Metagenomic study on the composition of culturable and non-culturable bacteria in tap water and biofilms at intensive care units

Maha Farhat, Khaled R. Alkharsah, Fatimah I. Alkhamis and Huda A. Bukharie

ABSTRACT

Bacterial community diversity of bulk water and corresponding biofilms of four intensive care units' (ICUs) drinking water systems were studied and compared using 16S rRNA gene amplicons and next generation sequencing. *Proteobacteria,* mainly *Alphaproteobacteria* and *Betaproteobacteria* were dominant in the bulk water and biofilms. Principal component analysis showed different bacterial communities characterizing each of the bulk water and the biofilms in three of the studied ICUs. Taxonomic classification and comparison of different genera between samples highlighted the dominance of *Aquabacterium* (80%) and *Novosphingobium* (72%) in bulk water while biofilms harbored different bacteria affiliated to *Pelomonas* (97%) and *Caulobacter* (96%), *Porphyrobacter* (78%) and *Staphylococcus* (74%). *Staphylococcus aureus* was the only possible pathogen found with low percentage (2.32%) in three of the ICUs' biofilm and only in one of the ICU's bulk water. This study sheds light on the prevalence of unculturable bacterial flora in the biofilm ignored by the microbiological standard methods. This study was performed on tap and bulk water from ICUs; however, it indicates the need for further studies to investigate the function and activity of the microbial diversity in order to assess the real risk presented by this water microflora on patients' health.

Key words | biofilm, ICU, next generation sequencing, water microbiology

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INTRODUCTION

Monitoring water supply networks in health care institutions remains a very critical issue in order to ensure a healthy environment for patients. Maintaining good water quality in water distribution systems requires regular maintenance, disinfection strategies and survey of the microbial flora in order to maintain microbial populations at the lowest numbers. Strict policies of water disinfection are usually well defined in hospitals, where patients have a high risk to develop hospital-acquired infections (HAI) (Nyamogoba & Obala 2002). In many countries, the water microflora is controlled by the use of disinfectants and monitored by analysing the bacterial flora in the end-product (tap water). Heterotrophic microorganisms are first indicators of water quality (WHO 2003). However, the most common microbial contaminants of water belong to enteric microorganisms predominantly of fecal origin from infected individuals or animals. These microorganisms include coliforms, *Escherichia coli* (faecal/thermotolerant coliform) and enterococci (Figueras & Borrego 2010). *Escherichia coli* is the chosen indicator in the WHO guidelines for drinking water quality in order to measure the effectiveness of disinfectants or to test for faecal contamination (WHO 2017). Although these parameters are the most commonly used to measure possible water quality deterioration, other

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pathogens (not transmitted by the faecal–oral route) could be present in water and also cause waterborne diseases. Monitoring of faecal indicators does not provide any information about these harmful microorganisms and no other indicators are currently known for such pathogens. Additionally, standard methods like membrane filtration followed by incubation on selective media or counting most probable number are used for water monitoring but have many disadvantages. Beside the false positive and false negative results, these methods limit our study to a low percentage of the real microbial diversity due to the viable but non-culturable state (VBNC) of many microorganisms (Amann *et al.* 1995; Albersten *et al.* 2013).

During the last decades, big improvements in molecular biology helped water microbiologists to discover new bacteria and a very rich diversity that would be unrevealed by culture methods. These molecular approaches rely on the analysis of nucleic acids extracted from water by a large array of techniques like polymerase chain reaction (PCR) or DNA fingerprinting (single strand conformation polymorphism (SSCP), fluorescence *in situ* hybridization (FISH), and the most up-to-date technique, next generation sequencing (NGS) (Waldor *et al.* 2015). This latter DNA-based technique assesses the presence and the relative abundance of every single phylotype of the entire microbial communities by targeting 16S rRNA genes or whole shotgun metagenomic DNA of a given sample.

Many comprehensive studies have been done in industrialized countries on waterborne infectious diseases aiming to assess microbial risk in relation to public water safety in order to reduce patient mortality rate and economical waste. However, few reports and premature studies have been carried out in developing countries. In this work, we collected water and biofilm samples from four different areas at the intensive care units (ICUs) at King Fahad Hospital of the University (KFHU), AlKhobar, in the Eastern province of Saudi Arabia. 16S rRNA gene sequencing using the Illumina platform was conducted to study the relative abundance and distribution patterns of the different encountered bacterial genera. A multivariate analysis of the bacterial community was also investigated exploring the diversity of genera between water and biofilm samples in different areas of the ICU.

MATERIALS AND METHODS

Sample preparation of water and biofilm

Water and biofilm samples were taken from four different locations at the ICU: (1) common area of the medical and surgical units (ICU1); (2) adult burn unit (ICU2); (3) pediatric burn unit (ICU3); and (4) neonatal care unit (ICU4). Biofilm samples were taken with flocked swabs from the inner surface of the faucets in three of the intensive care unit locations (ICU1, ICU2 and ICU4) and from the bathtub of the pediatric burn unit (ICU3). The surface area swabbed in each unit was noted for further interpretation of results. The swabs were performed with flocked nylon swabs (microRheologics, Italy) prior to the water sampling in order to avoid loosely adherent bacteria being flushed down the drain, possibly leading to a false negative result (Sun *et al.* 2013). After sampling, the swab was eluted in 6 mL of sterile/free of DNA water and transferred immediately to the laboratory for microbiological and molecular analysis. In order to extract the biofilm, the swab was vortexed for 5 seconds, sonicated (Grant, Cambridge) for 2 minutes and then discarded from the tube (Probst *et al.* 2010).

