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Review

Occurrence of Potentially Pathogenic Bacteria in Epilithic Biofilm Forming Bacteria isolated from Porter Brook River-stones, Sheffield, UK

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ABSTRACT

Biofilms in aquatic ecosystems develop on wet benthic surfaces and are primarily comprised of various allochthonous microorganisms, including bacteria embedded within a self-produced matrix of extracellular polymeric substances (EPS). In such environment, where there is a continuous flow of water, attachment of microbes to surfaces prevents cells being washed out of a suitable habitat with the added benefits of the water flow and the surface itself providing nutrients for growth of attached cells. When watercourses are contaminated with pathogenic bacteria, these can become incorporated into biofilms. This study aimed to isolate and identify the bacterial species within biofilms retrieved from river-stones found in the Porter Brook, Sheffield based on morphological, biochemical characteristics and molecular characteristics, such as 16S rDNA sequence phylogeny analysis. Twenty-two bacterial species were identified. Among these were 10 gram-negative pathogenic bacteria, establishing that potential human pathogens were present within the biofilms. *Klebsiella pneumoniae* MBB9 isolate showed the greatest ability to form a biofilm using a microtiter plate-based crystal violet assay. Biofilm by *K. pneumoniae* MBB9 formed rapidly (within 6 h) under static conditions at 37 °C and then increased up to 24 h of incubation before decreasing with further incubation (48 h), whereas the applied shear forces (horizontal orbital shaker; diameter of 25 mm at 150 rpm) had no effect on *K. pneumoniae* MBB9 biofilm formation.

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1. Introduction

Biofilms can exist in both natural and anthropogenic environments (Beściak and Surmacz-Górska, 2011). Although biofilms can develop on different exteriors, solid surfaces in aqueous environments are often susceptible (Ardré et al., 2015). The adhesion of bacteria onto submersed solid surfaces and subsequent microbial growth, followed by the production of extracellular polymeric substances (EPS) lead to the formation of biofilms (Pereira et al., 2002). Bacterial biofilms can be described as aggregations of cells attached to surfaces and surrounded by a matrix of EPS (Marvin et al., 2001).

In aquatic environments, such as surface waters, biofilms are ubiquitous (Mackowiak et al., 2018). River biofilms can be found in various compartments, such as benthic, hyporheic, and aquifer sediments (Sabater et al., 2002). In such environments, microorganisms that attach to surfaces and develop biofilms on submerged surfaces can be composed of bacteria, fungi, algae, and micrometozoa embedded in an organic polysaccharide matrix (Tien et al., 2011). Although biofilms in these places consist of allochthonous microorganisms, under contamination conditions, faecally derived bacteria can interact with these biofilms (Balzer et al., 2010). These biofilms which are niches for several pathogens may act as a reservoir for faecally derived and other bacterial pathogens in polluted waterways (Wolf-Rainer, 2011; Mackowiak et al., 2018). Hence, such organisms might be released, increasing risks to human health by exposure during water-related activities (Donovan et al., 2008). Pathogens likely enter rivers through various routes, including lateral inputs from pastures and riparian zones, influx of pathogen-contaminated groundwater, direct deposit of fecal matter from livestock and wildlife, discharge of contaminated sanitary sewer flows, wastewater treatment plant effluents, surface runoff from urban and agricultural areas and wildlife and mobilization of fecal organisms from the riverbed during sediment-disturbing events such as floods or heavy precipitation (Pandey et al., 2014; Mackowiak et al., 2018).

Establishment and development of bacterial biofilms are known to be dynamic and complex processes regulated by intrinsic biological properties and also by many environmental factors, since changes in the environment often trigger the formation of biofilm (Rühs et al., 2013; Gomes, 2012). The environmental factors that control attachment and formation of biofilm may also influence the ability of bacteria to grow and survive (Bjergbæk et al., 2006). Several factors, including incubation period, nutrient levels, temperature, pH, and ionic strength can have an impact on microbial biofilm formation; however, bacterial cell surface appendages and surface characteristics are also necessary for this process (Agarwal et al., 2011).

Attachment to surfaces and biofilm formation are mainly governed by electrostatic, van der Waals, hydrophobic, and contact interactions (Kurincic et al., 2016). Hydrophobic interactions are known to play a role in microbial adhesion to a variety of surfaces and facilitate the formation of biofilm. Surface hydrophobicity can influence bacterial colonization of materials, such that bacteria have evolved several various ways to use the hydrophobic effect in order to adhere to substrata (Van Loosdrecht et al., 1987; Tahmourespour et al., 2008; Tyfa et al., 2015). However, cell surface hydrophobicity may change as a function of the physiological state of bacteria (Tahmourespour et al., 2008).

To characterize the biofilm-forming bacteria from epilithic river biofilms as well as to assess the presence of potentially pathogenic bacteria in the biofilms present in the river, biofilm-associated bacteria were isolated from river-stones collected from the Porter Brook, Sheffield, and identified from their morphological, biochemical and molecular characteristics, such as 16S rDNA sequence analysis. The capacity of potentially pathogenic bacterial isolates to produce a biofilm and the effect of environmental factors, such as incubation time and hydrodynamic conditions on *Klebsiella pneumoniae* MBB9 biofilm were also assessed using a microtiter plate-based crystal violet assay.

2. Materials and methods

2.1. Source and sampling

River-stones with visible biofilms (thick, light brown, sticky growth) on the upper surfaces were collected on the same day (March 2015) in a sterile plastic container from the Porter Brook in Sheffield, United Kingdom, and were stored in a cool box with ice until analysis.

2.2. Isolation of bacteria from environmental biofilms

Epilithic biofilms were aseptically scraped from the stones and suspensions were serially diluted 1:100 in physiological saline (0.85%) using an aseptic technique (APHA, 1998). The suspensions were spread on various selective media: R2A agar, MacConkey agar, Eosin-methylene blue (EMB) agar, Violet Red Bile Agar, Xylose lysine deoxycholate (XLD) agar and nutrient agar. The plates were incubated at 37 °C for 24–72 h under aerobic conditions. Colonies with different colouration and morphologies were picked from the spread plates and streaked again on freshly agar plates to obtain pure cultures of the bacterial isolates.

