Composition and stability of bacterial communities associated with granular activated carbon and anthracite filters in a pilot scale municipal drinking water treatment facility

T. B. Shirey, R. W. Thacker and J. B. Olson

ABSTRACT

Granular activated carbon (GAC) is an alternative filter substrate for municipal water treatment as it provides a high surface area suitable for microbial colonization. The resulting microbial growth promotes biodegradation of organic materials and other contaminants from influent waters. Here, the community structure of the bacteria associated with three GAC and two anthracite filters was examined over 12 months to monitor changes in community composition. Nearly complete 16S rRNA genes were polymerase chain reaction amplified for terminal restriction fragment length polymorphism (T-RFLP) analyses. The identity of commonly occurring peaks was determined through the construction of five representative 16S rRNA clone libraries. Based on sequence analysis, the bacterial communities associated with both anthracite and GAC filters appear to be composed of environmentally derived bacteria, with no known human pathogens. Analysis of similarity tests revealed that significant differences in bacterial community structure occurred over time, with filter substrate playing an important role in determining community composition. GAC filters exhibited the greatest degree of bacterial community variability over the sampling period, while anthracite filters showed a lower degree of variability and less change in community composition. Thus, GAC may be a suitable biologically active filter substrate for the treatment of municipal drinking water.

Key words | anthracite, drinking water treatment, granular activated carbon, T-RFLP

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INTRODUCTION

Throughout the USA, municipal drinking water treatment facilities are faced with the increasingly difficult task of delivering safe, potable drinking water to their customers. Technologies such as flocculation, coagulation, aeration, physical filtration, disinfections, and ozone and UV treatment are often utilized by these facilities to combat the inherent contamination that exists in environmental influents (Edzwald 1993; Camel & Bermond 1998; Hitzfeld *et al.* 2000; Gaithuma *et al.* 2009; Moon *et al.* 2009; Nur *et al.* 2009; Fabris *et al.* 2010). Capital improvements to these drinking water treatment systems are costly, so as a consequence of new growth and development, novel technologies that may be sustainable and cost effective, while still complying with current regulations, are routinely investigated.

The application of granular activated carbon (GAC) or anthracite to filtration systems has become a treatment alternative to conventional water treatment technologies. Both filter substrates have demonstrated their effectiveness at adsorping unpleasant tastes and odorous compounds from influent waters (Chien *et al.* 2007). However, their capacity for adsorption is finite. For example, when either dissolved organic material is present in the influent water supply, or the carbon granules reach a point of complete saturation, GAC performance diminishes (Carter *et al.* 1992; Knappe *et al.* 1997; Shih *et al.* 2003). As a consequence,

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municipalities are burdened with the high costs associated with replacing the filter substrate. If, however, the GAC is allowed to become biologically active, the efficiency of filtration by the GAC can be increased, extending its use well beyond saturation (Karanfil *et al.* 1996; van der Hoek *et al.* 1999). While a recent study showed that GAC filtration is a more effective treatment alternative to traditional anthracite-sand filters at removing unwanted compounds (Chien *et al.* 2007), biologically active GAC could further increase drinking water filtration efficiency by providing a stable, sustainable substrate capable of removing performance-inhibiting organic compounds from the treated water (Wang *et al.* 1995). Before moving forward and fully employing this new technology, rigorous examinations investigating the efficacy and safety of GAC filters over other treatment technologies must be conducted.

For this study, a demonstration-scale GAC filtration facility was constructed by the Birmingham Water Works Board at the Shades Mountain Filter Plant (SMFP) in Birmingham, Alabama. In addition, this plant housed a mobile anthracite filtration unit that was used in parallel to comparatively examine GAC filtration and anthracite filtration. Both filtration systems were fed from a receiving basin of water pumped from the Cahaba River. The Cahaba River is one of the few remaining free flowing rivers in Alabama, and the exclusive water source for 25% of the population of Alabama (El-Kaddah & Carey 2004). It drains an urban watershed influenced by a variety of environmental and industrial processes and receives over 26 million gallons of treated wastewater effluent on an average day (Lalor & Pitt 1998). Combined with the many species of relatively harmless bacteria common to environmental habitats, the Cahaba River is home to a variety of pathogenic and parasitic bacteria (Mahbubani *et al.* 1998) that could pose risks to human health should they enter the drinking water supply.

As a means to examine the efficacy of GAC and anthracite as components of an comprehensive drinking water treatment system, the main objectives of this study were to firstly, survey the dominant bacteria within both GAC and anthracite filters to identify the types of bacteria colonizing the filters, and detect the presence of human pathogens, and secondly, to examine the stability of those bacterial communities over time. In order to identify abiotic factors influencing bacterial community composition and stability, we investigated the impacts of substrate type, colonization time, chlorination, and substrate depth on microbial community development in both GAC and anthracite filters. Molecular 16S rRNA gene-based techniques were applied during this study, which provided a mechanism to gain insight into the stability of the bacterial communities within the filter systems, and assess the latent health threats created by the potential colonization of the filters by human pathogenic bacteria.