Water samples (1 L) were taken in sterile glass bottles from the same faucets as the biofilm samples and from the bathtub outlet of the pediatric burn unit. Water samples and the resulting biofilm suspensions were subjected to microbiological analysis (conventional culture on R2A medium) and to 16S rRNA next generation sequencing.

Cultivable heterotrophic bacteria count

Heterotrophic plate count (HPC) for total bacteria was performed on R2A medium for the water and biofilm samples with direct and diluted (1/10 and 1/100) spread. After incubation at 28 °C for 7 days, the colonies were counted for HPC (Reasoner 2004).

DNA extraction

In order to extract the DNA from the water and biofilm samples, 500 mL and 3 mL of the water and the

concentrated biofilm suspension, respectively, were filtered on 0.22 μm PVDF (polyvinylidene flouride) filter membranes (GVWP04700, Millipore). The filters were stored in sterile Eppendorf tubes at −80 °C until DNA extraction. Roche High Pure PCR Template kit was used for DNA extraction. The frozen filters, maintained on dry ice, were crushed into very small pieces using sterile pestles. Debris filters were suspended in tissue lysis buffer and lysozyme and then heated at 70 °C in order to break the cell wall. Finally, the samples were washed three times with washing buffer and the DNA was eluted in 100 μL of elution buffer and stored at −80 °C until the time of analysis.

16S rRNA gene sequencing

Library construction

V3 and V4 regions of the 16S rRNA gene were studied for water and biofilm samples. The primer set used was F319/R806 (5′-ACTCCTACGGGAGGCAGCAG-3′/5′-GGACTACHVGGGTWTCTAAT-3′). The reverse primer R806 was attached to different barcode identifiers in order to enable sample multiplexing. Both primers contain Illumina adapters for sequencing on the MiSeq platform. PCR reaction mixture (25 μL) contained 12.5 μL of Taq PCR master mix (Qiagen), 0.5 μL of each forward and reverse primers (0.2 μM final concentration), 7 μL of genomic DNA and RNAase free water. Reaction conditions were set at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 sec, 55 °C for 60 sec, and 72 °C for 30 sec and a final extension of 72 °C for 5 min. PCR amplicons were run on a 1% gel and purified using AMPure XP bead kit following the manufacturer's instructions. PCR indexing was done using Nextera XT DNA sample preparation index kit (Illumina) followed by purification using the AMPure XP beads kit (Beckman Coulter). DNA concentrations were quantified by NanoDrop 2000 spectrophotometer. The Library was constructed using equimolar ratios of each amplicon and then quantified by real-time PCR using KAPA Library quantification kit (KK4601 and KK4808).

Purified and pooled library was then prepared for cluster generation and 250-bp paired-end sequencing on Illumina Miseq platform using Miseq Reagent Nano kit v2 (Illumina).

Phylogenetic analysis

After sequencing, the data were visualized by sequence analysis viewer and MiSeq reporter software. Two resulting fastq files representing two sets of reads (forward and reverse) for each sample were generated. These files were checked with FastQC (version 0.11.5) and high quality reads (>Q20) were processed using MOTHUR v.1.36.0 software as follows (Schloss *et al.* 2009); the two fastq files were combined in an individual fasta file, duplicates and short reads (<150 bp) were discarded and the unique sequences were kept, and the alignment was done using the SILVA bacteria database trimmed to the V3 and V4 regions (Yilmaz *et al.* 2014). Chimeric sequences were removed using the UCHIME algorithm. The sequences were then classified using the Bayesian classifier. The distances between sequences were calculated with a 0.2 cutoff and then clustered into operational taxonomic units (OTUs) at 97% similarity. Finally, each OTU was given the correspondent taxonomy. In order to minimize the sequencing error rate, only dominant OTUs were studied and the rest of OTUs representing <0.005% of the total sequence reads for each sample were discarded (Bokulich *et al.* 2013).

The coverage of the libraries was estimated by $1 - (n/N) \times 100$, where n is the number of unique OTUs in each sample and N is the total number of OTUs in the library.

Finally, 16S rRNA sequences of each representative OTU were merged into one fasta file. Principal component analysis (PCA) was performed to visualize the most abundant genera driving the relatedness of different samples from ICU water and biofilms by distance matrix along a correlation diagram.

Nucleotide sequence accession numbers

For each phylotype, one 16S rRNA gene representative sequence was deposited in the GenBank database. The accession numbers of the bacterial nucleotide sequences are from KX959627 to KX959684 for biofilm samples and from KX986802 to KX986851 for water samples.

RESULTS

Total bacteria quantification on culture media

Abundance of total heterotrophic bacteria on R2A medium was: W1 (1.25×10^3 CFU/mL), W2 (2×10^3 CFU/mL), W3 (2.4×10^4 CFU/mL), W4 (1.75×10^2 CFU/mL), B1 (9.73×10^3 CFU/cm^2), B2 (1.48×10^3 CFU/cm^2), B3 (2.3×10^1 CFU/cm^2) and B4 (4.08×10^4 CFU/cm^2).