2.3. Bacterial morphological and biochemical characterization

Isolated colonies from the agar plates were selected and characterized using various morphological and biochemical characteristics for preliminary identification (Bergey and Holt, 1994). Morphological parameters, included colony form, elevation, margin, surface, optical features, consistency and colour are used as well as biochemical tests, such as catalase and oxidase activities (Table 1).

2.4. Molecular identification of bacteria via 16S rRNA gene sequencing and phylogenetic analysis

Bacterial genomic DNA was extracted from all isolated bacteria using the GenElute™ Bacterial Genomic DNA Kit according to the manufacturer's instructions. The purity of the DNA preparations was assessed spectrophotometrically using a Nanodrop 1000 (A260/280) (NanoDrop Technologies, Wilmington, DE, USA). The amplification of 16S rRNA genes of the bacterial isolates was performed by PCR using the universal forward (27F) 5'-AGAGTTT

Table 1
Morphological and biochemical characterization of bacterial isolates. Isolated bacteria were first characterized for their morphological, cultural, and biochemical characteristics for preliminary assessment of diversity.

Bcaterial Isolate	Colony form	Colony elevation	Colony margin	Colony surface	Colony optical features	Colony consistency	Colony colour	Motility	Gram stain	Enzyme production	
										Oxidase test	Catalase test
1	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	+ve	+ve
2	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	+ve	+ve
3	Circular	Convex	Erose	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	+ve	+ve
4	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Non-motile	-ve	-ve	+ve
5	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	+ve	+ve
6	Circular	Convex	Erose	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	-ve	+ve
7	Circular	Convex	Erose	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	-ve	+ve
8	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Non-motile	-ve	-ve	+ve
9	Punctiform	Pulvinate	Entire	Smooth	Translucent	Viscid (slimy)	White	Non-motile	-ve	-ve	+ve
10	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	-ve	+ve
11	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	-ve	+ve
12	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	-ve	+ve	+ve
13	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Non-motile	-ve	-ve	+ve
14	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	Yellow	Non-motile	-ve	+ve	+ve
15	Irregular	Pulvinate	Undulate	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	+ve	+ve
16	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	-ve	+ve
17	Irregular	Convex	Filamentous	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	-ve	+ve
18	Circular	Umbonate	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	+ve	+ve
19	Circular	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	-ve	+ve
20	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	-ve	+ve
21	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	-ve	+ve
22	Punctiform	Convex	Entire	Smooth	Translucent	Viscid (slimy)	White	Motile	+ve	+ve	+ve

GATCCTGGCTCAG-3' and reverse (1492R) 5'-GGTTACCTTGTTAC GACTT-3' primers to amplify the V1-V9 region (1500 bp) of the16S rRNA gene. In brief, a PCR reaction mixtures of total volume 25 μl was prepared as follows: 12 μl of 2X master mix (BioLabs, England), 2 μl of each oligonucleotide primer (10 μM), 7 μl of Molecular Grade Water and 2 μl of template DNA. All reactions were run on a LabCycler (SensQuest, Germany) using the following parameters: initial denaturation at 98 °C for 30 s, 35 cycles of 95 °C for 1 min, 58 °C for 30 s, 72 °C for 5 min and a final extension at 72 °C for 5 min followed by a hold at 4 °C. The PCR products were separated by electrophoresis on a 1% (w/v) agarose gel electrophoresis (BIO-RAD, USA). Gels were stained with SYBR Safe® (Invitrogen). DNA bands were visualised using a UVI tech photodocumentation system to view the DNA fragments. Following the sequencing, each DNA sequence chromatogram was analyzed to assess its quality using the bioinformatic tool FinchTV software where the lower quality sequences were trimmed from both ends, and the remaining good-quality sequences were moved to a new file. Basic local alignment search tool (BLAST) in National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) were used to provide taxonomic information about the source species. The neighbor-joining method implemented in the program MEGA software was used for phylogenetic tree construction (Fig. 1).

2.5. Assessment of biofilm formation by the isolated bacteria using microtiter plate assay

The microtiter plate method of O'Toole and Kolter, 1998 was used with a few modifications. Briefly, bacterial isolates were grown overnight in nutrient broth at 37 °C. The OD_{600} of the bacterial suspensions was adjusted to 0.5 McFarland standards (approximately 10^8 CFU/ml). Aliquots (200 μl) were then used to inoculate the wells of a flat-bottomed polystyrene 96-well microtiter plate (Costar; Corning Incorporated., USA). Sterile nutrient broth was used as plate sterility controls. The plates were covered and incubated at 37 °C for 24 h (Gomes, 2012). Planktonic cells in the fluid were then removed by inverting the assay plate and decanting the liquid, followed by rinsing thoroughly three times with 200 μl of sterile deionised water (dH_2O) to remove any remaining planktonic cells (unattached bacteria). The microtiter plates were air-dried at 37 °C, and adherent bacteria were stained with 200 μl of 1% (w/v) crystal violet solution (CV) (crystal violet; Merck, Germany) for 25 min (Christensen et al., 1985; Christensen, Baldassarri and Simpson, 1995). Following the staining step, the supernatant was discarded, and the wells were rinsed by repeated washing with sterile deionised water (dH_2O) to remove any excess stain. Any CV incorporated by the biofilm was solubilized (eluted from stained biofilms) by adding 250 μl of 30% glacial acetic acid. The CV liberated from the attached material and control wells was assessed by measuring absorbance at 595 nm using a multiwell plate reader (BioTek FLx800, UK) (Saloni, Kusum and Sanjay, 2012). The mean absorbance for the blank was then subtracted from the mean absorbance of the test strain (Sonkusale and Tale, 2015).