MATERIALS AND METHODS

Pilot and demonstration filtration systems

The SMFP is an 80 million gallon a day (MGD) conventional water filtration plant owned and operated by The Water Works Board of the City of Birmingham (BWWB). SMFP is supplied by the Cahaba River and is supplemented by releases from Lake Purdy, a 5.7-billion gallon reservoir operated by the BWWB on the Little Cahaba River. Upstream of the raw water intake, the Cahaba River is influenced by both wastewater and industrial dischargers. Raw water is pumped from the Cahaba River System, via the Cahaba Pumping Station (CPS), to the receiving basin south of the SMFP. At the CPS, chemicals, including powder activated carbon, potassium permanganate ($KMnO_4$), and/or polymer, are added to the water prior to being pumped to the receiving basin as needed. Two facilities, a pilot plant facility and a demonstration facility located at the SMFP were utilized during the course of this project.

Five of ten temporary drinking water filtration systems constructed at the Birmingham Water Works SMFP were chosen for microbial characterization analyses. The filters selected for examination consisted of two anthracite filtration systems (P-series) located in the pilot plant facility, and three GAC filtration systems (D-series) located in the demonstration facility. The demonstration filter facility was comprised of four steel 4.65 m^2 carbon–coated filter cells housed in a semi-permanent shelter, capable of treating 1 MGD of water. Each filter was filled with 0.81 m of virgin Calgon 8×20 bituminous GAC and 0.3 m of sand. The pilot plant facility was housed in a 16 m trailer equipped

with a wet laboratory for water quality analysis, which permitted side-by-side comparisons under a wide range of treatment conditions. The two pilot filters, P5 and P6, were filled with 0.46 and 0.81 m of anthracite, respectively, and 0.3 m of sand. To evaluate the effects of backwash and chlorine disinfection, the filters were configured in a range of backwash and chlorination conditions (Table 1).

Sample collection

Samples were collected monthly by BWWB staff from GAC (D1, D2, and D4) and anthracite (P5 and P6) filters from April to December 2008 with a final sample collected in March 2009. Each of the five filters was sampled at two different depths immediately following their backwash treatment and samples were maintained at –20 °C until DNA extraction. Samples labeled 'Top' were collected approximately 5 cm below the filter substrate surface, and samples labeled 'Bottom' were collected approximately 5 cm above the bottom of the filter bed. In addition, samples were grouped into either 'Early' samples corresponding to the initial colonization and recruitment period (April–July 2008), or 'Late' samples corresponding to a period where the initial colonization and recruitment period had stabilized (August 2008–March 2009).

Environmental DNA extraction

Approximately 1 g of filter material was used for DNA extraction. DNA was obtained from filter substrate using the Qbiogene Fast DNA Spin Kit for Soil (Qbiogene, Carlsbad, CA) following the manufacturer's protocol with one exception. An overnight cell lysis step was used in place of the recommended 10 min to increase extraction efficiency. Eluted DNA was used immediately for polymerase chain reaction (PCR).

Table 1 | GAC and anthracite filter configurations

Parameters	D1	D2	D4	P5	P6
GAC depth (m)	0.81	0.81	0.81	–	–
Anthracite depth (m)	–	–	–	0.46	0.81
Chlorinated influent	No	No	Yes	No	No
Chlorinated backwash	No	Yes	Yes	Yes	No

PCR amplification of the 16S rRNA gene

Direct PCR amplification of the 16S rRNA gene from DNA extracts with universal bacterial primers 8F (5′- AGAGTTT GATCMTGGCTCAG-3′) (Edwards *et al.* 1989) and 1392R (5′-ACGGGCGGTGTGTACA-3′) (Lane 1991) yielded nearly complete (~1385 bp) genes. Each 50 μl reaction consisted of 1 μl template DNA, 2 U *Taq* DNA polymerase (5 Prime, Gaithersburg, MD), 1× PCR buffer, 1× TaqMaster PCR Enhancer, 1.25 mM $Mg(OAc)_2$, 0.06 mM deoxynucleoside triphosphates, and 25 pmol of each primer. Positive and negative reagent controls were run with each amplification reaction and each sample was amplified in triplicate. Reaction conditions in a PTC-200 Peltier Thermal Cycler (Bio-Rad Laboratories, Waltham, MA) consisted of an initial denaturation step at 85 °C for 5 min followed by 30 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 1.5 min, with a final extension step of 2 min at 72 °C. The amplified products were examined on 1% agarose gels containing GelRed™ Nucleic Acid Stain (Biotium, Hayward, CA) and visualized under UV transillumination. Excised bands of the predicted product size were cleaned with the QIAquick PCR purification kit (Qiagen) to remove unincorporated dNTPs and primers before quantifying the DNA on a ND-1000 spectrophotometer (NanoDrop™, Thermo Scientific). For terminal restriction fragment length polymorphism (T-RFLP) analyses, the 8F primer was fluorescently labeled at the 5′ end with a HEX label, and triplicate reactions were pooled for downstream T-RFLP analyses.