Bacterial diversity at the phylum level

V3 and V4 regions of the 16S rRNA gene were sequenced successfully for water and biofilm samples representing the four different ICUs. A library size of 211,328 raw sequences was obtained. After denoising, 133,637 high-quality 16S rRNA sequences remained where the number of sequences varied between 11,419 as the lowest and 28,988 as the highest between samples (Table 1).

Table 1 | 16S rRNA sequences in bulk water and biofilms and the corresponding coverages (%)

Sample	Location	No. of sequences	Coverage (%)
W1	ICU1	28,988	96
W2	ICU2	15,250	92
W3	ICU3	12,271	86
W4	ICU4	14,588	81
B1	ICU1	14,429	84
B2	ICU2	19,234	88
B3	ICU3	17,458	84
B4	ICU4	11,419	89

The taxonomic characterization of the bacterial community was conducted at the phylum level. Between the 52 well-known Phyla only a poor diversity was obtained including four taxonomic groups (Rappé & Giovannoni 2003) (Figure 1).

The Gram-negative *Proteobacteria* dominated the sequences (95.8%). The rest of the sequences included *Firmicutes* (W2 (0.55%), B1 (1.12%), B2 (0.85%), B3 (0.9%)); Bacteroidetes (B1 (0.27%), B2 (0.13%), B3 (0.12%)) and Gemmatimonadetes (W4 (0.24%)). Four subclasses of *Proteobacteria* were shared between the bulk water and biofilm communities with different percentages: *Alphaproteobacteria* (60%), *Betaproteobacteria* (37.2%), *Gammaproteobacteria* (0.85%) and *Deltaproteobacteria* (0.3%) (Figure 2). *Betaproteobacteria* dominated only in the water coming from the adult burn unit with a high percentage of 80.9%.

Bacterial diversity at the genus level

Among 133,637 sequences affiliated to the bacterial domain, 127,067 (95.1%) were assigned to known bacteria at the genus level based on ≥97% similarity while 4.9% were unclassified bacteria (Table 2). The sequences were grouped in 23 known phylotypes. Overall, *Aquabacterium*, *Novosphingobium* and *Porphyrobacter*, dominated the total number of

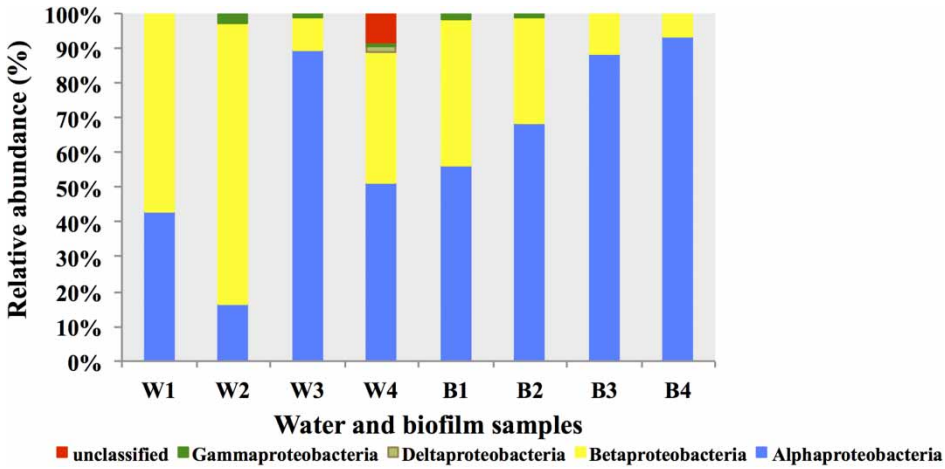

Figure 1 | Relative abundance of different phyla detected in water (W1, W2, W3, W4) and biofilm (B1, B2, B3, B4) samples in the four ICUs.