2.6. Cell surface hydrophobicity assay

Microbial adhesion to hydrocarbon (MATH) adapted from Rosenberg et al. (1980) was used with a few modifications. Bacteria were grown overnight in nutrient broth at 37 °C and were harvested by centrifugation at 6,000 rpm, 25 °C for 5 min. Bacterial cells were then washed and re-suspended in sterile deionised water (dH_2O). The OD_{600} of the bacterial suspensions was measured and adjusted spectrophotometrically at OD_{600} of 0.3 to 0.6 using a Unicam Spectrophotometer (Unicam, England). Two ml of bacterial suspensions were mixed in glass tubes for 2 min at room temperature with the same quantity of xylene using a vortex mixer (SciQuip, UK) (Bellon-Fontaine et al., 1996; Ngwai et al., 2006; Xu et al., 2010; Zoueki et al., 2010). Phases were allowed to separate (the aqueous phase from xylene) for 1 h at room temperature.

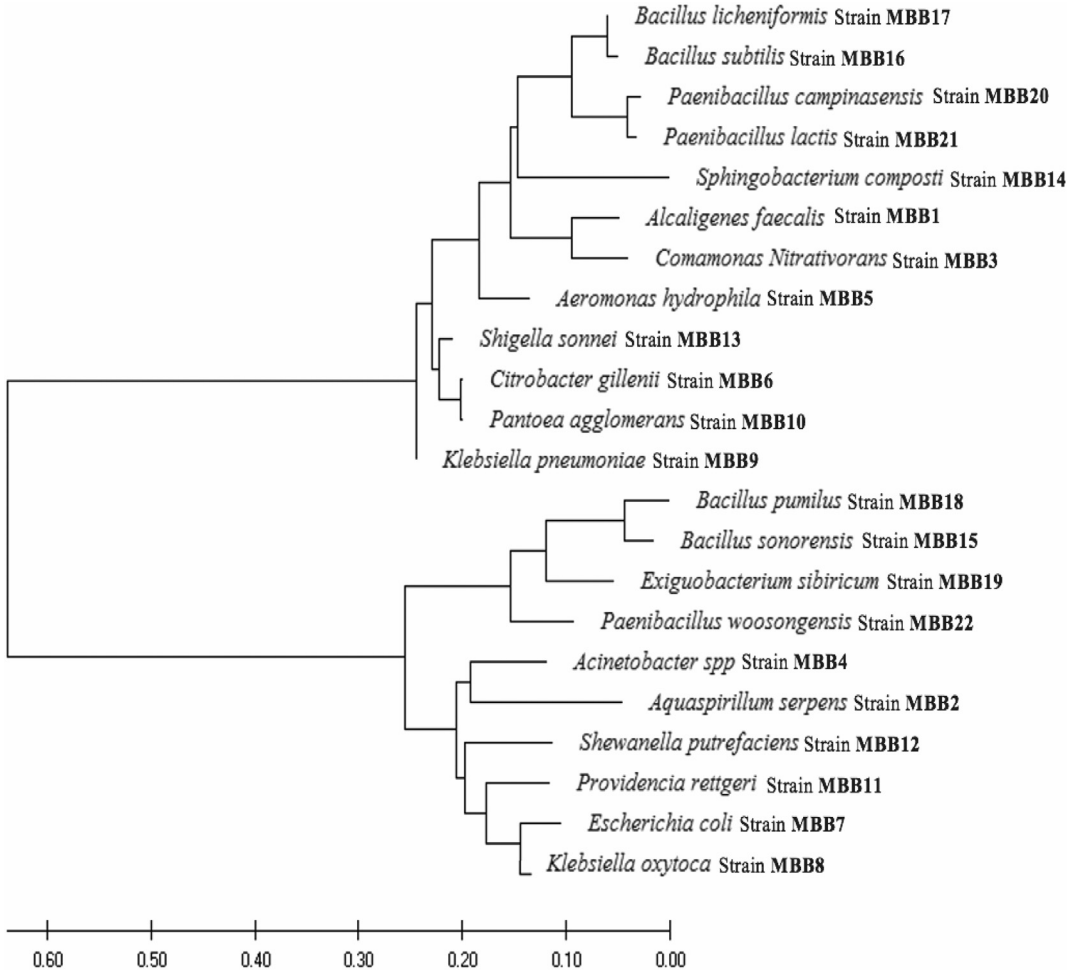

Fig. 1. Neighbor-joining phylogenetic tree of epilithic biofilm forming bacteria from the Porter Brook, Sheffield. Phylogenetic tree based on a comparison of the 16S ribosomal DNA sequences of bacterial isolates. The tree was created by the neighbor-joining method and the evolutionary distances analyses were conducted in MEGA X (Kumar et al., 2018).

Following incubation, the absorbance of the lower aqueous phase was measured as before. Hydrophobicity Index (the affinities to xylene) was calculated as:

$$[A_{\text{initial}} - A_{\text{aqueous phase}} / A_{\text{initial}}] \times 100$$

2.7. Effect of incubation time on biofilm formation by Klebsiella pneumoniae MBB9

Klebsiella pneumoniae MBB9 biofilm was developed under static conditions by incubation for 6, 12, 18, 24 and 48 h at an ambient temperature of 37 °C using microtiter plate-based crystal violet assay (Section 2.5).

2.8. Effect of hydrodynamic conditions on biofilm formation by Klebsiella pneumoniae MBB9

The microtiter plate-based crystal violet assay (Section 2.5) were used to test the effect of dynamic conditions (horizontal orbital shaking, 150 rpm, 25 mm throw) on the kinetics of *K. pneumoniae* MBB9 biofilm formation.

2.9. Statistical analysis

The mean and standard deviation (SD) were calculated from the results. A two-way analysis of variance (ANOVA) was used to evaluate the effect of hydrodynamic conditions on the physical structure of biofilm by *K. pneumoniae* MM9 p values of <0.05 were considered to be statistically significant. The error bars represent the standard deviation of the means.