Clone library construction and sequencing of 16S rRNA genes

Purified PCR products were ligated into pCR®2.1 vectors and used for direct cloning with the TA Cloning Kit (Invitrogen, Carlsbad, CA). Ligated DNA fragments were transformed into chemically competent *Escherichia coli* cells following the manufacturer's instructions. DNA from white colonies was extracted by adding cells to 50 μl of a sterile 5% Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA) in deionized water solution and vortexing vigorously followed by 1–2 freeze/thaw cycles. Following cell lysis, 10 μl of the DNA eluate was digested with *Hae*III for 8 h at 37 °C. Remaining enzyme was heat-inactivated

by a final incubation at 80 °C for 20 min. The DNA was electrophoresed in a 1% agarose gel containing GelRed™ Nucleic Acid Stain (Biotium, Hayward, CA) and visualized under UV transillumination to assess the banding patterns. Ninety-two clones demonstrating unique banding patterns were selected for sequencing. Overnight cultures of the transformants amended with glycerol were shipped to the Genome Center at Washington University for sequencing.

T-RFLP analysis

For digestion reactions, 400 ng of triplicate pooled PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen Sciences, Maryland) and digested with *Hae*III for 8 h at 37 °C. Remaining enzyme was heat-inactivated by a final incubation at 80 °C for 20 min. Resulting digestion products were ethanol precipitated overnight, dried and resuspended in 10 μl of deionized formamide and 0.5 μl GeneScan™ 500 TAMRA™ (Life Technologies California) size standard. The samples were analysed using an ABI 3100 Genetic Analyzer with a 50 cm capillary array (Applied Biosystems), and fragments determined using Applied Biosystems GeneScan software. Data matrices were constructed using peaks above a threshold of 50 fluorescence units, which was considered the background level. Peaks smaller than 100 bp and greater than 500 bp were removed from the data set to avoid uncertainties associated with fragment size determination. To determine which peaks were further analysed, the variable threshold method (Osborne *et al.* 2006) was employed.

Statistical analyses

Sample T-RFLP profiles were uploaded to the Internet-based data analysis program T-REX (Culman *et al.* 2009). The T-REX program generates a file output of all terminal restriction fragments (TRFs) identified and their relative fluorescent intensities. The corresponding matrix data file containing TRFs and calculated peak areas for all samples was analysed using the PRIMER v.6 software package (Clarke & Gorley 2006a). Using PRIMER, non-parametric multidimensional scaling plots (MDS) were generated to allow for visual identification of existing patterns between bacterial communities and the associated abiotic filter parameters, and analysis of similarity (ANOSIM), (Clarke & Gorley 2006b) used to examine the impacts of time, depth, substrate, and chlorination on bacterial community composition. An additional pairwise analysis compared TRF profiles between filters D1, D2, D4, P5, and P6. Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) was used to calculate mean TRFs identified in both GAC and anthracite filters.

Rarefaction curves for 16S sequences were generated using the Distance Based Operational Taxonomic Unit and Richness Determination (DOTUR) program (Schloss & Handelsman 2005) (Figure 3) with all of the clones that were screened for unique banding patterns. Clones demonstrating the same banding pattern following digestion with *Hae*III were considered to be a single operational taxonomic unit (OTU). Rarefaction curves were generated for OTU definitions of 99, 97, and 94% sequence homology.

RESULTS

Ninety-two clones were selected for sequencing from nearly complete 16S rRNA gene clone libraries constructed using DNA extracted from filters sampled in September 2008. The selected clones were pre-screened to minimise repeated sequencing and to optimise coverage of the bacterial diversity present within the filters. After running Mallard (version 1.02; Ashelford *et al.* 2006) to detect chimera formation within the sequenced clones, 67 sequences were submitted to NCBI (National Center for Biotechnology Information) and assigned GenBank accession numbers HQ642779 to HQ642845.