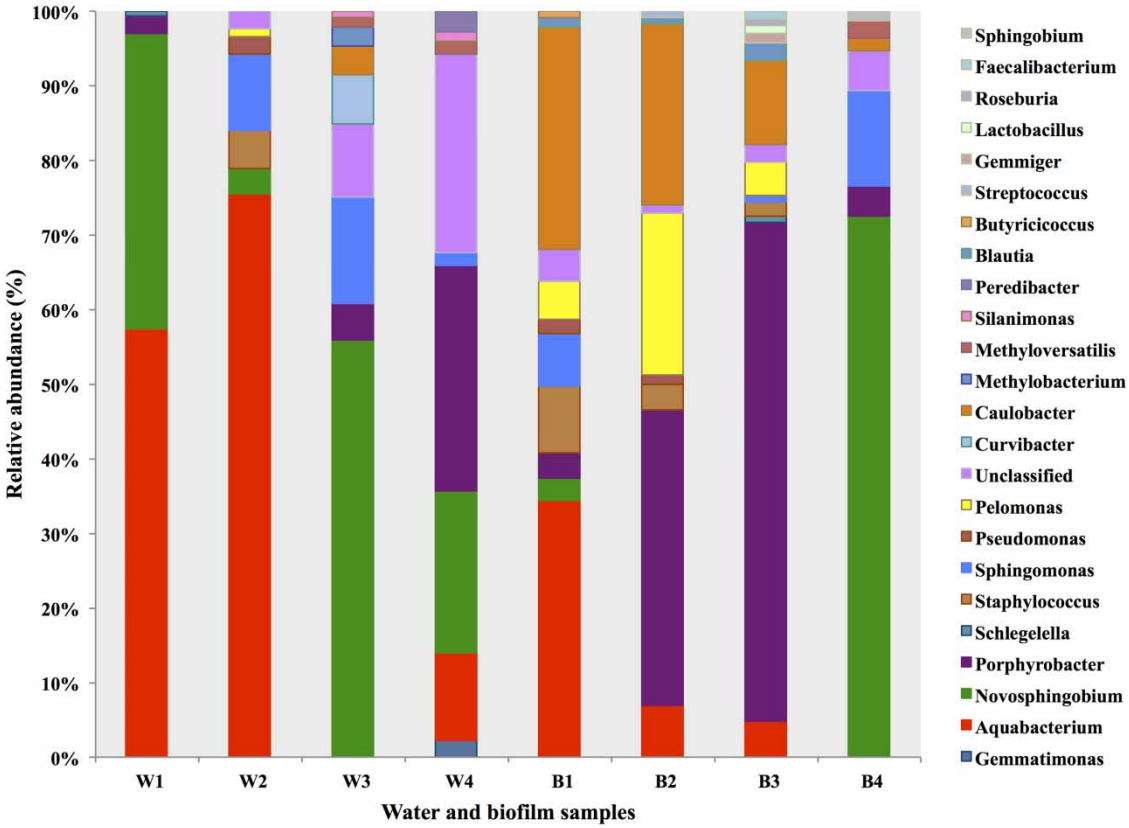

Figure 2 | Genera distribution in water (W1, W2, W3, W4) and biofilm (B1, B2, B3, B4) samples in the four ICUs.

the genera with 27.8, 22.97 and 19.48%, respectively. *Caulobacter*, *Sphingomonas*, *Pelomonas* and *Staphylococcus* were also detected with minor percentage of 8.76, 4.59, 4.47 and 2.33%, respectively. The rest of the bacterial groups (9.6%) included genera with very low percentages (<1%).

By calculating the percentage of the shared genera detected in bulk water versus biofilm, we observe that (1) *Novosphingobium* and *Aquabacterium* were detected mainly in bulk water with 72 and 80%, respectively, and (2) *Staphylococcus*, *Porphyrobacter*, *Caulobacter* and *Pelomonas* were identified mainly in the biofilm with 74, 78, 96 and 97%. respectively.

Bacterial community profiles between bulk water and biofilm

Two-dimensional PCA was adopted to study the relationship of bacterial core communities between ICU water and biofilm. The two principal axes highlighting the best multivariate differences between the bacterial communities are presented in Figure 3. These two selected factors accounted for more than 64% of the total variance.

According to the PCA plot, the different samples were separated into two major groups segregating water from biofilm samples except for samples W2 and B4, indicating an overall distinct bacterial diversity between bulk water and biofilm. Group 1 included three water samples (W1, W3, W4) and one biofilm sample (B4) and group 2 included three biofilm samples (B1, B2, B3) while W2 was distant from the two groups. Therefore, bacterial diversity is significantly comparable between the water samples coming from ICU1, ICU3 and ICU4 and different from the bacterial diversity present in the water of ICU2. Similarly, the three biofilm samples coming from ICU1, ICU2 and ICU3 shared in the majority the same bacterial diversity in opposition to the biofilm from ICU4. The different samples were in the majority separated according to the first component axis including mainly *Sphingobium*, unclassified bacteria,

Table 2 | List of genera identified in the different water and biofilm samples. All bacteria were also identified to species or genus level using BLAST alignment tool (GenBank)