3. Results

A total of 22 bacterial species were isolated and identified from biofilms formed on stones retrieved from the Porter Brook, Sheffield. From the results, Proteobacteria, Bacteriodetes and Firmicutes were the dominant detected bacterial phyla among biofilms. However, class γ-Proteobacteria was the largest bacterial group and class Bacilli was the second most common bacterial group followed by class β-Proteobacteria, while class Sphingobacteria was a minor component of the bacterial community in biofilm samples (Table 2). Several genera from diverse bacterial phyla were detected in samples, implying a high bacterial diversity in biofilms. Nevertheless, taxonomic classifications at the genus level revealed further diversity. Within class γ-Proteobacteria, genera *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Escherichia*, *Klebsiella*, *Pantoea*, *Providencia*, *Shewanella*, and *Shigella* were detected, while genus *Sphingobacterium* was only detected within class Sphingobacteria. Within class β-Proteobacteria, genera *Alcaligenes*, *Aquaspirillum*, and *Comamonas* and genera *Bacillus*, *Exiguobacterium*, and *Paenibacillus* within class Bacilli were also detected in biofilm samples. The α-Proteobacteria and β-Proteobacteria are often the most

Table 2
Sequence database homology search results for 16S rRNA genes of the isolated bacteria. The 16S rRNA amplicons were compared to sequences in GenBank, National Center for Biotechnology Information (NCBI), using the BLAST search engine to identify the origin of the DNA sequence.

Bacterial isolates	NCBI Best match (Genus species)	% Coverage	E value	% Identity	Accession number	Type of microorganism	Characteristics	Reference
	β-Proteobacteria							
1	*Alcaligenes faecalis*	100%	0.0	100%	KP224304.1	Gram-negative	• Causative agent of post-operative endophthalmitis in the human eye. Associated with development of peritonitis in peritoneal dialysis patients.	(Kaliaperumal et al., 2005) (Kahveci et al., 2011)
2	*Aquaspirillum serpens*	100%	0.0	99%	JN609336.1	Gram-negative		
3	*Comamonas nitrativorans*	100%	0.0	99%	KF530281.1	Gram-negative		
	γ-Proteobacteria							
4	*Acinetobacter* spp	99%	0.0	99%	KP747659.1	Gram-negative	• Some species cause; bacteremia, urinary tract infections, secondary meningitis, infective endocarditis, and wound and burn infections.	(Pratap et al., 2010)
5	*Aeromonas hydrophila*	100%	0.0	100%	KR052185.1	Gram-negative	• Human infections; cellulitis. Wound infections. Diarrhea	(McCracken and Barkley, 1972) (Skiendzielewski et al., 1990) (Janda et al., 1983)
6	*Citrobacter qillenii*	100%	0.0	100%	KR612001.1	Gram-negative	• Wound infection, urinary tract infections and bacteremia.	(Nayar et al., 2014)
7	*Escherichia coli*	100%	0.0	100%	CP014197.1	Gram-negative	• Certain serotypes can cause various intestinal and extra-intestinal diseases, such as urinary tract infections, diarrhea, neonatal meningitis and septicemia.	(Orskov and Orskov, 1992)
8	*Klebsiella oxytoca*	100%	0.0	100%	LC049180.1	Gram-negative	• Colitis and sepsis.	(Högenauer et al., 2006)
9	*Klebsiella pneumoniae*	100%	0.0	100%	LC049181.1	Gram-negative	• Opportunistic pathogen; cause infections of respiratory tract, pharynx and nasal mucosa. Nosocomial infection; Pneumonia	(Sikarwar and Batra, 2011) (Richards et al., 1999)
10	*Pantoea agglomerans*	100%	0.0	99%	KT075214.1	Gram-negative	• Opportunistic infections in humans; wound, blood, and urinary-tract infections. Endophthalmitis, periostitis, endocarditis and osteomyelitis in humans.	(Cruz et al., 2007) (Jacek et al., 2016)
11	*Providencia rettgeri*	100%	0.0	100%	KR091945.1	Gram-negative	• Opportunistic infections in humans; a major cause of traveller's diarrhea. Urinary tract infections. Eye infections.	(Yoh et al., 2005) (Jones and Mobley, 1987) (Koreishi et al., 2006)
12	*Shewanella putrefaciens*	100%	0.0	100%	HQ588327.1	Gram-negative		
13	*Shigella sonnei*	100%	0.0	100%	CP011422.1	Gram-negative	• Shigellosis Major causes of diarrhea Meningitis and bacteriemia	(Tajbakhsh et al., 2012) (Qu et al., 2012) (Puel et al., 2005)
	Bacteriodetes - Sphingobacteria							
14	*Sphingobacterium composti*	100%	0.0	100%	AB682399.1	Gram-negative		
	Firmicutes - Bacilli							
15	*Bacillus sonorensis*	100%	0.0	100%	KP282741.1	Gram-positive		
16	*Bacillus subtilis*	100%	0.0	100%	CP007173.1	Gram-positive		
17	*Bacillus licheniformis*	100%	0.0	100%	KP965752.1	Gram-positive		
18	*Bacillus pumilus*	100%	0.0	100%	EU244733.1	Gram-positive		
19	*Exiguobacterium sibiricum*	100%	0.0	100%	LK391534.1	Gram-positive		
20	*Paenibacillus campinasensis*	100%	0.0	100%	KF312290.1	Gram-positive		
21	*Paenibacillus lactis*	100%	0.0	100%	KF930955.1	Gram-positive		
22	*Paenibacillus woosongensis*	100%	0.0	100%	AB902951.1	Gram-positive		

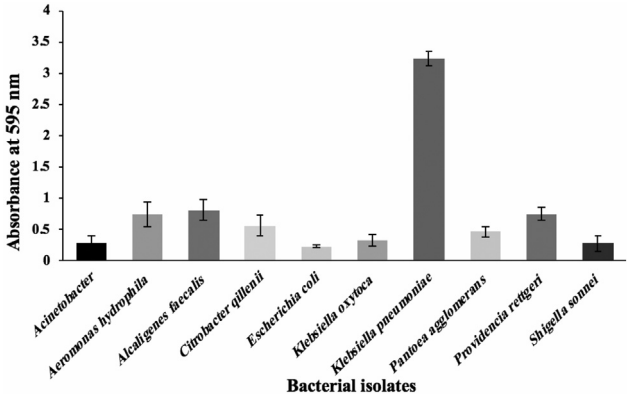

Fig. 2. Biofilm formation by different bacterial isolates. The microtiter plate-based crystal violet assay was used, and the optical density of the attached material and control wells were read at 595 nm using a multiwell plate reader (BioTek FLx800, UK). Bars are mean values, while error bars are the standard deviation (n = 6).