The sequence data showed that both GAC and anthracite filters were colonized by environmentally derived bacteria; none of the 67 sequences were closely related to common human pathogens (Table 2). Percent identity values to the closest GenBank match ranged from 78 to 99%. Sixty-six percent of clones exhibited greater than 5% sequence divergence to the closest match, which suggests that a large percentage of the bacterial species present within the filters likely represents currently uncharacterised, novel species and/or genera.

Only three clones (HQ642780, HQ642781 and HG642807) were most closely related to organisms obtained previously from drinking water treatment facilities.

Table 2 | Closest GenBank sequence matches for the sequenced clones. Also provided are the GenBank accession numbers, filters from which the clone was obtained (D or P series and top or bottom of filter), the percent identities for the closest GenBank matches, the expected terminal restriction fragment (TRF) size for the clone, and the percentage of samples in which that TRF was recovered

GenBank accession #	Filter	GenBank closest match	% Identity	TRF size	% Found
HQ642828	P6 Top	Acidobacteria bacterium	95	39	NA
HQ642829	P6 Top	*Microvirga* sp.	90	39	NA
HQ642830	P6 Top	*Nitratireductor* sp.	98	39	NA
HQ642832	P6 Top	*Rhodobacter* sp.	93	39	NA
HQ642833	P6 Top	*Rhizobium* sp.	93	39	NA
HQ642834	P6 Top	*Kofleria flava*	86	39	NA
HQ642836	P6 Top	*Claulobacter* sp.	94	39	NA
HQ642820	P6 Top	Verrucomicrobia bacterium	89	225	2.86%
HQ642821	P6 Top	*Ochrobactrum* sp.	94	39	NA
HQ642822	P6 Top	Planctomycete bacterium	92	401	2.86%
HQ642823	P6 Top	*Geobacter* sp.	91	419	1.43%
HQ642842	P6 Top	*Rhodobacter* sp.	96	39	NA
HQ642844	P6 Top	*Chloroflexus* sp.	90	220	4.29%
HQ642827	P6 Top	*Deinococcus* sp.	94	NA	NA
HQ642831	P6 Bot	*Phenylobacterium* sp.	98	40	NA
HQ642818	P6 Bot	*Candidatus Reyranella massiliensis*	95	296	7.14%
HQ642819	P6 Bot	*Methylocystis* sp.	89	193	18.57%
HQ642835	P6 Bot	*Nitratireductor* sp.	98	39	NA
HQ642837	P6 Bot	*Methylocystis parvus*	92	190	24.29%
HQ642838	P6 Bot	*Crenothrix polyspora*	93	194	1.43%
HQ642839	P6 Bot	*Hyphomicrobium* sp.	92	39	NA
HQ642840	P6 Bot	*Chloroflexus* sp.	90	282	2.86%
HQ642841	P6 Bot	*Sphingomonas* sp.	95	19	NA
HQ642824	P6 Bot	Myxococcales bacterium	85	201	8.57%
HQ642843	P6 Bot	*Nitratireductor* sp.	98	39	NA
HQ642845	P6 Bot	Cyanobacterium	94	294	8.57%
HQ642825	P6 Bot	*Steroidobacter denitrificans*	90	406	2.86%
HQ642826	P6 Bot	Planctomycete bacterium	89	39	NA
HQ642795	D1 Top	*Candidatus Reyranella massiliensis*	97	383	1.43%
HQ642779	D1 Top	Acidobacteria bacterium	90	228	38.57%
HQ642797	D1 Top	*Parvularcula* sp.	95	189	27.14%
HQ642798	D1 Top	*Rhodobacter* sp.	95	39	NA
HQ642781	D1 Top	*Parvularcula bermudensis*	88	189	27.14%
HQ642801	D1 Top	*Sinorhizobium* sp.	90	189	27.14%
HQ642804	D1 Top	Acidobacteria bacterium	98	215	15.71%
HQ642790	D1 Top	*Sphingomonas* sp.	93	NA	NA
HQ642806	D1 Top	*Microvirga* sp.	89	39	NA
HQ642807	D1 Top	*Sphingomonas* sp.	96	293	15.71%

(*continued*)