	Operational taxonomic unit		The closest phylotype (GenBank)	
Sample	**Affiliation (% similarity)**	**No. of seq.**	**Organism name**	**Accession nb**
W1	*Aquabacterium* (100)	16673	*Aquabacterium fontiphilum* strain CS-6	NR_044322.1
	Novosphingobium (100)	11,448	Uncultured *Novosphingobium* sp.	HQ330760.1
	Porphyrobacter (99)	664	*Porphyrobacter* sp. OTB63	KX022854.1
	Schlegelella (100)	203	Uncultured *Schlegelella* sp.	HF912284.1
W2	*Aquabacterium* (100)	11,324	Uncultured *Aquabacterium* sp. clone YJQ-2	AY569280.1
	Staphylococcus (100)	797	*Staphylococcus aureus* subsp. *aureus* T0131	CP002643.1
	Sphingomonas (99)	655	*Sphingomonas leidyi* strain SS-88	KX959972.1
	Sphingomonas (100)	530	*Sphingomonas ginsenosidimutans*	HF930757.1
	Novosphingobium (100)	505	*Novosphingobium* sp. SG76	KR856395.1
	Pseudomonas (100)	376	*Pseudomonas yamanorum* strain LMG 27247	LT629793.1
	Sphingomonas (100)	354	*Sphingomonas hankyongensis* strain W1-2-4	KT309084.1
	Unclassified (98)	341	Uncultured bacterium clone LIB078_B_B04	KM852191.1
	Aquabacterium (100)	193	*Aquabacterium citratiphilum* strain B4	NR_024871.1
	Pelomonas (100)	175	*Pelomonas* sp. enrichment culture clone 35Fe00	KF287732.1
W3	*Novosphingobium* (100)	6,209	Uncultured *Novosphingobium* sp. clone B4	KX959672.1
	Sphingomonas (100)	1,741	*Sphingomonas sanxanigenens* strain T12AR28	JF459934.1
	Curvibacter (100)	805	Uncultured *Curvibacter* sp. clone B4-2	KX959673.1
	Porphyrobacter (100)	598	Uncultured *Porphyrobacter* sp. clone B4-5	KX959676.1
	Caulobacter (100)	476	Uncultured *Caulobacter* sp. clone B2-2	KX959645.1
	Novosphingobium (100)	406	Uncultured *Novosphingobium* sp. clone B4-6	KX959677.1
	Unclassified (100)	388	Uncultured bacterium clone B3-9	KX959664.1
	Methylobacterium (100)	312	*Methylobacterium isbiliense*	AB302929.1
	Novosphingobium (100)	259	Uncultured *Novosphingobium* sp. clone B4-9	KX959680.1
	Sphingobium (100)	217	Uncultured *Sphingobium* sp. clone B4-11	KX959682.1
	Unclassified (100)	207	Uncultured bacterium clone B4-13	KX959684.1
	Unclassified (100)	197	Uncultured *Curvibacter* sp. clone B4-2	KX959673.1
	Unclassified (100)	194	Uncultured bacterium clone B4-12	KX959683.1
	Methyloversatilis (100)	158	Uncultured *Methyloversatilis* sp.	KF956449.1
	Silanimonas (100)	104	*Silanimonas* sp. PVC(72 hr)9 partial	AM421790.1
W4	*Porphyrobacter* (97)	4,235	*Porphyrobacter* sp. HIN1 gene	AB599864.1
	Unclassified (100)	1,722	Uncultured bacterium clone eff52	JN245746.1
	Novosphingobium (100)	1,576	*Novosphingobium* sp. SG75	KR856391.1
	Novosphingobium (98)	1,297	*Novosphingobium* sp. CB 286424	LN833301.1
	Unclassified (100)	885	Uncultured bacterium clone 299	KF830576.1
	Aquabacterium (100)	870	Uncultured *Aquabacterium* sp. clone C638	FJ890906.1
	Aquabacterium (100)	837	*Aquabacterium citratiphilum* strain B4	NR_024871.1
	Unclassified (100)	592	Uncultured bacterium clone TW_2_6	JQ905989.1
	Unclassified (100)	500	Uncultured bacterium clone EV818CFSSAHH60	DQ337000.1
	Peredibacter (100)	406	*Peredibacter starrii* strain A3.12	NR_024943.1
	Gemmatimonas (100)	344	Uncultured *Gemmatimonadetes* bacterium clone R2-32	KC994663.1
	Novosphingobium (100)	292	Uncultured *Novosphingobium* sp. clone C04B74	KT731553.1
	Methyloversatilis (100)	246	Uncultured *Methyloversatilis* sp. clone NS-OTU20	KF956449.1
	Sphingomonas (100)	246	*Sphingomonas sanxanigenens* strain T12AR28	JF459934.1
	Silanimonas (100)	193	*Silanimonas* sp. JK13	KF206369.1
	Porphyrobacter (100)	174	*Porphyrobacter* sp.	LC094484.1
	Unclassified (100)	173	*Sterolibacterium* sp. TKU1	AM990454.1
	Porphyrobacter (97)	4,235	*Porphyrobacter* sp. HIN1 gene	AB599864.1

(*continued*)