abundant bacterial phyla in freshwater habitats, reflecting the planktonic bacteria in the river water, while Firmicutes and γ-Proteobacteria are also commonly found to dominate these habitats (Katharina et al., 2012). However, among the latter were 10 gram-negative pathogenic bacteria that can be associated with human infections. Most of the isolated potentially pathogenic bacterial strains belonged to the family *Enterobacteriaceae* amongst which *Providencia rettgeri* MBB11, *Escherichia coli* MBB7, *Pantoea agglomerans* MBB10, *Citrobacter qillenii* MBB6, *Klebsiella oxytoca* MBB8, *Shigella sonnei* MBB13, *Klebsiella pneumoniae* MBB9. Others included *Acinetobacter* spp MBB4, *Alcaligenes faecalis* MBB1, and *Aeromonas hydrophila* MBB5 (Table 2).

Ten gram-negative potential pathogens were selected from the 22 isolates and screened for biofilm production (Table 2). The results of the modified microtiter-plate test as a quantitative assay showed that almost all tested bacteria produced biofilms where the amount of crystal violet stain retained on the test plate indicated the presence of biofilms (Fig. 2). Among these isolates, *Klebsiella pneumoniae* MBB9 showed the greatest biofilm production in the CV microtiter plate assay followed by *Alcaligenes faecalis* MBB1, *Providencia rettgeri* MBB11 and *Aeromonas hydrophila* MBB5.

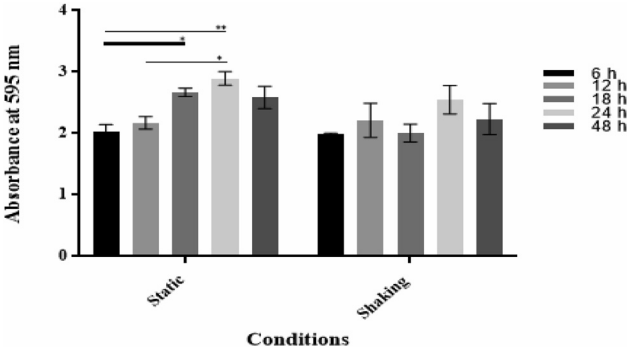

Fig. 3. Effect of incubation time and orbital shaking on biofilm formation by *K. pneumoniae* MBB9. Biofilm was developed under either static (left dataset) or horizontal orbital shaking (right dataset) conditions. The latter experienced higher shear forces with a diameter of 25 mm at 150 rpm imposed by a horizontal orbital shaker. Cultures were grown in nutrient broth (peptone 15 g L^{-1}, yeast extract 3 g L^{-1}, sodium chloride 6 g L^{-1} and D (+) glucose 1 g L^{-1}) and were incubated for 6 h, 12 h, 18 h, 24 h and 48 h at 37 °C under both static and shaking conditions. Data shown are the mean values, error bars represent standard errors (n = 3). The data were analyzed by one-way ANOVA Holm-Sidak's multiple comparisons test. $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

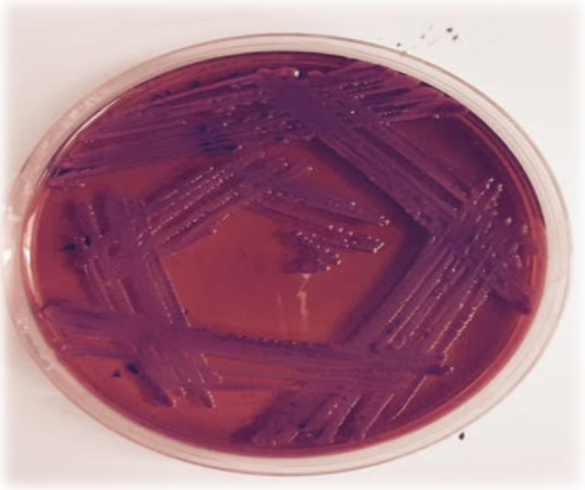

Fig. 4. Photograph presenting the growth of *Klebsiella pneumoniae* MBB9 on Eosin Methylene Blue (EMB) agar medium. *Klebsiella pneumoniae* is a gram-negative bacterium, rod-shaped, facultative anaerobic and nonmotile (Sikarwar and Batra, 2011).

However, microbial adhesion to hydrocarbon (MATH) using xylene as the hydrophobic agent was used to evaluate the hydrophobic surface characteristic of planktonic *K. pneumoniae* MBB9. From the results, *K. pneumoniae* MBB9 was moderately hydrophobic with a rating of 42% (Fig. 4). In addition, *K. pneumoniae* MBB9 was able to form biofilm under static conditions and the biofilm density increased significantly ($p < 0.05$) with increasing incubation time from 6 h to 24 h, whereas a reduction in biofilm biomass was observed after 48 h of incubation. Although the biofilm mass increased with the incubation period, there was no significant difference ($p < 0.05$) in the amount of biofilm formed by *K. pneumoniae* MBB9 under static and shaking conditions (Fig. 3).

4. Discussion

Environmental sampling, bacterial isolation and 16S rRNA gene sequencing identified 22 bacterial species present within biofilms formed on stones retrieved from the Porter Brook, Sheffield. Amongst these were 10 gram-negative pathogenic bacteria. Therefore, it was established that potential human pathogens were present within the biofilms attached to stones of the Porter Brook. The presence of such bacteria raises the possibility of infection arising from exposure to these pathogens at this location.

