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Early bacterial biofilm colonizers in the coastal waters of Mauritius

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ABSTRACT

Background: The past years have witnessed a growing number of researches in biofilm forming communities due to their environmental and maritime industrial implications. To gain a better understanding of the early bacterial biofilm community, microfiber nets were used as artificial substrates and incubated for a period of 24 h in Mauritian coastal waters. Next-generation sequencing technologies were employed as a tool for identification of early bacterial communities. Different genes associated with quorum sensing and cell motility were further investigated.

Results: Proteobacteria were identified as the predominant bacterial microorganisms in the biofilm within the 24 h incubation, of which members affiliated to Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria were among the most abundant classes. The biofilm community patterns were also driven by phyla such as Firmicutes, Bacteroidetes, Chloroflexi, Actinobacteria and Verrucomicrobia. The functional analysis based on KEGG classification indicated high activities in carbohydrate, lipid and amino acids metabolism. Different genes encoding for luxI, lasI, agrC, flhA, cheA and cheB showed the involvement of microbial members in quorum sensing and cell motility.

Conclusion: This study provides both an insight on the early bacterial biofilm forming community and the genes involved in quorum sensing and bacterial cell motility.

1. Introduction

The term 'biofouling' refers to the undesired accumulation of microorganisms, plants and animals on any artificial structures, which are exposed to aquatic environments [1]. The establishment of the fouling community has been characterized in terms of several stages and some of these stages can overlap or occur in parallel. In the development of biofouling, any submerged surface rapidly becomes coated by a conditioning film comprising of organic and inorganic molecules which may act as source of nutrients for microorganisms [2]. Formation of this film is immediately followed by the accumulation of microorganisms, which secrete extracellular polymeric substances (EPS) during attachment, colonization, population growth and the resulting layer is termed as the biofilm (microfilm) [3].

Biofilms are complex assemblage of microbial cells encapsulated within a self-produced EPS. The primary biofilm community is formed through specific and/or nonspecific interaction between initial colonizer [4]. The primary colonizers of any surface are predominantly bacteria, whereby the bacteria transit to an aggregated biofilm layer, hence forming the primary biofilm community [2]. Primary colonization triggers the accumulation of bacteria through growth and reproduction, which result in the modification of the characteristics of the surface and makes it suitable for subsequent colonization by secondary microorganisms [5].

Bacteria are a diverse group of organisms that are ubiquitous in most aquatic habitats. Bacteria exhibit both planktonic and benthic life strategies, and occupy a range of diverse habitats [6]. Bacterial biofilm plays an important role in biodegradation of organic matter, environmental pollutants, and nutrient recycling in nature [4]. On the other hand, bacterial biofilm presents a serious problem for the maritime shipping industry where they are a major component of the microbial slime layers. Microbial slime layers increase hydrodynamic drag, increasing fuel costs as well as costs associated with hull cleaning, corrosion and maintenance [7]. Several studies have investigated the microfouling component of the adhered microbial film after several weeks or months of immersion in the ocean [5,8,9,10,11,12,13,14], reviewed by Molino and Wetherbee [15]. The techniques utilized therein have generally involved the study diversity

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of the whole population of organisms from the substratum surface by using fingerprinting techniques [10,16,17], however low taxonomic resolution was recorded. As a result, little is known of the initial bacterial population that is established within the 24 h after submersion of the substrata and the genes involved in the early colonization process.

Advances on the capabilities of characterizing the microbial community, interaction within their niche and their response to environmental parameters have significantly increased through the use of high-throughput next generation gene sequencing techniques. The advent of next generation sequencing makes it possible for more detailed documentation of bacterial biofilm within a 24 h period. Metatranscriptomics and metagenomics which are emerging powerful technologies for the functional characterization of their microbiomes were used [18]. Metagenomics allows us to investigate the composition of a microbial community. By focusing on what genes are expressed by the entire microbial community, metatranscriptomics sheds light on the active functional profile of a microbial community. The metatranscriptome also provides a snapshot of the gene expression in samples at a given moment and under specific conditions by capturing the total mRNA.

In this respect, the objectives of this study were to use both metagenomics and metatranscriptomics to investigate the bacterial communities to gain insights into bacterial diversity during the early stage of biofilm formation as well as genes involved in quorum sensing and cell motility within the 24 h of biofilm layer formation in the marine samples.

