Evaluation of denaturing gradient gel electrophoresis for the detection of mycobacterial species and their potential association with waterborne pathogens

G. Aboagye and M. T. Rowe

ABSTRACT

The versatility of denaturing gradient gel electrophoresis (DGGE) protocol provides enough grounds for its wide application over an array of microorganisms. This work was designed to evaluate DGGE for the detection and confirmation of mycobacteria and their association, if any, with waterborne pathogens. A total of 76 samples comprising raw untreated water, schmutzdecke, floccules and final treated water obtained from a common water source, and two water treatment works (WTW1 and WTW2), were analysed. Thirty-five species were identified from the overall samples, with 7% (5/76), 13% (10/76) and 26% (20/76) from the common raw water source, WTW1 and WTW2 respectively. The majority of the species were Cyanobacteria, with high dominance in the raw water entering WTW2. In the final treated water of WTW1 *Eutreptiella braarudii* was found, and that of WTW2 contained *Anabaena nereformis*, *Anabaena torulosa* and *Podocarpus nerrifolius*. Furthermore, one *Mycobacterium* species was found in the raw water of WTW1 aside from the detection of *Mycobacterium avium* ssp. *paratuberculosis* by the technique. No association between mycobacteria and the other species was observed. This implies DGGE may be employed to study the diversity of other akin mycobacterial species from various sources, and not as a direct means of elucidating microbial associations.

Key words | denaturing gradient gel electrophoresis, diversity of microorganisms, potable water, waterborne pathogens, water treatment works

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INTRODUCTION

Mycobacteria reside in the environment and are known to survive for protracted periods in soils (Heitkamp & Cerniglia 1989; Whittington *et al.* 2003), sediments (Iivanainen *et al.* 1999; Daane *et al.* 2001), groundwater, surface water, wastewater, recreational water and tap water (USEPA 2002; Lee *et al.* 2008; Thomson *et al.* 2008). The species of *Mycobacterium* are characterised by genotypic resemblance, wide differences in phenotypic traits (Alexander *et al.* 2009), and exhibition of nucleotide polymorphisms that allow their differentiation from other microflora based on the genomic sequence of their DNA (Green *et al.* 2015; Filliol *et al.* 2006). This is demonstrated by *Mycobacterium avium* ssp. *paratuberculosis* (Map) which is 90% similar at the nucleotide level to its close relatives (the *M. avium* complex), yet members of this complex differ widely by their pathogenic potentials and disease phenotypes (Jaykus *et al.* 2009). It has been shown that mycobacteria interact with other organisms including protozoa to enhance virulence, as well as facilitate their transfer between locations (Cirillo *et al.* 1997). The study of microbial diversity and their interrelationships has employed denaturing gradient gel electrophoresis (DGGE) to elucidate bacterial community structure (Kawai *et al.* 2004), degradation of polycyclic aromatic compounds in soils with diverse microbial populations (Leys *et al.* 2005;

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Uyttebroek *et al.* 2007), and profiling of microbial communities in water samples (Williams *et al.* 2009; Kumar *et al.* 2013; Ma *et al.* 2014; Otten *et al.* 2016). Based on 1% of species in a total microbial population, detectable bands can be visualized on a DGGE gel (Green *et al.* 2015). Kalle *et al.* (2014) reported DGGE as a simple, rapid method able to detect mutations and polymorphisms, insertions and deletions, and Li *et al.* (2012) indicated its usefulness in the detection of mutations associated with antibiotic resistance in *Mycobacterium tuberculosis*. While findings in research have indicated environmental persistence and virulence of pathogens through associations and interactions they manifest with other species (Primm *et al.* 2004; Biet *et al.* 2005; King *et al.* 2016), employing DGGE to determine the diversity of slow-growing environmental mycobacteria and their associations with other waterborne pathogens remains a gap to be filled. In this study, preliminary work was carried out to determine the capacity of DGGE to detect slow growing mycobacteria from a population of other microbial species in pure cultures, before being applied to a wide range of samples such as raw water, schmutzdecke, floccules and final water that were obtained from requisite routes of water treatment operations.

MATERIALS AND METHODS

Sampling

Samples of raw water and sediment, schmutzdecke, floccules and final water were taken from the common raw water source (abstraction site), WTW1 and WTW2, as shown in Table 1.