Biofilms have been shown to be diverse communities that include algae, archaea, bacteria, fungi, protozoa and viruses (Katharina et al., 2012). The α-Proteobacteria and β-Proteobacteria tend to be the predominant bacteria present in freshwater biofilms, reflecting the planktonic bacteria in the river water, however Firmicutes and γ-Proteobacteria are also common (Katharina et al., 2012). Investigation of the assembly of biofilms on glass beads placed in three streams in Sweden led to the suggestion that the capacity to make an initial attachment to the surface might have led to the dominance of β-Proteobacteria in the biofilms (Katharina et al., 2012). However, by comparing the results obtained from 16S rRNA gene sequencing (bulk biofilm) and 16S rRNA sequencing (active biofilm) α-, β-, γ-Proteobacteria and Bacilli were more abundant in the active biofilm, including *B. subtilis* and *E. coli*, which were identified here. In the Swedish river biofilms, the Bacteroidetes, *Sphingobacterium* spp, was found mostly in the bulk biofilm and not in the active biofilm (Katharina et al., 2012). This was interpreted as suggesting that

growth conditions for these bacteria were more favourable during early biofilm formation. The isolation of *S. composti* MBB14 here might suggest that this strict aerobe might also be most active during the early stages of biofilm formation in the Porter Brook. Previous studies of biofilms formed on polycarbonate slides placed in Kanzaki river (0.5 m below the water surface) in Japan have also shown that β-Proteobacteria were the dominant group in biofilms in each month of the study period (Aug, Oct and Dec) (Araya et al., 2003). The dominance was interpreted as β-Proteobacteria might attach to surfaces more easily during initial formation of biofilms. The isolation of *Aquaspirillum serpens* MBB2, *Comamonas nitrativorans* MBB3 and *Alcaligenes faecalis* MBB1 here might suggest that these motile aerobes might tend to make an effective initial attachment to the surfaces of the stones during the early stages of biofilm development. In the Japan river study, the ratio of metabolic activity was found to be higher in bacterial cells associated with biofilms than in free-living bacterial populations during the study period (Araya et al., 2003). This was thought to reflect the greater availability of surface-associated carbon compared to the water column. In general, biofilm-associated bacteria are able to collect and concentrate nutrients, such as organic and inorganic compounds that accumulate at higher concentrations at the solid-liquid interface leading to higher metabolic activity than their planktonic counterparts (Konhauser et al., 1994; Araya et al., 2003). Flagella are thought to be one of the initial factors used by some bacterial species for cell-to-surface adhesion and biofilm formation (Ratthawongjirakul et al., 2016). In several gram-negative bacteria, flagella play essential roles in early biofilm formation (Lemon et al., 2007). Most of the isolated bacteria (~77%) here are motile, suggesting that possession of such appendages might increase the probability of adhesion to these substrata and the subsequent growth. In general, the physical appendages of bacteria, such as flagella, fimbriae and pili can also overcome the repulsive physical forces of the electrical double layer, contact the bulk lattice of the conditioning layer and consolidate the bacteria–surface bond, as these repulsive forces have shown to prevent the bacterial cells from making a direct contact with the surface (Kumar and Anand, 1998; Garrett et al., 2008).

The presence of *E. coli* MBB7 in the biofilms is not surprising as it is a common member of human and animal intestinal flora and has been used as an indicator of faecal contamination of water (Amirat et al., 2012). Although *E. coli* bacteria are part of the healthy flora of the gut, some serotypes can cause intestinal and extra-intestinal infections, such as meningitis, nosocomial bacteremia and urinary tract infection (Von Baum and Marre, 2005; Cabral, 2010). In various European Rivers, human origins have been identified as the primary source of faecal contamination of urban rivers, whereas the farmed animal sources were the main source of faecal pollution in rural rivers (Amirat et al., 2012). Study of distribution of *E. coli* in water, epilithic biofilms and sediments of Ruhr river in German has shown that the presence of *E. coli* in all epilithic biofilms collected from stones (Mackowiak et al., 2018). The number of *E. coli* in epilithic biofilms was found three orders of magnitude higher than in the flowing water, suggesting that *E. coli* may grow at favourable temperatures in river biofilms and can persist in these environments. Similarly, Balzer et al. (2010) have found that the number of *E. coli* in epilithic biofilms retrieved from the Ruhr was higher compared with the overlying water. This was interpreted as suggesting that presence of high densities of the authochthonous microflora in epilithic biofilms may also help their survival (Balzer et al., 2010). In the German river biofilms, an increased number of *E. coli* in water was found to depend on the flow rate, whereas the level in epilithic biofilms was independent of the flow rate, suggesting that epilithic biofilms may act as a reservoir for fecal indicators. A previous study showed the existence of these strains in water samples collected from the river Thames (Dhanji et al., 2011). Another study showed that heavy rainfall during the study period (spring, summer and autumn) increased the numbers of *E. coli* in water implying that such conditions might result in an increased level of pathogens (Amirat et al., 2012). The Porter Brook rainfall might influence the concentrations of *E. coli* MBB7 in the water, whereas the moderate-flow rate may have no effect on the survival and persistence of these bacteria in biofilms attached to stones. Different mechanisms may underline the existence of *E. coli* MBB7 here, such as the input of external sources, transport and incorporation into biofilms and the adoption of survival strategy to persist (Mackowiak et al., 2018). Besides, EPS can protect biofilm cells against the harmful external factors (Gupta and Diwan, 2017).