Table 2 | continued

GenBank accession #	Filter	GenBank closest match	% Identity	TRF size	% Found
HQ642808	D1 Top	*Leptothrix ginsengisoli*	94	219	4.29%
HQ642810	D1 Top	Cyanobacterium	94	294	8.57%
HQ642786	D1 Top	*Leptothrix ginsengisoli*	96	219	4.29%
HQ642813	D1 Top	*Hyphomicrobium* sp.	95	39	NA
HQ642787	D1 Top	*Candidatus Nitrotoga arctica*	91	197	25.71%
HQ642815	D1 Top	*Thermomonas* sp.	96	406	2.86%
HQ642799	D1 Bot	*Methylibium aquaticum*	98	217	7.14%
HQ642789	D1 Bot	Acidobacteria bacterium	90	NA	NA
HQ642802	D1 Bot	*Parvularcula* sp.	96	189	27.14%
HQ642803	D1 Bot	Acidobacteria bacterium	93	227	51.43%
HQ642805	D1 Bot	Planctomycete bacterium	90	63	NA
HQ642784	D1 Bot	*Parvularcula* sp.	94	189	27.14%
HQ642785	D1 Bot	*Planctomycete bacterium*	90	603	NA
HQ642811	D1 Bot	*Methylobacter whittenburyi*	91	69	NA
HQ642814	D1 Bot	*Sphingomonas* sp.	99	293	15.71%
HQ642788	D1 Bot	Thiotrichales bacterium	90	69	NA
HQ642816	D1 Bot	*Acidobacterium* sp.	97	239	7.14%
HQ642791	D1 Bot	Acidobacteria bacterium	80	NA	NA
HQ642817	D1 Bot	*Acidobacterium* sp.	93	NA	NA
HQ642796	D4 Top	*Bradyrhizobium* sp.	99	195	4.29%
HQ642792	D4 Top	*Alterierythrobacter* sp.	78	283	4.29%
HQ642780	D4 Top	*Crenothrix polyspora*	86	117	20.00%
HQ642800	D4 Top	*Rhodomicrobium* sp.	93	199	24.29%
HQ642782	D4 Top	*Bosea* sp.	91	23	NA
HQ642783	D4 Top	Cyanobacterium	90	260	7.14%
HQ642809	D4 Top	*Sphingomonas sp.*	97	227	51.43%
HQ642793	D4 Top	*Rhodopseudomonas* sp.	87	NA	NA
HQ642812	D4 Top	Acidobacteria bacterium	99	265	1.43%
HQ642794	D4 Top	*Methylobacillus* sp.	94	NA	NA

NA = not applicable as the TRF was <100 bp, >500 bp, or no restriction site was identified within the sequence.

From those earlier studies, *Sphingomonas* sp. were recovered from a sand filter, and *Crenothrix polyspora* was found in a GAC water treatment facility. Interestingly, although the source water for the treatment facility in this study was obtained from a local river, only 13.4% of the sequences showed that the closest sequence match was derived from a freshwater environment.

T-RFLP methodology was used to examine the bacterial communities in all sampled filters ($n = 70$). Analysis of individual TRFs present in each substrate type showed a mean TRFs per sample number of 38.72 for GAC filters, and 87.08 for anthracite filters. The number of TRFs ranged from 6 to 313 and 27 to 126 for GAC and anthracite filters, respectively. Great variation was apparent in the samples over the sampling period but no trends were noted for the number of TRFs recovered over time, depth, substrate, or treatment (e.g., chlorination).

In silico digestion was performed on the sequences derived from the 16S rRNA clone library. Digests resulting in fragment sizes outside the TAMRA size standard (below

100 or above 500 bp) were excluded from analysis. Restriction fragments from the remaining 37 clones were detected in at least one of the 70 filter samples collected. Of those 37 clones, *Sphingomonas* sp. and a member of the Acidobacteria were most prevalent, detected in 51.4% of samples. In addition, six sequences (most closely related to Verrucomicrobia, Chloroflexi, *Methylibium aquaticum*, *Bradyrhizobium* sp., and two Cyanobacteria) corresponded to bacteria found only in GAC filters, while no sequences were found exclusively in anthracite filters.

ANOSIM tests based on T-RFLP generated fragment patterns over the entire sampling period revealed that both time ($p = 0.001$, $R = 0.170$) and filter substrate ($p = 0.027$, $R = 0.098$) were significant factors influencing bacterial community composition (Table 3). When the sampling period was divided into an early (April–July 2008) colonisation period, and a late (August 2008–March 2009) colonisation period, shifts in significant effects on community structure were revealed. Substrate type loses its significance as the filters transition from the period of early ($p = 0.001$, $R = 0.481$) colonisation to late ($p = 0.405$, $R = 0.01$). While chlorination is not significant during any sampling period, the effect of depth on community composition becomes significant during the late colonisation period ($p = 0.017$, $R = 0.082$). The ANOSIM test statistic, R, was included in the analysis as a comparative measure of the degree of separation of sites (Clarke & Gorley 2006a). The R test statistic can lie within a range of $R = 0$ (no difference within the group) and $R = 1$ (dissimilarities across sample parameters are larger than any dissimilarity within the tested parameter).