Table 1 | Sample location and number of samples obtained from the respective sites

Sample location	Sample type	Number of samples taken
Common raw water source	Raw water and sediment	26
WTW1	Raw water	10
	Schmutzdecke	5
	Final water	10
Total for WTW1		25
WTW2	Raw water	10
	Floccule	5
	Final water	10
Total for WTW2		25
Grand total		76

Extraction of bacterial DNA from samples

One millilitre each of raw untreated water, schmutzdecke, floccules and final treated water samples (a grand total of 76 samples) were subjected to centrifugation (7,558 × g) for 30 min before the upper aqueous layer was decanted. To the remaining pellets at the bottom of the Eppendorf tubes, 700 TEN lysis buffer containing 25 μg proteinase K was added. The resulting sample was vortexed until the contents were completely mixed before being incubated at 25 °C overnight. Following overnight incubation, the lysis solution containing pellets was transferred into FastProtein Blue tubes containing matrix B (a further lysing agent) and subjected to disruption at 6.5 m sec^{-1} for 45 s before being placed on ice for 15 min to settle the foam generated. The settled solution containing the crude DNA of bacteria in the original sample was subjected to further DNA extraction and purification procedures as outlined in the following section.

DNA extraction and purification

Seven hundred microlitres of phenol: chloroform: isoamylalcohol (25:24:1), pH 8.0 (Sigma) was added to each FastProtein Blue tube containing the disrupted Map cells. The tubes were centrifuged (Biofuge Pico Heraeus, Osterode, Germany) for 10 min at 7,826 × g. The top aqueous layer of the sample was transferred into a micro-centrifuge tube containing 400 μL of isopropanol to precipitate the Map DNA. The tubes were then placed in a –20 °C freezer for 30 min. The DNA was recovered by centrifuging again at 7,826 × g for 10 min and washing once with 500 μL of 70% v/v ethanol. The ethanol was decanted and the DNA pellet allowed to air dry for 30 min at room temperature before being re-suspended in 50 μL Tris-EDTA (TE) buffer pH 8.0 and stored at –20 °C until required.

Polymerase chain reaction

The polymerase chain reaction employed here involved the use of forward and reverse universal primers (Invitrogen

Biosciences) with the following sequences: *Forward primer (338GC)* Sequence: CGC CCG CCG CGC CCC CGC CCC GGC CCG CCG CCC CCG CCC ACT CCT ACG GGA GGC AGC. *Reverse (530R)* Sequence: GTA TTA CCG CGG CTG CTG. The DGGE-PCR reaction using the above two primers was performed using quantities of requisite materials and template DNA as follows: $MgCl_2$ (2.5 μL), PCR buffer without Mg, 10× (5.0 μL), dNTP mixture (10 mM, 0.5 μL), Forward primer (338GC) 0.5 μL, Reverse primer (530R) 0.5 μL, Taq DNA polymerase (5 U/μL) 0.25 μL, Template DNA (2.5 μL), Sterile nuclease free water (38.25 μl). The PCR reaction was performed in a Thermocycler with cycle conditions as follows: 1 cycle of 95 °C for 2 min, 95 °C for 1 min followed by 35 cycles of 42 °C for 1 min, 72 °C for 1 min followed by a final extension of one cycle of 72 °C for 5 min and held at 4 °C before analysis.

DGGE profiling

Preparation, running and analysis of DGGE gradient

Twenty and 80% DGGE gradients with 6% denaturant (calculated by adding 7.5 mL of 40% of acrylamide to a final gradient composition of 50 mL) were prepared by adding known volumes and weights of 40% acrylamide, urea, formamide and 50× TAE buffer all from Sigma, to a sterile graduated tube and adding distilled water to a final volume of 50 mL as follows: 40% acrylamide at 20% gradient: 7.5 mL; 80% gradient: 7.5 mL; Urea; 4.2 and 16.8 g; Formamide: 4.0 and 16.0 mL; 50× TAE buffer; 1 mL each of the 20 and 80% gradients; distilled water: all the gradients to a final volume of 50 mL. To 11.5 mL of the 20 and 80% gradients in sterile graduated tubes on ice was added 50 μL 10% ammonium persulphate (APS, Sigma) and 5 μL N,N,N,N-Tetramethylethylene-diamine-catalyst, TEMED (Sigma), vortexed to mix and immediately poured onto separate columns of a gradient maker at 80 and 20% to the right and left holes of the gradient respectively. Excess liquid droplets were dried with blotting paper and a Pasteur pipette was used to fill the comb chamber with stacking gel consisting of 5 mL 0% gradient prepared by adding 7.5 mL, 40% acrylamide and 1 mL, 50× TAE buffer, to 50 mL distilled water), 5 μL TEMED and 50 μL APS. The stacking gel was allowed to attain firmness for at least 1 h before the comb was removed, and the gel was flushed with TAE buffer to fill and demarcate the wells before loading, running and analysing the samples.