Table 2 | continued

	Operational taxonomic unit		The closest phylotype (GenBank)	
Sample	**Affiliation (% similarity)**	**No. of seq.**	**Organism name**	**Accession nb**
B1	*Aquabacterium* (100)	4,801	Uncultured *Aquabacterium* sp. clone C638	FJ890906.1
	Caulobacter (100)	4,375	*Caulobacter* sp. B2.29.1	KX442638.1
	Staphylococcus (100)	1,314	*Staphylococcus aureus* subsp. *aureus* T0131	CP002643.1
	Sphingomonas (98)	792	*Sphingomonas leidyi* strain SS-88	KX959972.1
	Pelomonas (100)	771	Uncultured *Pelomonas* sp. clone CNY_01033	JQ401068.1
	Novosphingobium (100)	435	*Novosphingobium* sp. SG76	KR856395.1
	Unclassified (100)	383	*Asinibacterium* sp. ZJ6106	KP301113.1
	Pseudomonas (100)	282	*Pseudomonas yamanorum* strain LMG 27247	LT629793.1
	Aquabacterium (100)	252	*Aquabacterium* sp. Aqua3	AF089859.1
	Porphyrobacter (100)	245	*Caulobacter* sp. B2.29.1	KX442638.1
	Sphingomonas (98)	237	*Sphingomonas hankyongensis* strain W1-2-4	KT309084.1
	Blautia (100)	165	*Blautia* sp. GD8	LN890282.1
	Butyricicoccus (100)	140	Uncultured bacterium clone PCS406_74	JX851442.1
	Porphyrobacter (99)	120	*Porphyrobacter* sp. HIN1	AB599864.1
	Porphyrobacter (100)	117	*Porphyrobacter* sp. W14	LC094481.1
B2	*Porphyrobacter* (100)	7,604	*Porphyrobacter* sp. OTB63	KX022854.1
	Caulobacter (100)	4,661	*Caulobacter segnis* strain LEM09	KU180331.1
	Pelomonas (100)	4,213	Uncultured *Pelomonas* sp. clone CNY	JQ401068.1
	Aquabacterium (100)	803	Uncultured *Aquabacterium* sp. clone C638	FJ890906.1
	Staphylococcus (100)	675	*Staphylococcus aureus* subsp. *aureus* T0131	CP002643.1
	Aquabacterium (100)	532	*Aquabacterium fontiphilum* strain CS-6	NR_044322.1
	Pseudomonas (100)	222	*Pseudomonas migulae*	AY605698.1
	Unclassified (100)	192	Uncultured bacterium clone CP33c07	JN196170.1
	Blautia (100)	188	*Blautia wexlerae*	LC037229.1
	Streptococcus (100)	144	*Streptococcus salivarius* strain PUA082	KX661124.1
B3	*Porphyrobacter* (100)	11,399	*Porphyrobacter* sp. SLCR_2	LN681617.1
	Caulobacter (100)	2,000	*Caulobacter* segnis strain LEM09	KU180331.1
	Aquabacterium (100)	869	Bacterium 'niu b1'	KJ950444.1
	Pelomonas (100)	816	Uncultured *Pelomonas* sp. clone HL-D40	KU588028.1
	Porphyrobacter (100)	393	*Porphyrobacter* sp. OTB63	KX022854.1
	Staphylococcus (100)	326	*Staphylococcus aureus* subsp. *aureus* T0131	CP002643.1
	Blautia (100)	254	*Blautia wexlerae* gene	LC037229.1
	Gemmiger (100)	212	Uncultured bacterium clone B5_509	EU766446.1
	Faecalibacterium (100)	205	Uncultured organism	HQ751613.1
	Blautia (100)	182	*Blautia* sp. GD8	LN890282.1
	Lactobacillus (97)	175	*Lactobacillus salivarius* strain CICC 23174	CP017107.1
	Unclassified (100)	174	Bacteroidetes bacterium OR-43	HM163267.1
	Schlegelella (100)	169	Uncultured *Schlegelella* sp.	HF912284.1
	Roseburia (100)	149	*Lachnospiraceae* bacterium MC_35	LN907763.1
	Sphingomonas (98)	135	*Sphingomonas leidyi* strain SS-88	KX959972.1
B4	*Novosphingobium* (100)	7,711	*Novosphingobium* sp. SG75	KR856391.1
	Sphingomonas (100)	792	*Sphingomonas sanxanigenens* strain T12AR28	JF459934.1
	Sphingomonas (100)	652	*Sphingomonas alpina*	HF930754.1
	Porphyrobacter (100)	480	*Porphyrobacter* sp. LM 6	CP017113.1
	Novosphingobium (99)	356	*Novosphingobium* sp. CB 286424	LN833301.1
	Unclassified (99)	355	*Blastomonas natatoria* strain A2.41.1	KX442630.1
	Methylobacterium (100)	251	Uncultured *Methylobacterium* sp. clone 4LB10	JF460964.1
	Novosphingobium (100)	205	Uncultured *Novosphingobium* sp. clone C04B74	KT731553.1
	Caulobacter (100)	198	*Caulobacter* sp. B2.29.1	KX442638.1
	Sphingobium (99)	152	*Sphingobium* sp. RAC03	CP016456.1
	Unclassified (100)	135	*Phreatobacter oligotrophus* strain U274	KT345641.1
	Unclassified (100)	132	Uncultured *Rhodobacter* sp. clone DWIIA07	HQ711905.1