2. Materials and methods

2.1. Study area and sampling strategy

This study was carried out in coastal waters around Mauritius (Fig. 1) during the month of May 2016. Among the sites, sea water temperature varied from 28 to 30°C, salinity varied from 26.4 to 36.0‰, conductivity varied from 58.2 to 57.6 mS/cm and dissolved oxygen content varied from 6.25 to 7.89 mg/l. Salinity and temperature were measured in situ using a conductivity/temperature/depth sensor (CTD) and a DO meter (YSI, USA). The samples were collected at the 12 selected regions at depths of 2 m. The fiberglass screen was used as artificial substrate [19]. The fiberglass screens (10.2 cm × 15.2 cm, with mesh size of 20 mm) were used for collecting the bacterial communities. Each screen was attached to monofilament fishing line and suspended in the water column within 20 cm of the seabed using a weight and small subsurface float. The subsurface floats were used to limit the length of monofilament line and avoid disturbance to the screen. After placement, the screens were allowed to incubate for a defined period of 24 h before being retrieved. For retrieval, a 775 ml plastic wide-mouth jar filled with ambient seawater was positioned beside each screen before the screen was gently removed from the monofilament line and transferred to the jar (underwater) without being folded. The jar was then capped and stores on ice and returned to the laboratory for processing. The screens were placed in 1 liter plastic jar with ambient seawater. The jars were vigorously shaken for approximately 1 min,

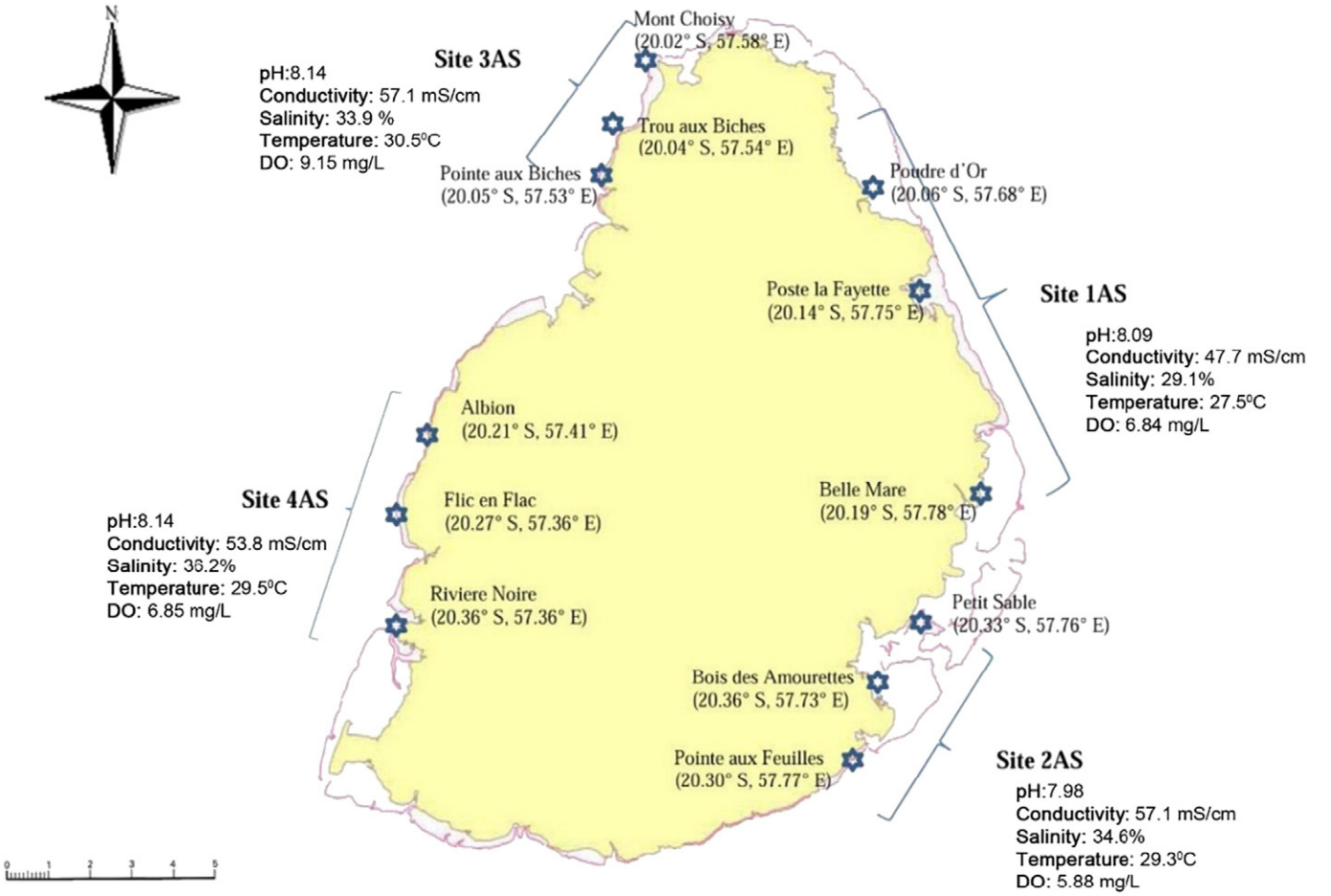

Fig. 1. Base map of Mauritius with sites of study.