Sequencing PCR

Single bands were cleaned to avoid contamination by DNTP and other artefacts resulting from the PCR reaction. The cleaning process was performed by employing the charge-switch kit (Invitrogen Biosciences), containing purification buffer (N5), magnetic beads, wash buffer (W12) and elution buffer (E5). The resulting DNA was stored at –20 °C until required. Multiple bands resulting from amplified DNA fragments of excised DGGE bands were subjected to cloning, employing the *Topo TA* cloning kit (Invitrogen Biosciences), which enabled separation of the individual fragments prior to sequencing. The sequencing PCR employed Terminator ready reaction mix (TRRM), TRRM buffer, M13 forward and reverse primers, nuclease free water and template DNA, all in a final reaction volume of 20 μl as follows: TRRM (v 3.1) 2.0 μL; TRRM buffer 6.0 μL, M13 combined forward and reverse primer set 1.0 μL, Template DNA 1.0 μL; Sterile nuclease free water 10.0 μL. Positive and negative controls were set by adding 1 μL each of pGEM (3Zf; 0.2 μg μL^{-1}) control template supplied as part of the Big Dye terminator kit (BDTK v3.1, Applied Biosystems), and nuclease free water respectively, to 19 μL of the sequencing PCR mix containing M13 forward primer (0.8 pmol μL^{-1}, supplied as part of the BDTK). The following conditions were employed for the sequencing PCR: one cycle at 96 °C for 1 min, 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min, then one cycle at 4 °C before analysis. The resulting products were subjected to ethanol extraction that served to purify the amplified DNA fragments produced, which were then analysed in a Genetic Analyser (Applied Biosystems) and subjected to base alignment analysis using the NCBI GenBank comparison database.

RESULTS

DGGE profiling

Figure 1 is an example of the many DGGE-PCR performed before the DGGE profiling. The figure also shows the

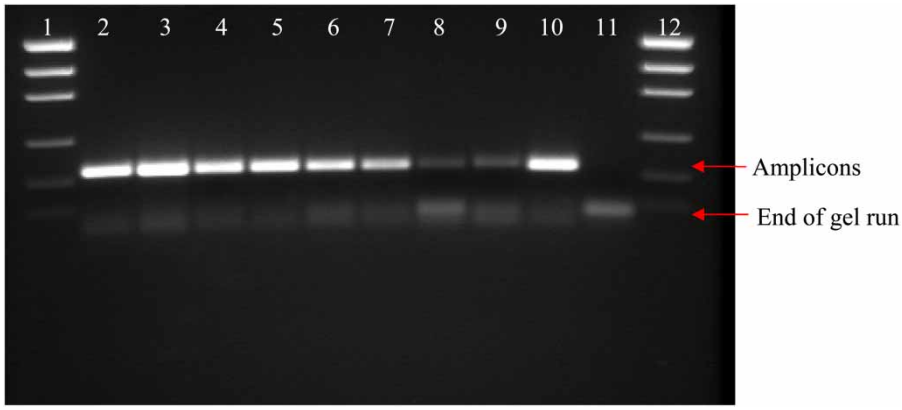

Figure 1 | DGGE-PCR of DNA isolated from Map (from different sources) and raw water. Lanes 2 and 3: bovine Johne's Disease (JD), lane 4: raw milk isolate, lanes 5 and 6: same source raw and pasteurised milk isolates, lane 7: Crohn's disease (CD) patient isolate, lanes 8 and 9: raw water, lane 10: positive control (Map isolates obtained from bovine JD, milk and CD patient), lane 11: negative control (nuclease free water).

amplified DNA fragments of Map isolates from bovine JD, milk, CD patient, and DNA isolate from raw water samples. The amplified DNA (lanes 2–7) was obtained from Map isolates from different sources cultured under optimum growth conditions (37 °C and in M-7H9 supplemented with mycobactin J), and amplified DNA (lanes 8 and 9) was obtained from raw water samples also cultured under optimum growth conditions as given for the Map isolates. The resulting bands of the Map isolates appeared as intense bands while bands of the raw water samples were faint.