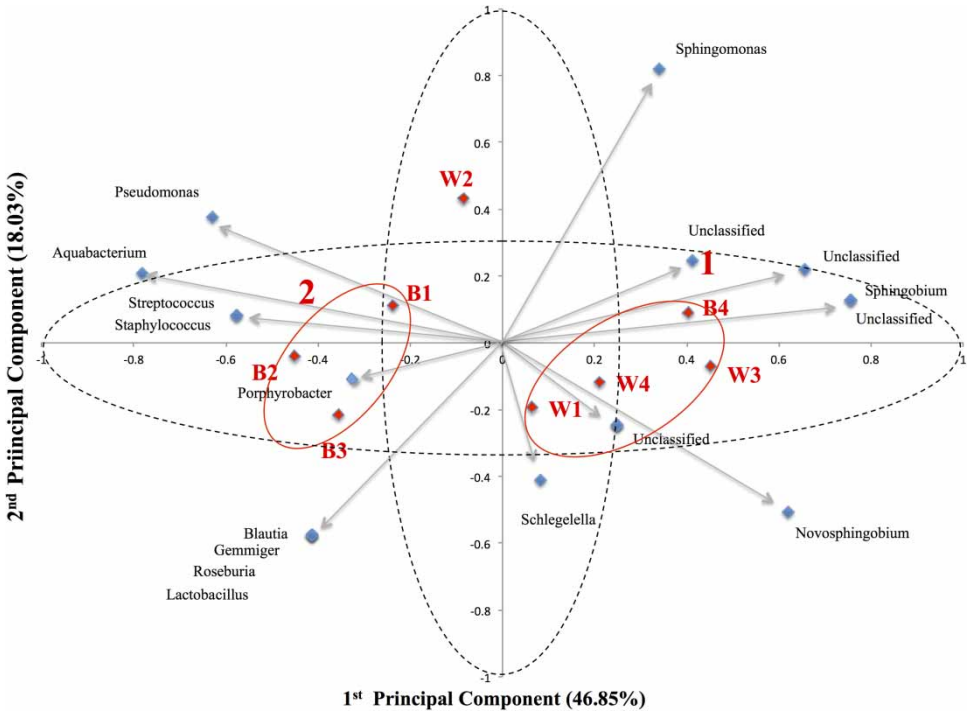

Figure 3 | Principal component analysis of different genera obtained in the water and biofilm samples from the different ICUs. Samples that cluster more closely together share similar bacterial diversity. Correlation diagram with genera influencing the plot ordination were added to the PCA plot.

Novosphingobium, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Porphyrobacter* and *Aquabacterium*. However, and because it was difficult to interpret how these genera separated the two groups 1 and 2, Venn diagrams were created for each of the similar water samples (W1, W3, W4) and biofilm samples (B1, B2, B3) in order to highlight the shared phylotypes between the samples in each group (Figure 4(a) and 4(b)).

According to the Venn diagrams, water samples (W1, W3 and W4) shared two genera affiliated to *Novosphingobium* and *Porphyrobacter* whereas biofilm samples (B1, B2 and B4) had in common six genera that belonged to

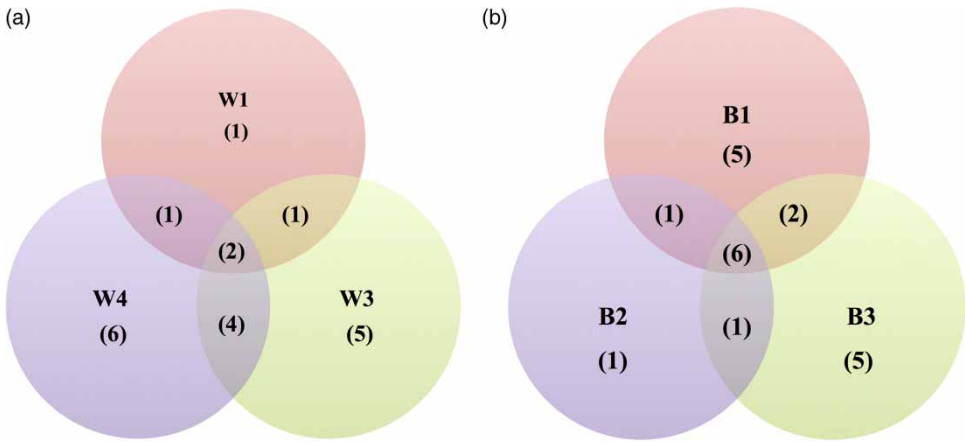

Figure 4 | (a) Venn diagram of the shared genera (at ≥97% level of sequence similarity) between water samples of ICU1, ICU3 and ICU4. (b) Venn diagram of the shared genera (at ≥97% level of sequence similarity) between biofilm samples of ICU1, ICU2 and ICU3.

Aquabacterium, *Porphyrobacter*, *Caulobacter*, *Staphylococcus*, *Pelomonas* and unclassified bacteria.

DISCUSSION

Phylogenetic analysis based on the 16S rRNA gene was used in this research work to study the bacterial communities in water and biofilm of a Saudi hospital water distribution network. Despite the high rates of HAI in this region (Abdel-Fattah 2005), drinking water-related diseases are still occurring due to the lack of information regarding the ecology of these organisms.

This study focused on drinking water in ICUs, where patients are at high risk. Beside bulk water, bacterial core communities were studied in the biofilm – as opposed to many of the published studies worldwide – because microorganisms attached to the surface constitute the main biomass proportion in water distribution systems (Berry & Raskin 2006; Declerck *et al.* 2009) and can harbour opportunistic pathogens (Anaissie *et al.* 2002; Kusnetsov *et al.* 2003; Angelbeck *et al.* 2006) and bacteria resistant to disinfectants (Farhat *et al.* 2011, 2012).

Total bacteria quantification on culture media

Knowing that water networks in the four studied ICUs were fed by the same water supply, cultivable heterotrophic bacterial count ranged from 10^2 to 10^4 CFU/mL in the bulk water with the highest being detected in the bathtub outlet of the pediatric burn unit. Total cell count in biofilm samples ranged from 10^1 to 10^4 CFU/cm^2 with the lowest count coming from the pediatric burn unit biofilm. This low count is normal and can be explained by the accessibility to the bathtub biofilm and its mechanical removal by cleaning as opposed to the rest of the biofilms extracted from the inner surface of the tap faucets. Overall, these concentrations are within the normal range found in drinking water and biofilm (Lu & Zhang 2005).