Pairwise tests of filter columns revealed differences between several of the columns (significance value adjusted for 10 post-hoc treatments using a Bonferroni correction to 0.005; Table 4) (Abdi 2007). When examined over the entire sampling period, significant differences were found between columns D2 and P6, D2 and P5, and D4 and P6. When sampling was divided into early and late periods, significant differences were found between columns D1 and P5, D2 and P5, D2 and P6, D4 and P5, and D4 and P6 during the early

Table 3 | ANOSIM of bacterial communities. 'All' signifies samples composed of both early and late periods of colonization

Effect	Comparison	*R* statistic (All)	*p* (All)	*R* statistic (Early)	*p* (Early)	*R* statistic (Late)	*p* (Late)
Time	Early vs. Late	0.170	0.001[a]	NA	NA	NA	NA
Media	GAC vs. Anthracite	0.098	0.027[a]	0.481	0.001[a]	0.01	0.405
Depth	Top vs. Bottom	−0.006	0.288	−0.054	0.967	0.082	0.017[a]
Chlorine	Yes vs. No	0.007	0.967	0.003	0.428	0.02	0.168

NA = not applicable.
[a]Significant at $\alpha = 0.05$.

Table 4 | Pairwise tests between columns. 'All' signifies samples composed of both early and late periods of colonization

Column comparison	*R* statistic (All)	*p* (All)	*R* statistic (Early)	*p* (Early)	*R* statistic (Late)	*p* (Late)
D1, D4	0.025	0.753	0.003	0.456	0.012	0.363
D1, D2	0.042	0.270	−0.068	0.728	0.015	0.414
D1, P5	0.097	0.141	0.415	0.005[a]	−0.128	0.766
D1, P6	0.092	0.022	0.250	0.026	0.102	0.068
D2, D4	0.066	0.183	0.113	0.120	−0.001	0.423
D2, P5	0.378	0.002[a]	0.672	0.002[a]	0.448	0.029
D2, P6	0.512	0.001[a]	0.566	0.002[a]	0.582	0.007
D4, P5	0.278	0.006	0.938	0.001[a]	0.073	0.324
D4, P6	0.235	0.001[a]	0.811	0.001[a]	0.25	0.004[a]
P5, P6	0.084	0.132	0.368	0.026	−0.21	0.969

[a]Significant at $\alpha = 0.005$.

months; however, the only significant difference in the late months occurred between filters D4 and P6. We anticipate that these significant values likely reflect differences in substrate (GAC vs. anthracite).

Non-metric multidimensional scaling (MDS) plots were constructed to display relationships between bacterial community composition and abiotic components of the filter systems, revealing potential determinants influencing the bacterial communities within the filters (Figures 1 and 2). With visually distinct clustering of samples by substrate type and time (Figure 1), and column (Figure 2), the MDS plots appear to be in agreement with ANOSIM results suggesting that a more variable bacterial community existed within both GAC and anthracite filters during the early colonisation period. The MDS plot for column (Figure 2) suggests a higher degree of variability exists for GAC filters than in anthracite filters. No clustering patterns were apparent for chlorination or depth (data not shown). MDS plots were constructed using data from the entire sampling period (April 2008–March 2009) and have stress values of 0.22, indicating that these data were not readily represented in two-dimensional space.

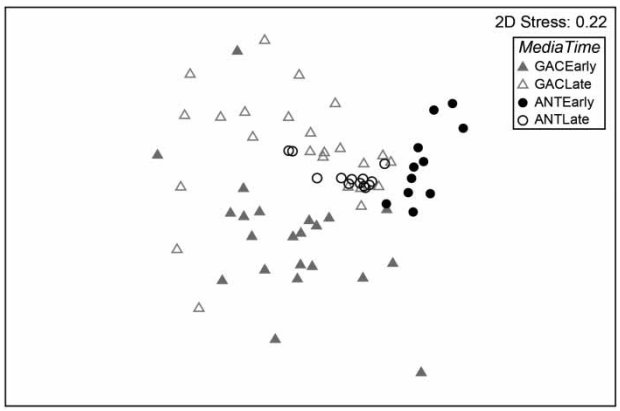

Figure 1 | MDS plot of the effects of filter substrate and time on community composition over the entire sampling period. This cluster plot, represented in two-dimensional space, provides a graphical representation of bacterial community composition where less distance between points indicates greater similarity in bacterial communities.

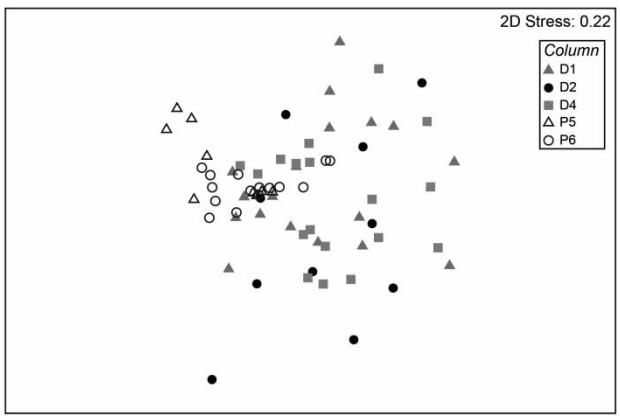

Figure 2 | MDS plot of bacterial community composition in filters over the entire sampling period. The clustering of samples within this two-dimensional plot provides a graphical representation of the stability of bacterial communities within specific filters over time.