The DGGE-PCR resulted in bands >200 and less than 400 base pairs (band sizes; positions 1 and 12 from top down to bottom corresponds to 2,000, 1,200, 800, 400, 200 and 100 of low mass DNA ladder, Invitrogen Biosciences).

Following the DGGE-PCR results (Figure 1), the amplified bands were analysed by DGGE profiling as shown in Figure 2.

In Figure 2 above, the DGGE profiling of the DNA amplified products obtained from Map isolates (from bovine JD, milk and CD patient) and raw water showed

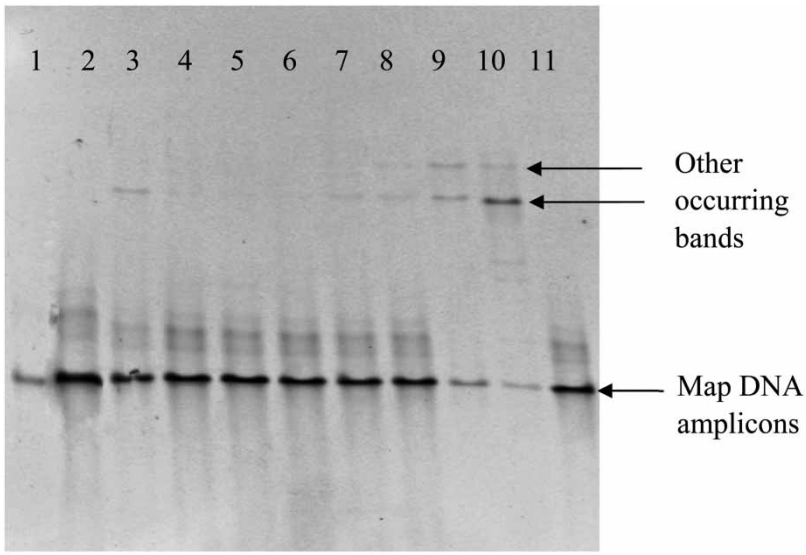

Figure 2 | DGGE profiling of DNA of Map (from different sources including bovine JD), milk, and CD patient) and raw water samples. The samples were arranged as follows: lane 1: positive control (Map isolates obtained from bovine JD, milk and CD patient), lanes 2 and 3: bovine JD, lane 4: raw milk isolate, lane 5: raw milk isolate, lane 6: positive control (Map isolates obtained from bovine JD, milk and CD patient), lane 7: pasteurised milk isolate from raw milk of lane 5, lane 8: CD patient isolate, lanes 9 and 10: raw water, lane 11: positive control (Map isolates obtained from bovine JD, milk and CD patient).

intense bands corresponding to the position of the positive control amplicon. The results for the raw water could be a confirmation that Map was present, since the profile also contained DNA amplicons that perhaps occupied the same position as those of the Map isolates and the positive control. Other faint bands were also observed with the samples, which could be due to other bacterial species in the raw water samples. The results, as shown in Figures 1 and 2, indicated the successful application of DGGE for determining mycobacterial species including Map in different sources including water. Using this successful procedure, other water samples were analysed. In Figures 3–5, raw untreated water profiles from a water treatment works (WTW1, Figure 3, lanes 10 and 11; Figure 5, lanes 7–9) showed similar band profiles for the two samples loaded into the respective lanes. Raw untreated water samples were taken from another water treatment facility (WTW2, Figure 5, lanes 1–5). Between both WTWs, the profiles were not exactly the same between the said lanes even though both had their raw untreated water from one source. The schmutzdecke and floccules from slow sand filtration, SSF (Figure 3, lane 4) and dissolved-air flotation, DAF (Figure 4, lanes 6 and 7) respectively, however, showed differences in profile. Since the SSF and DAF operate differently, with the former being a biological and the latter a physical process, it is not unusual to have different profiles for samples lifted from those places (SSF and DAF). The resulting bands in Figure 3, lanes 5 and 6, from WTW1, and Figure 4, lanes 8 and 9, from WTW2, looked similar. However, Figure 4, lanes 10 and 11, showed two similar final treated water profiles with samples taken on two different dates while the other two (lanes 12 and 13) showed dissimilar profiles, also with samples taken on different dates. The final treated water profiles for WTW2 (Figure 3, lanes 3, 7 and 9; Figure 4, lanes 8 and 9 and Figure 5, lane 6) were also different. The different times of sampling could have played a role with the differences in profiles observed here.