the screens were rinsed with 0.2-μm-filtered seawater, and scraped with a razor blade [20]. The harvests were then size-fractionated by filtering sequentially through 300, 125 mm mesh sieves, 20 μm pore size nylon mesh, and finally filtered through 0.22 μm pore sized white polycarbonate filters to keep all free-living organisms. The membrane filters were immersed in extraction buffer.

2.2. Nucleic acid extraction

The DNA and RNA extraction procedures were performed as described by Chen et al. [21] and Tripathy et al. [18] respectively. Total RNA and DNA were isolated using RNA power soil and DNA power water kits (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to manufacturer's instructions. Following extraction, RNA was treated with DNase using the TURBO DNA-free kit (Ambion, Austin, TX). In order to obtain mg quantities of mRNA, approximately 500 ng of RNA was linearly amplified using the MessageAmp II-Bacteria Kit (Ambion) according to the manufacturer's instructions. Finally, the amplified, antisense RNA (aRNA) was converted to double-stranded cDNA Universal RiboClone cDNA Synthesis System (Promega, Madison, WI). The cDNA was purified with the Wizard DNA Clean-up System (Promega).

DNA/RNA concentrations (100 ng of total RNA and DNA) were assessed based on Fluorometer absorption (Qubit™ 3.0 Fluorometer, products by Thermo Fisher Scientific). Further DNA integrity was confirmed by gel electrophoresis. The samples were sent to Inqaba biotech™ (Inqaba Biotechnical Industries (Pty) Ltd. Pretoria, South Africa) for next generation sequencing on the Illumina MiSeq. The primer sets (341F and 805R) used were to amplify the V3–V4 regions of 16S rRNA genes [22]. The PCR conditions were as follows: 3 min at 96°C, followed by 30 cycles of denaturation at 95°C for 50 s, annealing at 58°C for 50 s, primer extension at 72°C for 1 min and a final extension at 72°C for 10 min.

2.3. Metagenomic and metatranscriptomic sequencing and de novo assembly

For metagenomic sequencing, two libraries with insert sizes of 500 bp and 1500 bp were independently generated. For metatranscriptomic sequencing, a library with an insert size of 300 bp was generated from the cDNA sample. Raw Illumina reads from both libraries were then assembled using the CLC Genomics Workbench (version 4.0.3; CLC Bio, Cambridge, MA, USA). Paired-end reads were assembled using the following parameters: mismatch cost 2, insertions cost 3, deletion cost 3, length fraction 0.5 and similarity 0.8. Sequences were further processed to remove primer, linker, and barcode sequences. Reads were merged using PEAR (options: $P < 0.001$) and low quality reads size lower than 30 were discarded and trimmed from the dataset. Sequences with ambiguous nucleotides or shorter than 180 bp were discarded. Chimeric reads were detected and discarded using the de novo chimera detection algorithm UCHIME [23]. Assembled data were annotated using CAMERA (v2.0.6.2) [24] and duplicated counting from multiple assembly was removed. Reads from metagenomic and metatranscriptomic samples were taxonomically classified using BLASTn against databases derived from SILVA, GreenGenes, Ribosomal Database Project II and NCBI.

2.4. Functional annotation of genes

Functional annotation of the predicted genes was conducted based on analysis against the proteins in the databases of Kyoto Encyclopedia of Genes and Genomes (KEGG) [25]. The matched genes were then assigned to KEGG orthologs (KOs), KEGG pathways, KEGG categories, clusters of orthologous groups of proteins (COG) catalogs and COG categories for further analysis. Genes selected for the present work based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology classification of proteins (http://www.genome.jp/kegg) were further subjected to BLASTp searches against the Swiss-Prot [26]. Annotation was done by utilizing the hierarchical structure of the KEGG Brite database. The KEGG analysis was detailed on functional features related to the quorum sensing and biofilm development in bacteria. The cDNA sequences were assigned functional protein or pathway predictions based on the COG database or KEGG database. The cut-off criteria for functional protein prediction based on orthologous groups using BLASTX analysis against the COG database were established using the same in silico approach with 100 bp fragments of known functional genes as E-value <0.1, identity >40% and overlapping length >23 aa to the corresponding best hit. The COG cut-off criteria were also applied to the KEGG database for pathway prediction because of the similarity in database size.