Bacterial diversity at the phylum level

The different phyla found in our water and biofilm samples were very similar to those detected normally in drinking water systems (Poitelon *et al.* 2009; Baron *et al.* 2014) where *Proteobacteria* was the most abundant phylum followed by *Firmicutes*, *Bacteroides* and *Gemmatimonadetes*. Additionally, Gram-positive *Alpha-* and *Betaproteobacteria* subclasses dominated the rest of the phyla (Williams *et al.* 2004; Tokajian *et al.* 2005). A higher abundance of *Alphaproteobacteria* was observed in all samples except for W1 and W2 where *Betaproteobacteria* had the highest percentage belonging to *Aquabacterium* genus. Studies explained *Betaproteobacteria* predominance in some drinking water networks by a higher level of total organic carbon (TOC) and lower disinfectant residual (Kalmbash *et al.* 2000), in addition to other factors like the age and pipe material (Norton & LeChevallier 2000).

Bacterial diversity at the genus level

On the other hand, water and biofilms had low genera diversity compared to other studies (Williams *et al.* 2004; Tokajian *et al.* 2005; Oberauner *et al.* 2013; Vaz-Moreira *et al.* 2013). Our phylotypes included bacteria frequently detected in drinking water distribution systems. The highest in percentage belonged to *Aquabacterium* (27.7%), *Novosphingobium* (22.9%), *Porphyrobacter* (19.41%), *Caulobacter* (8.73%) and *Sphingomonas* (4.57%).

Novosphingobium, *Porphyrobacter, Sphingomonas* and *Sphingobium* belong to the Sphingomonads. They are widespread in natural environments (like soil, plants and clinical samples) and man-made environments (like drinking water systems, reservoirs, tap water and bathtubs) (Hong *et al.* 2010). The presence of Sphingomonads in drinking water distribution systems is undesirable due to the pathogenic status of some of the species such as *Sphingomonas paucimobilis* and *Sphingomonas parapaucimobilis* (Nandy *et al.* 2013). According to our BLAST affiliation results, *Sphingomonas* species detected in the four ICUs belonged to non-pathogenic species (*S. leidyi, S. ginsenosidimutans, S. hankyongensis, S. sanxanigenens* and *S. alpine*). However, detection of the Sphingomonadaceae family at higher counts in drinking water can be related to resistance to the disinfection process used, like chlorination and formation of new biofilms, as these bacteria are known as pioneers in the initial steps of biofilm formation in drinking water networks (Sun *et al.* 2013; Vaz-Moreira *et al.* 2013).

Gammaproteobacteria, which is possible to be included as opportunistic pathogens, represented a very low percentage of our drinking water microflora (0.85%). Only sequences affiliated to *Pseudomonas*, *Methylobacterium* and *Streptococcus* were detected with a very low percentage of 0.66%, 0.23% and 0.11%, respectively, and correspondent species were revealed as non-pathogenic according to BLAST results (*Pseudomonas yamanorum*, *Pseudomonas migulae, Methylobacterium isbiliense* and *Streptococcus salivarius*). On the other hand, skin-associated bacteria *Staphylococcus* was present in three of our studied areas (common area of the medical and surgical units, adult burn unit and pediatric burn unit) and absent in the neonatal care unit. *Staphylococcus* was further affiliated to the opportunistic *S. aureus* with a percentage of 2.32%. This species is a common member of human microflora and a causative agent of a growing number of health care-associated infections (Tong *et al.* 2015). Interestingly, *Staphylococcus aureus* was absent in the bulk water and only detected in the corresponding biofilm of three of the ICUs (1, 2 and 3), except for the adult burn unit where this species was identified in both water and biofilm. Because this bacterium is known as part of the human skin microflora, it is likely that it could be transmitted from patients or health care providers by direct contact with the faucet. Compared to the countable studies done on the microbial ecology of water distribution systems in hospitals, our water distribution system harboured much less diversified pathogens or genera containing possible opportunistic pathogens. Many authors have described complex bacterial communities including possible pathogenic genera such as *Legionella* spp., *Acinetobacter* spp., *Stenotrophomonas* spp., *Mycobacterium* spp., *Burkholderia*, *Escherichia/Shigella, Flavobacterium, Propinobacterium, Pseudomonas* spp., *Staphylococcus* spp. and *Methylobacterium* (Oberauner *et al.* 2013).

Bacterial community profiles between bulk water and biofilm

The multivariate analysis showed us distinctive genera between each of the bulk water and its corresponding biofilm except for the NICU. This finding was also highlighted by the taxonomic results where different bacteria dominated the bulk water (*Novosphingobium* and *Aquabacterium*) and biofilms (*Staphylococcus, Porphyrobacter*, *Caulobacter* and *Pelomonas*). In contrast, the other water and biofilm samples, W4 and B4, were close on the PCA plot revealing a similar bacterial diversity shared between the bulk water and biofilm of the NICU. This resemblance between bulk water and biofilm microflora might be explained by the frequent detachment of biofilm structure into the bulk water due to the intermittent pressure applied usually in water supply systems. The presence of a high percentage of Sphingomonads in this unit (86% of the total genera found) enforces this hypothesis where new biofilm could be starting to form.

CONCLUSIONS

Surveillance for nosocomial infections is the keystone of prevention and control. Our research study has provided evidence of the prevalence of particular bacterial genera in the biofilm. Our future objective is to study gene expression patterns of water and biofilm bacteria in order to accentuate their function and impact in drinking water distribution systems. New perspectives should be defined in taking the attached bacterial flora into consideration in order to assess and address the health impact risks of unsafe drinking water distribution systems. Moreover, hospitals are required to refine a quantitative microbial risk assessment for identification and selective detection of life-threatening microorganisms.

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