Species identified

After sequencing the excised bands, a total of 35 species were identified from the three sites as shown in Table 2 by similarity sequence match with the NCBI BLAST database.

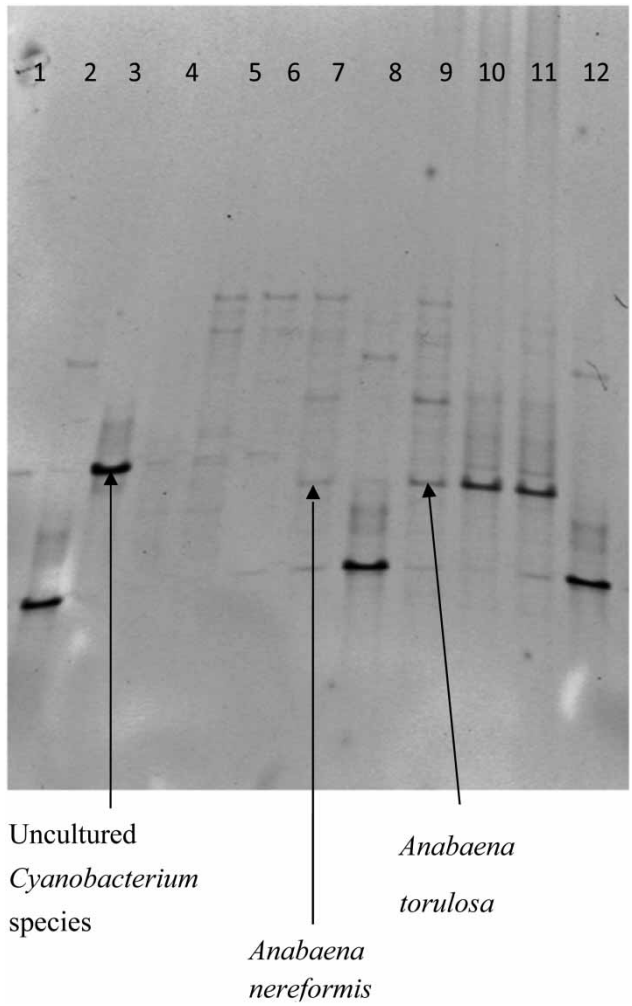

Figure 3 | DGGE profiling of water samples obtained from WTW1 and WTW2. Lane 1: incomplete lane of positive control, lane 2: positive control (Map isolates obtained from bovine JD, milk and CD patient), lane 3: final treated water from WTW2, lane 4: schmutzdecke of SSF from WTW1, lanes 5 and 6: final treated waters from WTW1, lane 7: final treated water from WTW2, lane 8: positive control (Map isolates obtained from bovine JD, milk and CD patient), lane 9: final treated water from WTW2, lanes 10 and 11: raw untreated waters from WTW1, lane 12: positive control (Map isolates obtained from bovine JD, milk and CD patient).

DISCUSSION

DGGE is a molecular assay employed to study the diversity of microorganisms in any given sample or medium (Hurtado-Alarcon & Polania-Vorenberg 2014). The DGGE principles were applied on a limited number of environmental samples obtained from a common raw water source used as an abstraction site by WTW1 and WTW2. Corresponding raw water, schmutzdecke, floccules and