The occurrence of other members of *Enterobacteriaceae* in Ruhr, Moersbach, Anrathskanal river biofilm was also reported, such as *Klebsiella pneumoniae, Klebsiella oxytoca, Citrobacter* spp, and *Pantoea* spp. as the family contains a large number of genera that are part of the intestinal flora of humans and animals (Kallen et al., 2013). Coliforms, which are *Enterobacteriaceae* members, can also be used as bacterial indicators of the faecal pollution and thus the potential presence of pathogens in the aquatic environment (Lin and Ganesh, 2013). Previously, the presence of *K. pneumoniae* has been found in water samples collected from the river Thames (Amirat et al., 2012) and *K. oxytoca* was present in river Thames sediment and water samples (Boden et al., 2008). *Enterobacteriaceae* members have been detected in a number of studies of freshwater habitats (Paulse et al., 2012). The predominant bacterial species in water and epilithic biofilms retrieved from Berg and Plankenburg rivers, South Africa, identified by 16S rRNA PCR and DNA sequencing included fecal indicators, confirming the introduction of pollutants into these habitats (Paulse et al., 2012). In South Africa rivers samples, phylogenetic analyses have shown the dominance of gram-negative bacteria, suggesting that these microbes possess different mechanisms to survive and tend to be more resistant to various lipophilic and amphiphilic inhibitors than gram-positive bacteria. In addition to these species, *Acinetobacter* species along with *Aeromonas hydrophila, Alcaligenes faecalis* were also isolated which are all indicators of faecal contamination (Paulse et al., 2012). The isolation of these bacteria from the Porter Brook biofilms is therefore not unusual, suggesting that Porter Brook might have received surface waters containing materials from different origins, e.g., water of urban or industrial effluents, agricultural activities, or rain. The presence of such bacteria here could lead to major health concerns and might indicate the existence of potential disease-causing bacterial strains. Although faecal indicator bacteria are part of the normal intestinal flora, they can include some species that are known to be opportunistic pathogens that may cause a range of human infections (Balzer et al., 2010). Characteristics of these diseases are shown in Table 2. However, investigation of the occurrence of the *Shigella* spp. in water and riverbed sediments samples retrieved from the Apies River, South Africa suggest the need to investigate the genetic relatedness of *Shigella* spp in water and riverbed sediments at the genetic level (Ekwanzala et al., 2017). In the Apies River samples, *Shigella sonnei*, *Shigella dysenteriae, Shigella boydii* and *Shigella flexneri* were found more abundant in both water and sediments, suggesting that these species could have a common origin (99% genetic relatedness) and the bacteria could possibly be moved between the water column and the sediments. *Shigella* spp. are commonly found in water polluted with human excreta, indicating the human faecal contamination (Ekwanzala et al., 2017). The isolation of *Shigella sonnei* MBB13 here might suggest the possible movement of these species and other isolated bacteria through the water column and the potential interaction with and resuspend from epilithic biofilms. However, in the Apies River samples, both culture-based and culture-independent methods were used for the

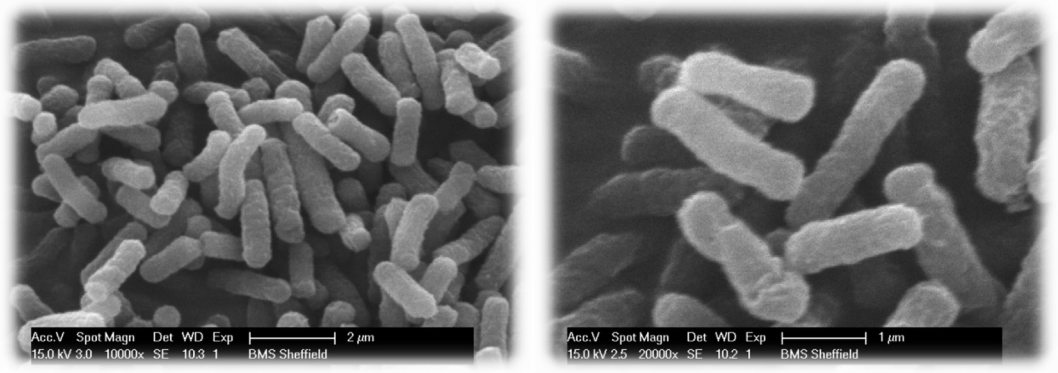

Fig. 5. Scanning electron micrographs of *Klebsiella pneumoniae* MBB9 cells retrieved from biofilms formed on stones recovered from the Porter Brook, Sheffield. Magnifications: ×10,000 left picture and × 20,000 right picture. Scale bars indicate 2 and 1 μm, respectively.

determination of bacterial loads, and it was found that the amounts of *Shigella* spp. by qPCR were 30 times higher than that obtained using culture-based techniques, suggesting that culture-based methods might not reflect the exact number of bacteria in these environments. Similarly, quantification of *E. coli* using culture-based procedures has been reported to underestimate their actual numbers in natural waters as some bacteria can be in a viable but non-culturable state (VBNC) (Amirat et al., 2012). Entering into VBNC state can be as a result of an insufficient supply of nutrients (uptake ceases) and/or under some environmental stresses that might inhibit natural activities for growth and cell division, such as extremes of temperature, pH, toxic chemicals, UV irradiation, water limitation or desiccation and fluctuating oxygen concentrations, whereas cells can recover from this situation when provided with the essential nutrients and environmental conditions, however some complex biofilm matrices might have both viable and non-viable cells (Trevors, 2011). Using only culture-based techniques for microbial isolation here limits the analysis to viable bacteria only and likely underestimates the actual diversity present in the Porter Brook.

Nevertheless, 10 gram-negative pathogenic strains were selected from the 22 isolates and screened for the production of biofilm. Amongst the 10 potentially pathogenic isolates, the *K. pneumoniae* MBB9 isolate showed the highest ability to form a biofilm in the microtiter plate assay (Figs. 2 and 5). *In vitro, K. pneumoniae* have shown the ability to form biofilms in polystyrene microtiter plates (Adriana Marcia Nicolau et al., 2013). *Klebsiella pneumoniae* are frequently found in a variety of environmental niches, including water, soil, and vegetation, and are gram-negative, encapsulated, nonmotile, rod-shaped opportunistic pathogens that can cause a wide range of nosocomial infections, including pneumonia, bacteremia, and urinary tract infections (Chen et al., 2014; Seidel et al., 2016). It has been shown that type 1 and type 3 pili that promote adhesion, and biofilm formation, as well as capsular polysaccharides, can be the most important virulence factors of *K. pneumoniae* (Seidel et al., 2016). Biofilm formation and its subsequent development have been suggested to play a significant role in the pathogenesis of *K. pneumoniae* (Maldonado et al., 2007).

