in such environments (Willoughby 1974). *Micromonospora* are generally more abundant in lake environments than *Streptomyces*, where they have been regarded as a truly indigenous group of microbial inhabitants (Willoughby 1969; Johnston & Cross 1976a; Jiang & Xu 1996). Supporting this, Johnston & Cross (1976a) found significant amounts of *Micromonospora* mycelium in lake sediments using homogenisation and sonication experiments. They have been regarded as non-producers of T&O in the literature, but recently one species (*M. olivasterospora*) was reported by Citron *et al.* (2012) to possess the gene encoding 2-MIB synthase and was confirmed to be a 2-MIB producer.

Species of *Actinoplanes* have been isolated directly from river and lake water and have been found associating with allochthonous decomposing vegetation around lake edges and shallow streams (Willoughby 1971; Willoughby *et al.* 1972; Johnston & Cross 1976a; Makkar & Cross 1982). Makkar & Cross (1982) demonstrated that they have motile zoospores and Willoughby *et al.* (1972) found a phage specific to this genus from stream and lake water, which both serve as evidence for aquatic activity. There are currently no reports that species in this genus can produce T&O. *Nocardia* have been isolated from streams, rivers and lakes in low numbers, and have been assumed to have originated from inactive spores of terrestrial origin (Willoughby 1969; Johnston & Cross 1976a). Three *Nocardia* isolates have been shown to produce geosmin in the laboratory (Gerber 1979; Schrader & Summerfelt 2010). A strain of *Microbispora rosea* was shown by Gerber & Lecheval (1965) to produce geosmin in much higher concentrations (5.8 μg mL^{-1}) than the greatest yielded by a *Streptomyces* (1.75 μg mL^{-1}), but this genus has only been detected in very low numbers in lake sediments (Johnston & Cross 1976a). Gerber (1969) showed that a strain of *Actinomadura* could produce 2-MIB in culture, but the genus is not regarded as aquatic. Similarly, species of other genera including an *Actinomyces* from lake sediment (Kikuchi *et al.* 1973) and, more recently, strains of *Actinosynnema, Kitasatospora, Rothia* and *Saccharopolyspora* (Citron *et al.* 2012) have demonstrated geosmin and/or 2-MIB production in culture but have not been identified in T&O source tracking studies. It may be that conventional isolation and enumeration procedures have overlooked the occurrence of these

non-*Streptomyces* and their potentiality to contribute to T&O events (Wood *et al.* 1983).

Mere isolation of *Actinobacteria* from a water source is not evidence that they caused a T&O event (Zaitlin & Watson 2006). The advent of culture-independent molecular techniques has made it possible to differentiate between actively growing *Actinobacteria* and dormant spores in aquatic environments. Fluorescence *in situ* hybridisation (FISH) targeting ribosomal RNA has been used to indicate the abundances of active filamentous *Actinobacteria* (Sekar *et al.* 2003; Klausen *et al.* 2005; Nielsen *et al.* 2006). The increased understanding of the genes involved in geosmin and 2-MIB biosynthesis may provide a more relevant tool to detect and track the emergence of T&O producing *Actinobacteria*, particularly as methods such as plate counting and FISH cannot distinguish between producers and non-producers of these compounds. Auffret *et al.* (2011) reported the use of primers targeting *Streptomyces* geosmin and 2-MIB synthase genes which combined with techniques such as real-time polymerase chain reaction (qPCR), may be an effective means to measure the level of T&O metabolite producers prior to the occurrence of a T&O episode. More recently Du *et al.* (2013) developed a qPCR protocol using *Streptomyces*-specific primers targeting the geosmin synthase gene (*geo*A) to quantify and monitor the presence of geosmin producers in the Chinese-liquor making processes. Similarly, qPCR has been applied to quantify geosmin producing *Anabaena* species in freshwater systems (Su *et al.* 2013). In both cases, geosmin concentrations were correlated with the bacterial populations detected by qPCR, demonstrating the application of such protocols for monitoring potential T&O development.

Any attempt to source track and control T&O necessitates the identification of the causal organisms, their growth sites and conditions which trigger their production of these odourous secondary metabolites. A review of current knowledge regarding *Actinobacteria* within freshwater environments and their association with T&O is presented below.