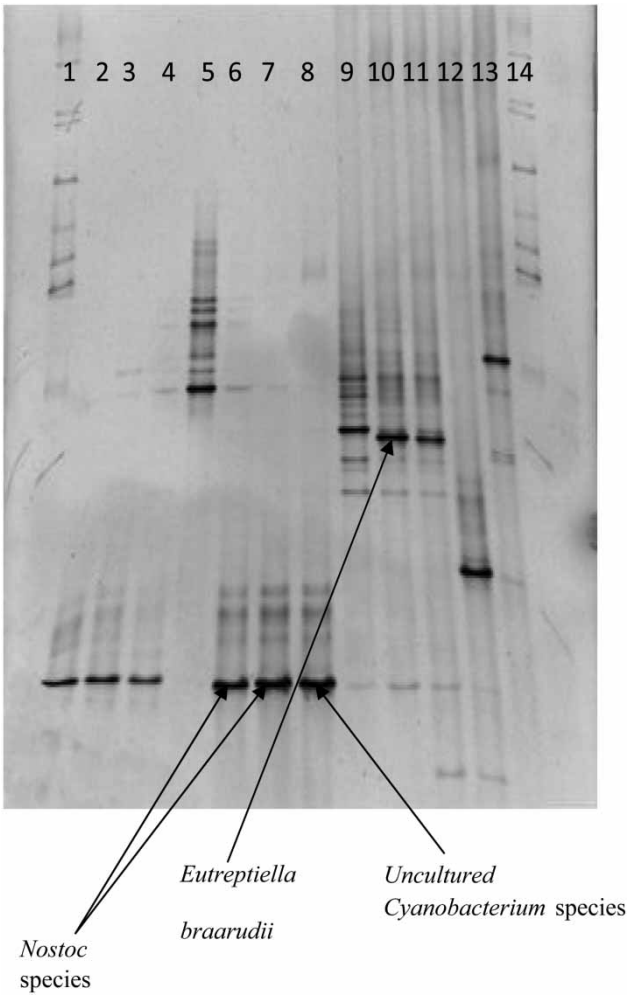

Figure 4 | DGGE profiling of water samples obtained from WTW1 and WTW2 including water (L 85 and L 87) and CD isolate (ATCC 43015). Lanes 1 and 14: molecular weight marker, lanes 2: Map strain ATCC 43015, lane 3: L 85, lane 4: L 87, lane 5: *Campylobacter* species (strain A75), lanes 6 and 7: DAF samples (floccules) from WTW2, lanes 8 and 9: final treated water from WTW2, lanes 10–13: final treated water from WTW1.

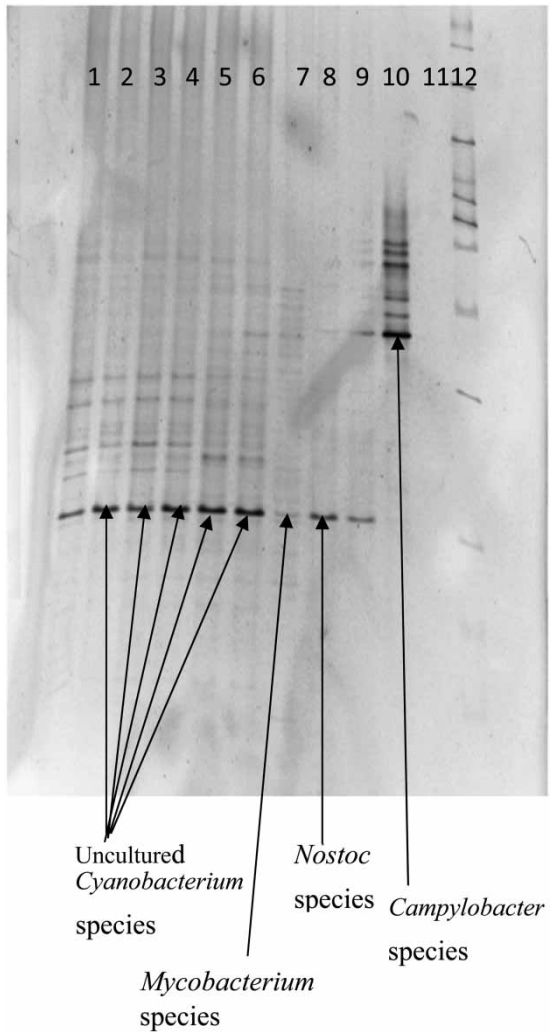

Figure 5 | DGGE profiling of water samples obtained from two WTWs. Lanes 1–5: raw untreated water from WTW2, lane 6: final treated water from WTW2, lanes 7–9: raw untreated water from WTW1, lane 10: strain A75, lane 11: empty lane 12: molecular weight marker.