Rarefaction analysis with OTU definitions of 99, 97 and 94% sequence similarity was used to compare the relative 16S rRNA gene diversity between GAC and anthracite filters. In contrast to ANOSIM results, the rarefaction curves indicated a higher level of bacterial diversity existed within the GAC as compared to anthracite filters (Figure 3).

DISCUSSION

Molecular techniques were employed in this study to examine the bacterial communities within both GAC and anthracite filters. Although inherent biases associated with DNA extraction and PCR amplification exist, the molecular approach is generally preferred over traditional cultivation techniques as it has previously been shown that isolation studies often grossly underestimate the actual diversity present in the sample (Pace 1997; Head *et al.* 1998; Theron & Cloete 2000; Olson & McCarthy 2005). Furthermore, Amann *et al.* (1995) outlined the advantages to using molecular applications to assess bacterial community composition over a wide range of environmental samples including marine sediments, soils and biofilm communities.

Few studies utilizing clone libraries to examine GAC and anthracite associated bacterial communities exist; however, past cultivation studies have revealed considerable variability in the bacteria associated with biofilters or filter effluents. Camper *et al.* (1986) found that a large percentage of GAC samples was colonised by coliform bacteria. In contrast, Wilcox *et al.* (1983) recovered only small numbers of coliform bacteria in GAC effluents while the dominant isolates included *Achromobacter*, *Acinetobacter*, *Bacillus*, *Chromobacterium*, *Micrococcus*, *Paracoccus* and

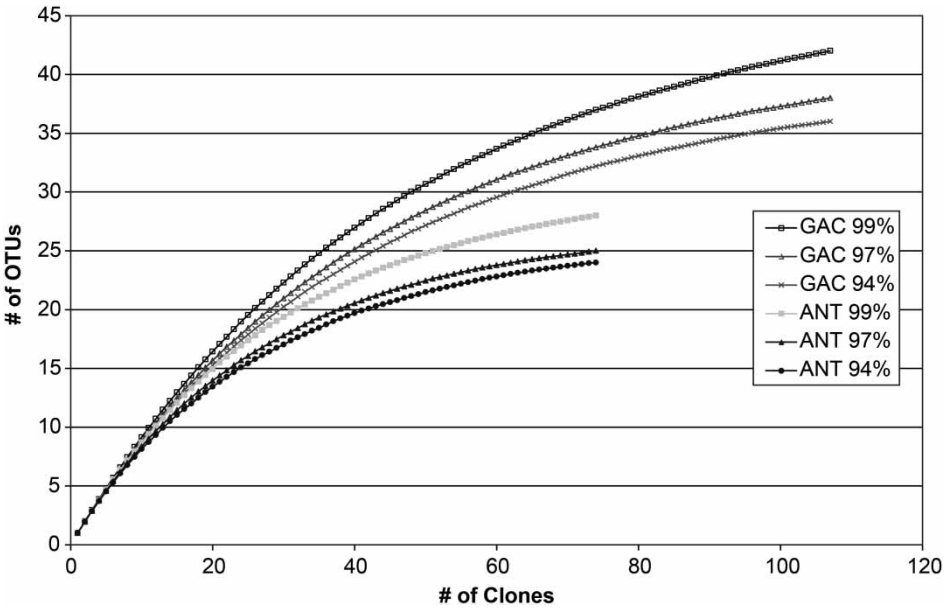

Figure 3 | Rarefaction curves for granular activated carbon (GAC) and anthracite (ANT) 16S sequences. Rarefaction curves plot the total number of operational taxonomic units estimated as a function of number of clones examined. At the point on the *y*-axis where the curves flatten, the maximum number of operational taxonomic units has been reached and an increased sampling effort would yield no new taxonomic groups. Steep rarefaction curves indicate a more diverse community, while flatter curves indicate less diversity.

Pseudomonas. Similarly, we failed to identify any coliform bacteria in our study and instead found the bacterial communities to be dominated by aquatic and terrestrially derived microorganisms. It is likely that a variety of factors, including differences in environmental conditions of waters supplying the treatment facilities, treatment effects and methodologies employed, contributed to the variation found in these studies. Additional molecular-based research may help to further elucidate the composition of the bacteria associated with biofilter substrates.