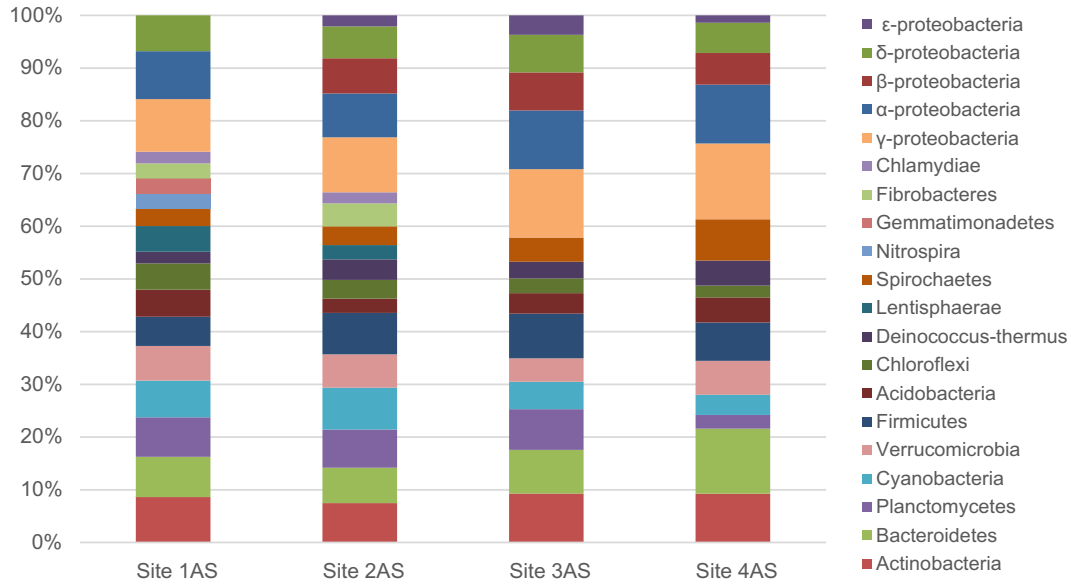

Fig. 2. Relative abundances of and phylogenetic groups detected by 16S rRNA analysis of biofilm samples derived from the 4 sites.

Table 1
Percentage abundance of most common OTUs classified in their respective orders and families according to the sites of collections.

Percentage abundance of OTUs					Most common	
Site 1AS	Site 2AS	Site 3AS	Site 4AS	Order	Families	Genera
1.8	7.1	3.5	3.1	Acidithiobacillales	Acidithiobacillaceae	*Acidithiobacillus*
3.8	5.2	1.5	2.8	Actinomycetales	Actinomycetaceae	*Actinobacteria*
5.0	4.0	4.4	4.2	Aeromonadales	Aeromonadaceae	*Aeromonas*
3.6	7.0	5.9	5.9	Alteromonadales	Alteromonadaceae	*Alteromonas*
3.8	0.0	4.7	2.4	Alteromonadales	Shewanellaceae	*Shewanella*
3.5	3.0	3.3	3.4	Bacteroidales	Bacteroidaceae	*Bacteroides*
3.0	5.1	2.9	3.2	Burkholderiales	Burkholderiaceae	*Burkholaria*
4.1	3.3	4.1	4.6	Chloroflexales	Chloroflexaceae	*Thermoflexus*
3.9	4.7	2.8	3.2	Enterobacteriales	Enterobacteriaceae	*Escherichia*
2.0	1.0	1.2	2.0	Chitinispirillales	Chitinispirillaceae	*Chitinispirillia*
4.1	3.1	3.1	4.1	Flavobacteriales	Flavobacteriaceae	*Polaribacter*
1.4	3.9	4.1	4.6	Gemmatimonales	Gemmatimonaceae	*Gemmatimonas*
3.0	3.8	2.9	3.0	Oceanospirillales	Halomonadaceae	*Halomonas*
3.3	0.0	3.2	3.4	Alteromonadales	Idiomarinaceae	*Idiomarina*
3.5	3.5	2.9	4.6	Lentisphaerales	Lentisphaera	*Lentisphera*
3.4	3.2	1.9	5.8	Actinomycetales	Micrococcaceae	*Micrococcus*
2.3	3.3	4.1	2.9	Neisseriales	Neisseriaceae	*Neisseria*
3.2	2.7	2.1	0.0	Nitrospirales	Nitrospiraceae	*Nitrospira*
4.2	4.1	6.2	6.9	Pseudomonales	Pseudomonadaceae	*Pseudomonas*
4.4	2.9	4.5	2.8	Spirochaetales	Spirochaetaceae	*Spirochaeta*
3.2	2.9	3.2	2.6	Verrucomicrobiales	Verrucomicrobiaceae	*Prosthecobacter*
5.8	5.1	5.2	5.2	Vibrionales	Vibrionaceae	*Vibrio*
2.7	4.5	3.6	4.4	Myxococcales	Myxococcaceae	*Myxococcus*
3.2	3.7	3.3	3.3	Bacillales	Bacillaceae	*Exiguobacterium*
3.5	3.9	4.0	3.3	Lactobacillales	Streptococcaceae	*Streptococcus*
7.3	3.6	5.2	4.0	Bacillales	Staphylococcaceae	*Staphylococcus*
6.8	5.4	6.1	4.3	Rhodobacterales	Rhodobacteraceae.	*Roseobacter*