final water along the entire water treatment process were also taken from respective sites and analysed by the DGGE technique. This was a follow up of preliminary data that succeeded from DGGE application on pure milk and Crohn's isolates of Map that confirmed the suitability of the method to study mycobacteria and their association, if any, with waterborne pathogens (Figures 1 and 2). Of the entire DGGE profiling of 76 samples, 35 species were positive from all the sampled sites. The profiles obtained from WTW1 showed similar band profiles (Figures 3 and 4, lanes 5 and 6 and 10 and 11 respectively) except for lanes 12 and 13 (Figure 4), which looked different. In WTW1, however, all band patterns of final treated water looked different (Figures 3–5, lanes 3, 7 and 9, lanes 8 and 9, and lane 6 respectively).

As can be seen in Table 2, only one type of organism proximal to the respective sampling sites was identified from the bands in the raw water source in contrast to WTW1 and WTW2, where more than just one type of species was identified at the sampled sites. It is not uncommon though, for all species to not be identified by the 16S sequencing; for example, the unculturable *Cyanobacterium*

Table 2 | Species identified from the common raw water source, WTW1 and WTW2

Site of sampling	Name of species	Areas sampled and no. of positives detected from these sites	Total no. of species from site (N)
Common raw water source	*Podocarpus macrophyllus*	Proximal to WTW1	1
	Sciadopitys verticillata	Proximal to WTW1	1
	Uncultured Synechococcus	Proximal to WTW1	1
	Dacrydium pierrei	Proximal to WTW1	1
	Coleodesmium species	Proximal to WTW2	1
WTW1	*Eutreptiella braarudii*	Final treated water	1
	Mycobacterium species	Raw water	1
	Anabaena species	Raw water (2), SSF (1)	3
	Nostoc species	Raw water (4), SSF (1)	5
WTW2	Uncultured *Cyanobacterium* species	Raw water (4), final treated water (4), DAF (2)	10
	Nostoc species	Raw water (2), DAF (2);	4
	Planktothrix agardhii	Raw water	1
	Anabaena sp.	DAF	1
	Anabaena nereformis	DAF (1), final treated water (1)	2
	Anabaena torulosa	Final treated water	1
	Podocarpus nerrifolius	Final treated water	1

sp. An agreement to this finding was reported by Bland *et al.* (2005), who after 16S sequencing obtained one-third of their results as unidentified species following their research work on the ecology of mycobacteria of the Rio Grande. The *Anabaena* and *Nostoc* species were present in the raw untreated water, SSF and DAF, thus confirming both WTWs shared similarities in microbial profile due to the fact that both obtain raw water from one source. Aside from *Anabaena* and *Nostoc* species, unculturable *Cyanobacterium* species were the dominant and persistent species (Metcalf & Codd 2009; Chatziefthimiou *et al.* 2016); many of them were identified in raw untreated water, DAF and also in final treated water obtained from WTW2 (Ho *et al.* 2012; Funari *et al.* 2017; Lee *et al.* 2017). Since the SSF and DAF represent a microcosm of the raw water source due to the fact that both WTWs receive the same natural water from that source, the microbial diversity reported here could be due to the amounts and kinds of available substrates required for their metabolism, and also by seasonal changes (Taniguchi & Hamasaki 2008; Yin *et al.* 2009).

Yin *et al.* (2009) also reported that seasonal change affected the diversity and composition of microbial communities in constructed wetlands. It is known that in constructed wetlands microorganisms do interact and metabolise many compounds including both organic and inorganic ones, with nitrogen fixation being pivotal and caused by *Anabaena* spp. (Saralov *et al.* 1980). Since a similar microbial activity occurred in the SSF and DAF, due to the concentration effect caused by these two different water clarification processes (Downing *et al.* 2002), the availability and variability of required substrates for metabolism by the microorganisms present, and seasonal changes, are pivotal to the findings reported here. The DGGE profiling also detected *Mycobacterium* species (Figure 2; Table 2), which confirms the viability and efficacy of the technique in detecting mycobacteria. However, the technique had no definitive response on associations between microorganisms that were detected and identified in the study.

CONCLUSIONS

The results from this study demonstrate that the DGGE technique is capable of detecting mycobacteria, including the slow growing species, as well as diverse groups of other microbial species from the environment. The technique was reproducible on DNA fragments that were sequenced for species identification. However, no direct association was detectable between the species identified.

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