The water mass

The water mass of lakes, rivers and reservoirs has often been regarded as an unsuitable environment for the growth of *Actinobacteria* with counts considered too low to contribute

to T&O production (Sugiura & Nakano 2000; Juttner & Watson 2007). However, a source of nutrient enrichment may allow them to become metabolically active and important producers of T&O in the water mass (Bays *et al.* 1970). Wood *et al.* (1985), after demonstrating that oligotrophic reservoir water supported sparse growth of *S. albidoflavus*, showed that growth and geosmin production increased (3,900 ng L^{-1}) with the supplementation of carbon, nitrogen and phosphorus. Such nutrients could be obtained from suspended sediment material, plant debris, algae or from soil leachates. Some authors have detected high abundances of *Actinobacteria* in the water mass during periods of low cyanobacterial biomass and have identified coincidences with T&O (Henatsch & Juttner 1990; Lanciotti *et al.* 2003).

Molecular techniques are providing convincing evidence to support that *Actinobacteria* are active and abundant microbial inhabitants in the water column and may contribute more to T&O problems than previously expected, 16S rRNA sequencing of water samples from the cold and oligotrophic Lake Baikal (Russia) showed that up to 30% of the microbial population were *Actinobacteria* (Denisova *et al.* 1999). A RNA hybridisation protocol (FISH-CARD) also demonstrated high abundances of *Actinobacteria* (32–55%) relative to the total bacterial planktonic communities in lakes of different trophic status (Sekar *et al.* 2003). Using this technique, Nielsen *et al.* (2006) detected higher activity of *Actinobacteria* (33–49%) compared to other bacteria (27–39%) in the surface and bottom water of the North Pine River Dam (Australia). Furthermore, high numbers of filamentous *Actinobacteria* (1.3×10^8 cells L^{-1}) were detected, which suggests that *Streptomyces* may be metabolically active in water. In the same reservoir, rRNA analyses revealed that *Actinobacteria* comprised 18–24% of all bacteria during a summer period of detectable geosmin and 2-MIB with 5–10% of the population estimated to be filamentous *Actinobacteria* (Klausen *et al.* 2004). The low T&O producing cyanobacterial biomass (<1%) during this period suggested that *Actinobacteria* were major T&O producers.

Recently, qPCR using genus specific primers was used to determine the abundance of *Streptomyces* in rivers and reservoirs in southeast Queensland, Australia. *Streptomyces* were detected in all locations (average of 225 cells L^{-1} in surface waters and up to 45,650 cells L^{-1} at 8.5 m depth)

aside from the deepest bottom waters (35–40 m depth) which the authors attributed to anoxic conditions. However, based on the densities obtained and cell-specific production rates of geosmin and MIB by *Streptomyces* determined by Klausen *et al.* (2005), the authors concluded that *Streptomyces* may not to be a major source of these odourous compounds (Lylloff *et al.* 2012).

Vegetation

Decaying vegetation and macrophyte communities along the margins of lakes, reservoirs and rivers have been considered to be a potential site of T&O production by *Actinobacteria* (Raschke *et al.* 1975; Cross 1981; Makkar & Cross 1982; Goodfellow & Williams 1983). This seems feasible given that their role as decomposers of complex plant polymers is firmly established. High counts of *Streptomyces*, *Actinoplanes* and *Micromonospora* have been obtained from plant debris adjacent to lakes and rivers (Raschke *et al.* 1975; Persson & Sivonen 1979; Makkar & Cross 1982). Wood *et al.* (1985) found that significant concentrations of geosmin were produced when *S. albidoflavus* was grown on sterilised plant debris (180–6,700 ng kg^{-1}) collected from the margins of English reservoirs. The wet-dry regime that this marginal habitat experiences may provide an ideal situation for their synthesis of T&O metabolites in aerobic conditions during dry periods, followed by their subsequent wash-in by the action of rain, waves and rising water levels (Goodfellow & Williams 1983; Wood *et al.* 1983).

Many rivers, natural lakes and reservoirs have large areas of shallow water which support extensive communities of submerged, emergent and floating macrophytes (Silvey & Roach 1975). Besides their beneficial ecosystem services such as supplying oxygen, macrophytes provide an ideal habitat for *Actinobacteria*, where they can obtain nutrients and grow and sporulate due to aerobic conditions as water levels fluctuate (Silvey & Roach 1975; Cross 1981). *Actinobacteria*, predominately *Streptomyces*, have been found to be particularly associated with dying macrophytes, with numbers increasing as vegetation begins to decompose (Silvey & Roach 1975; Wohl & McArthur 1998). Large populations have been observed growing on aquatic emergent vegetation such as *Typha* and *Lemna* in various reservoirs

(Silvey *et al.* 1950; Bartholomew 1958). Silvey & Roach (1975) refer to observations in a water reservoir in East Texas, which experienced intense T&O and supported a high population of *Chara* and *Najas*, with *Actinobacteria* readily isolated from these macrophytes. Once the macrophyte populations were controlled at low densities, T&O problems disappeared. Zaitlin *et al.* (2003b) also detected *Actinobacteria* associated with macrophytes (*Valisnerai* and *Myriophyllum* spp.) and overlying water samples (10–340 cfu mL^{-1}) collected in Lake Ontario (Canada), with some *Streptomyces* isolates capable of producing geosmin and 2-MIB.