The microbial adhesion to hydrocarbons (MATH) assay has been used widely to characterize microbial cell hydrophobicity and/or the degree of cell adhesion to hydrophobic liquids since the hydrophobicity of microbial cell can influence the microbial adhesion to various surfaces, where adhesive properties and hydrophobic/hydrophilic interactions have been proposed to participate in the process of biofilm formation (Zoueki et al., 2010; Tyfa et al., 2015). Hydrophobic nature of the outermost surface of different microbial cells has been involved in such biological phenomena as attachment of bacteria to host tissue, bacteria and phagocytes interactions, non-wettable solid surfaces, partitioning of bacteria at liquid:liquid and liquid:air interfaces, and the capability of microbial cells to grow on hydrocarbons through direct contact with the immiscible substrate (Rosenberg et al., 1980). *Klebsiella pneumoniae* MBB9 exhibited moderate hydrophobicity 42% according to the classification used by Tyfa et al. (2015), suggesting that the hydrophobic nature of these bacteria might contribute to the initial adhesion and facilitate the formation of biofilm, since the adhesion of bacteria is thought to be the first step of biofilm formation (Cerca et al., 2005). In general, the lower the degree of microbial cell hydrophobicity, the lower the adhesive capacity (Tyfa et al., 2015). Seventy eight percent of *K. pneumoniae* strains isolated from patients with urinary tract infections were found to have hydrophobic properties (Ljungh and Wadström, 1984). However, the level of hydrophobicity required to promote adherence is unknown and may vary depending on the nature of the surface (Schneider and Riley, 1991). The potential presence of fimbriae, which are known to consist of hydrophobic amino acids, in *K. pneumoniae* might also contribute to the surface hydrophobicity of these bacteria and consequently result in a highly hydrophobic surface (Meno and Amako, 1991).

Biofilm formed by *K. pneumoniae* MBB9 increased with increasing incubation time (Fig. 3). It has been shown that the length of incubation period influences the amount of biofilm produced as the density of biofilm increases with the extension of incubation time, where the incubation time can be defined as the contact time between the bacterial cells and the surface (Agarwal et al., 2011; Tang et al., 2012). It is probable that *K. pneumoniae* MBB9 had a high capability to adhere and required only a short contact time for attachment as it was able to attach to microtiter plates within the first 6 h (Fig. 3). These results are in agreement with a previous study showing that *K. pneumoniae* can form biofilm on the surface of microtiter plates within the 6 h of incubation, and biofilm continued to increase until 24 h (Maldonado et al., 2007). Similarly, the yields of *K. pneumoniae* biofilm formed on 96-well microtiter plate increased with time to a certain point, after which the biofilm present decreased gradually (Saloni et al., 2012). A previous study determining the relationship between contact time and biofilm formation by *Salmonella typhi* showed that the attachment strength of this species increased with increased incubation time (Tang et al., 2012). This was interpreted as suggesting that more bacteria might have time to attach and form biofilm as EPS production increased to facilitate attachment. Also, investigation of biofilm formed by *Listeria monocytogenes* and *Listeria* spp. has shown that the production of biofilm increased with incubation period on a range of hydrophilic and hydrophobic surfaces (Adetunji and Isola, 2011). After 24 h, *K. pneumoniae* MBB9 biofilm density

under static condition was slightly decreased, suggesting that the biofilm possibly had entered into a phase of slow or no-growth. The expected decrease in biofilm biomass may occur as a result of nutrients or dissolved oxygen depletion at the base of the biofilm and the bacterial cells may begin to detach after 48 h and return to suspension in the final step of biofilm maturation (Donlan, 2002; Beściak and Surmacz-Górska, 2011).

Higher shear stress leads to detachment of the biofilm cells when the shear forces are greater than the inner strength of the biofilm matrix as the velocity of the fluid in contact with microbial layer can have an enormous effect on the structure and behavior of biofilm (Qi et al., 2008; Teodósio et al., 2011). Here, no significant difference in the amount of biofilm formed by *K. pneumoniae* MBB9 under both static and shaking conditions was observed (Fig. 3). This might suggest that shear forces applied are not sufficient to overcome biofilm or that the adhesion is sufficient to enable cells to resist shear stress. Flagellation and motility of organisms may also influence microbial attachment to surfaces, and thus relevant to biofilm formation (Chavant et al., 2002; Gorski et al., 2003; Ngwai et al., 2006). Surface-exposed components, such as flagella, fimbriae and curli are found to drive the adhesion of planktonic cells to the surface in various bacteria (Guilhen et al., 2016). It is possible that the presence of type 3 pili in *K. pneumoniae* strengthen attachment to the surface, which has been shown to be necessary for biofilm formation on abiotic surfaces (Schroll et al., 2010; Murphy et al., 2013). Conversely, investigation of *Pseudomonas aeruginosa* biofilm growth using planar flow cell device has shown that high hydrodynamic shear might lead to cell detachment (Zhang et al., 2011). In the study of *P. aeruginosa* biofilm, a positive relationship was observed between liquid phase velocity and biomass accumulation as higher nutrient influx promoted faster microbial growth, but this relationship eventually reversed when higher shear stresses led to detachment of cells from the surface, substantially hindering biofilm development. Shear stress has also been found to slow down *Pseudomonas putida* biofilm maturation (Rochex et al., 2008).

5. Conclusion

Characterization of the biofilm-forming bacteria attached to stones in the Porter Brook, Sheffield revealed the presence of some potential pathogenic bacterial genera enclosed in a protective matrix of extracellular polymeric substances (EPS). Such bacteria might have the potential to release into the bulk water, raising hazards to human health by exposure during water-related activities. *Klebsiella pneumoniae* MBB9 biofilm formed rapidly (within 6 h) under static conditions at 37 °C and then increased up to 24 h of incubation before decreasing with further incubation (48 h). Although there was no significant difference ($p < 0.05$) in the amount of biofilm formed by *K. pneumoniae* MBB9 under static and shaking conditions (applied shear stress), shear dispersion due to the presence of a horizontal or vertical velocity shear might enhance the bacterial dispersion when the shear stress due to current and turbulent velocity fluctuations reaches a critical level.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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