The majority of the bacterial clones recovered from our GAC and anthracite filters were most closely related to common soil or aquatic bacteria (Table 2). Interestingly, 79% of the clones exhibited greater than 3% sequence divergence from the closest reference sequence and 66% exhibited greater than 5% sequence divergence. This suggests that our filters were predominantly colonized by novel species or genera, which is not surprising considering that the vast majority of bacteria remain uncharacterised. As a result, minimal information regarding the true genetic affiliations of the clones was obtained. Substantial amounts of additional isolation and phenotypic characterisation of environmental bacteria are needed in order to provide a more robust database for sequence comparison.

In addition to being colonised by environmentally derived bacteria, GAC can become colonised by a suite of human pathogenic bacteria (LeChevallier *et al*. 1981; Camper *et al*. 1985; Stewart *et al*. 1990). Our limited clone library analysis indicated that no common human pathogens were detected in these filters (Table 2); however, two clones most closely related to the species *Leptothrix gensengisoli* were identified in one of the GAC filters. Although *L. gensengisoli* is not known to be a human pathogen, a related species of *Leptothrix*, *L. buccalis*, is known to be pathogenic to humans. Additional work needs to be performed in order to more completely characterise the associated bacterial communities.

T-RFLP analyses were employed to provide temporal information on the abundant members of the bacterial communities within the filters. This method has proven to be a useful tool in investigating bacterial communities from a variety of environments (Schutte *et al*. 2008), and was used here to comparatively examine factors influencing bacterial community structure. To our knowledge, this is the first application of T-RFLP methodology to characterise the bacteria colonising GAC or anthracite filters for drinking water treatment.

Analysis of T-RFLP data showed filter substrate significantly influenced bacterial community structure.

Anthracite filters demonstrated less bacterial variability over the entire sampling period than GAC filters. Mean TRF peak number was significantly greater in anthracite filters (87.08 vs. 38.72 for GAC), suggesting that these filters maintained a diverse but relatively consistent bacterial community. Rarefaction curves generated from clone library sequence analysis indicated that GAC filters supported a greater number of OTUs than anthracite filters, at least in September 2008.

Time was also a significant factor shaping bacterial community structure within the filters. As expected, all filters exhibited highly variable profiles during the 'Early' period of colonisation. During this period, many bacteria entered the filter matrix in the source water and attached themselves to the filter substrate. After the initial acclimation and colonisation period had passed, overall community structure became less variable. This was likely due to the presence of certain types of bacteria that were more adept at colonising the filters and able to out-compete other species both temporally and spatially. As a result, when 'Early' samples were excluded from analyses, time ceased to be a significant factor regulating community structure.

Depth was the only studied parameter that exhibited a significant effect on bacterial community structure in the 'Late' samples. It is thought that this is the result of bacteria becoming spatially partitioned within the filter columns as time progressed. Factors influencing this distribution may include the availability of organic materials, as the communities associated with the substrate material closest to the influent source water may be able to utilise those compounds that are readily broken down while bacteria further down in the substrate may have to utilise the remaining more recalcitrant compounds. No analyses of functional genes were conducted in this study but will be important in future studies to determine which organisms are capable of utilising the various organic compounds.

Subjecting the filter bed to chlorinated backwash did not appear to significantly contribute to changes in community structure. A previous study by LeChevallier *et al.* (1984) showed that, when attached to GAC, bacteria exhibit a high degree of resistance to chlorine disinfection. Moreover, it has been shown in drinking water systems treated with chlorine, a strong selection exists for chlorine-tolerant bacteria that are more resistant to chlorine disinfection; an effect not found in unchlorinated systems (Ridgway & Olson 1982). Our sample size was not sufficient to address interactive effects between chlorination, substrate type time, and depth in shaping community structure within the filters, which likely occurred.

Although subjected to identical source groundwater, the two treatment facilities used for this study differed in both construction and treatment parameters. Also, the filter backwash rate in the pilot facility differed from that of the demo facility due to programming restraints within the automatic backwash function, and it can be assumed that water flow through the media was altered by differences in construction. It is unclear whether differences in construction contribute to differences in bacterial colonisation, but community composition did differ between GAC and anthracite filters.

CONCLUSIONS

In summary, a number of factors appear to contribute to the variation found in the bacterial species colonising biofilters including media composition, colonisation time, and depth. Although biofilters can become colonised by an array of human pathogenic bacteria, the molecular genetic techniques employed here revealed that the majority of bacteria found colonising both the anthracite and GAC filters in this study were consistent with common water and soil bacteria. The data indicated that both GAC and anthracite have the potential to be effective substrate types for the recruitment of diverse communities of non-pathogenic, environmentally derived bacteria.

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