Sediments and muds

Benthic sediments and muds in freshwater environments have been recognised as a possible habitat for Actinobacterial growth and T&O production for some time (Adams 1929; Thaysen 1936; Issatchenko & Egorova 1944; Romano & Safferman 1963; Bays *et al.* 1970; Willoughby *et al.* 1972). Sediments are an important reservoir of nutrients potentially available to *Actinobacteria*, with their abundance often correlated with sediment nutrient status (Johnston & Cross 1976b; Sugiura *et al.* 1987; Jiang & Xu 1996). Under anoxic conditions in sediments, they are unlikely to be metabolically active and produce T&O. Wood *et al.* (1985) found that the exposure of reservoir sediment during maintenance work led to earthy odour development, previously being undetectable in unexposed sediment. Furthermore, sterilised sediment was found to produce geosmin (460 ng kg^{-1}) when inoculated with *S. albidoflavus*. Shallow lakes with high summer temperatures and a supply of degradable organic matter would indeed provide ideal conditions for Actinobacterial growth, particularly those with fluctuating levels, which provides aeration and therefore enables sporulation and secondary metabolism to occur (Cross 1981). Silvey & Roach (1975) suggest that the replenishment of oxygen to sediments during lake overturn may activate dormant *Actinobacteria*, leading to T&O production. The authors claim that this phenomena has been observed in many water supplies in the USA, but such a potential mechanism of T&O production is yet to be systematically investigated (Zaitlin & Watson 2006). Interestingly, Guttman & Rijn (2008) showed that although aerobic conditions supported

higher geosmin and 2-MIB production, low concentrations were also observed in anaerobic cultures of *Streptomyces* spp. which were thought to be using nitrate as an electron acceptor.

T&O source tracking studies using RNA hybridisation techniques have found that *Actinobacteria* may be metabolically active components of sedimented communities. Klausen *et al.* (2004) found a coincidence between high geosmin and 2-MIB concentrations and high abundance of *Actinobacteria* ($20–60 \times 10^6$ cells g^{-1}) in the upper 2 cm of surface sediment in the North Pine Dam (Australia). Nielsen *et al.* (2006) also detected a 35–80% higher proportion of active Actinobacterial cells in the surface sediment of the same reservoir and both authors suggest that the aeration systems deployed in the dam combined with organic matter availability provides an ideal habitat for *Actinobacteria* and therefore T&O production. In hypereutrophic Lake Kasumigaura (Japan), T&O episodes have been associated with *Actinobacteria* growing in benthic sediments. Sugiura *et al.* (1987) found that counts correlated with increasing musty (2-MIB) odour and organic carbon content and Sugiura *et al.* (1994) attributed the Actinobacterial odour production to their utilisation of sedimented algae and cyanobacteria as carbon substrates. In the same lake, Sugiura & Nakano (2000) found that sedimented *Actinobacteria* (up to 16,000 cfu g^{-1}) correlated with geosmin concentration with 40 isolates confirmed producers. Several other studies have isolated geosmin and/or 2-MIB producing *Actinobacteria* from sediment and have regarded them as the cause of earthy-musty odour (Zaitlin *et al.* 2003b; Tung *et al.* 2006; Zuo *et al.* 2009, 2010; Lee *et al.* 2011). In order to evaluate their contribution to T&O, it is critical to understand whether they are metabolically active in this habitat and elucidate the environmental factors which stimulate their production of these secondary metabolites.

Other aquatic habitats

Drinking water distribution systems may be oligotrophic habitats, however oxygenated water and areas of slow flow where sediments and organic debris accumulate, may provide safe havens for biofilm establishment and optimal conditions for chronic T&O production by *Actinobacteria*

(Geldreich 2002; Lanciotti *et al.* 2003). Counts of up to 4.4×10^5 cfu L^{-1} have been found in old soft deposits, three orders of magnitude greater than in water leaving the waterworks (Zacheus *et al.* 2001) and Silvey & Roach (1975) reported high numbers (180,000–135,000 cfu mL^{-1}) in distribution systems during warm temperatures alongside elevated T&O. *Streptomyces* spores, being relatively resistant to chlorination, readily breakthrough to the distribution system which may be an important site of T&O production (Jensen *et al.* 1994).

Evidence suggests that *Streptomyces* may live in association with filter feeding mussels and produce T&O. In the North American Great Lakes, T&O problems have coincided with the occurrence of heavy exotic Zebra Mussel (*Dreissena*) infestations. Geosmin and 2-MIB have been detected in concentrated *Dreissena* faeces/pseudo-faeces and interstitial material which also supported the growth of *Streptomyces* (Lange & Wittmeyer 1997). Similarly, Zaitlin *et al.* (2003b) detected geosmin and several isolates of *Streptomyces* in mussel and overlying water samples in Lake Ontario. The organic material provided by mussel filter feeding and dissolved oxygen from siphon activity may provide ideal conditions for *Streptomyces* to thrive in mussel beds and produce T&O.

Actinobacteria have been isolated from periphyton and this complex mixture of microorganisms and detritus growing attached to submerged surfaces has been identified as a source of geosmin and 2-MIB by several authors (Zaitlin *et al.* 2003b; Watson & Ridal 2004; Ridal *et al.* 2007). Its major consitituents (algae and cyanobacteria) can act as a growth substrate for *Actinobacteria* which may support geosmin and 2-MIB production (Schrader & Blevins 1999). T&O problems have been ascribed to *Streptomyces* growing in the decaying filaments of algal blooms in surface waters and lyophilised cyanobacteria have been shown to support odour production by benthic *Streptomyces* isolates (Silvey & Roach 1953; Sugiura *et al.* 1994). However, as cyanobacteria can also produce these compounds, their cell-bound odourous metabolites may be the major sources of T&O upon decomposition (Juttner & Watson 2007).

In addition to drinking water supplies, geosmin and 2-MIB are problematic in aquaculture where they bioaccumulate in the lipid rich tissues of fish creating great economic losses (Guttman & van Rijn 2008). Several studies

have pointed to *Actinobacteria* as the likely offenders of this tainting including *Nocardia* and *Streptomyces* species isolated from the water and sludge of aerobic sections in recirculating aquaculture systems (RAS) (Guttman & van Rijn 2008; Schrader & Summerfelt 2010). Pan *et al.* (2009) isolated species of *Streptomyces*, *Nocardia*, *Micromonospora* and *Actinomadura* (counts up to 1.5×10^6 cfu mL^{-1}) from the odour affected brackish waters of intensive cultivation fishponds in Tianjin (China), with one *Streptomyces* isolate capable of producing large amounts of 2-MIB. Given the significant positive correlation between Actinobacterial biomass and 2-MIB concentration in the ponds, they were regarded as responsible for the T&O. Recently, Auffret *et al.* (2013) monitored the abundance of the *Streptomyces* geosmin synthase gene (*geo*A) using qPCR in two RAS units. Although geoA was detected and coincided with higher geosmin concentrations in fish flesh, *Streptomyces* 16S rRNA gene sequences were not identified. Sequences associated with other geosmin producers including *Sorangium* and *Nannocystis* (*Myxococcales*) were detected, and *geo*A qPCR fragments generated were more related to the amino acid sequences from these taxa. Thus *Myxobacteria* may be an underestimated source of off-flavour compounds in aquaculture and indeed, drinking water supplies.

CONCLUSION

Evidence for the widespread distribution, abundance and diversity of *Actinobacteria* in natural and man-made aquatic environments has been appraised. Cultivars isolated from freshwater habitats including sediment, vegetation, the water mass or more specialized substrates (e.g. mussels or periphyton) readily demonstrate geosmin and 2-MIB producing abilities *in vitro* and may indeed be potent sources of earthy-musty T&O. To elucidate the contribution of these bacteria to this aesthetic water quality problem, more research is required to verify their capability to be metabolically active in such habitats. Culture-dependent techniques alone cannot achieve this, being constrained by both the unculturability of many *Actinobacteria* and the inability to discriminate between active and dormant forms. The development and application of nucleic-acid molecular methods such as the analysis of rRNA using hybridisation methods

can enable the identification of the active population of microbial communities, such as *Streptomyces* in aquatic environments. In particular, the PCR together with recent advances in our understanding of the genes responsible for geosmin and 2-MIB biosynthesis in *Actinobacteria* provides a research avenue to enormously extend our knowledge regarding the presence and activity of these bacteria in drinking water supplies and their potentiality to contribute to T&O episodes.

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