2.5. Diversity indices

Data were prepared by logarithmic transformation using the R® software package (www.r-project.org) and Microsoft Excel®. Hierarchical cluster analysis of functional genes related to quorum sensing was generated using the unweighted pairwise average-linkage clustering algorithm with R®, using complex heatmap and circlize stats packages. Diversity Indices including operational taxonomic unit (OTU) richness, Shannon, Simpson and Chao1were calculated using Microsoft Excel®.

Variations in the bacterial community composition at the different sites were examined using clusplot analysis (principal coordinate analysis (PCoA)) conducted with both Bray–Curtis and weighted UniFrac distance matrices in R's "stats" package. Variation in the relative abundance of different bacterial phyla associated with river, zone, and habitat levels was calculated using a correlation component model with the 4 sites as random factors. Analysis of similarity (ANOSIM, R's *anosim* function) based on both Bray–Curtis and UniFrac distances, was then performed to assess differences among groups within each hierarchical level. Most statistical tests were done using R 1.0.44 (R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/).

3. Results

The average rate of successful amplifications ranged between 50 to 90%. The number of high quality useable sequences was retained for further analysis. Clustering of sequences from the samples combined produced 18,247 bacterial OTUs. Bacterial sequences were assigned to 21 bacterial taxonomic groups. The most abundant and highly diverse phylum of the biofilm community was represented by the Proteobacteria (43.3%). Large proportions of reads were also assigned to Actinobacteria (8.6–12.2%), Bacteroidetes (8.3–12.2%) and Deltaproteobacteria (5.5–7.8%) across the four sites. Nitrospirae (0.8%) and Gemmatimonadetes (0.8%) and Epsilonproteobacteria (1.6%) on the other hand, were the least abundant phyla recorded and were more site-specific. Furthermore, the abundance of the phylogenetic groups detected by 16S rRNA gene analysis of the four samples was compared with respect to location of the sampling sites (Fig. 2). Across the 4 collection sites Gammaproteobacteria (11.7%) and Alphaproteobacterial (9.7%) dominated the early biofilm population. However, a significant change in population density was observed in phyla such as Gemmatimonadetes and Nitrospirae which was present only at site 1AS, as compared to Epsilonproteobacteria and Deltaproteobacteria which were completely absent. Other site specific groups were Fibrobacteres and Chlamydiae present only at 1 and 2 AS. Hence, these observations depicted a variation between site of collection and the relative abundance of bacterial phyla.

The bacterial community was mostly dominated by known bacterial biofilm producers (Table 1). Alteromonadales (44.3%), Bacillales (33.2%), Vibrionales (21.3%), and Neisseriales (19.0%). *Alteromonas* exhibited an average abundance of approximately 33% of the overall bacterial community recovered across the 4 sites. *Vibrio* was the second highest genus which accounted for 21.3% of whole biofilm population. Halotolerant bacteria such as *Exiguobacterium*, Gram-positive and rod shaped bacteria *Bacillus*, Gram-negative bacteria Enterobacteriaceae, were also present in the biofilm layers.

Table 2
Diversity indices of all OUT's in marine samples.

	Sites			
	1AS	2AS	3AS	4AS
Diversity indices				
Shannon	2.20	2.02	2.58	0.94
Simpson	4.41	3.06	7.85	1.67
Richness	1.32	1.08	1.13	0.29
Chao 1	111.75	125.05	131.75	128.04

*Shannon–Weiner indices, higher number represents higher diversity. *Reciprocal of Simpson's indices, higher number represents higher diversity.

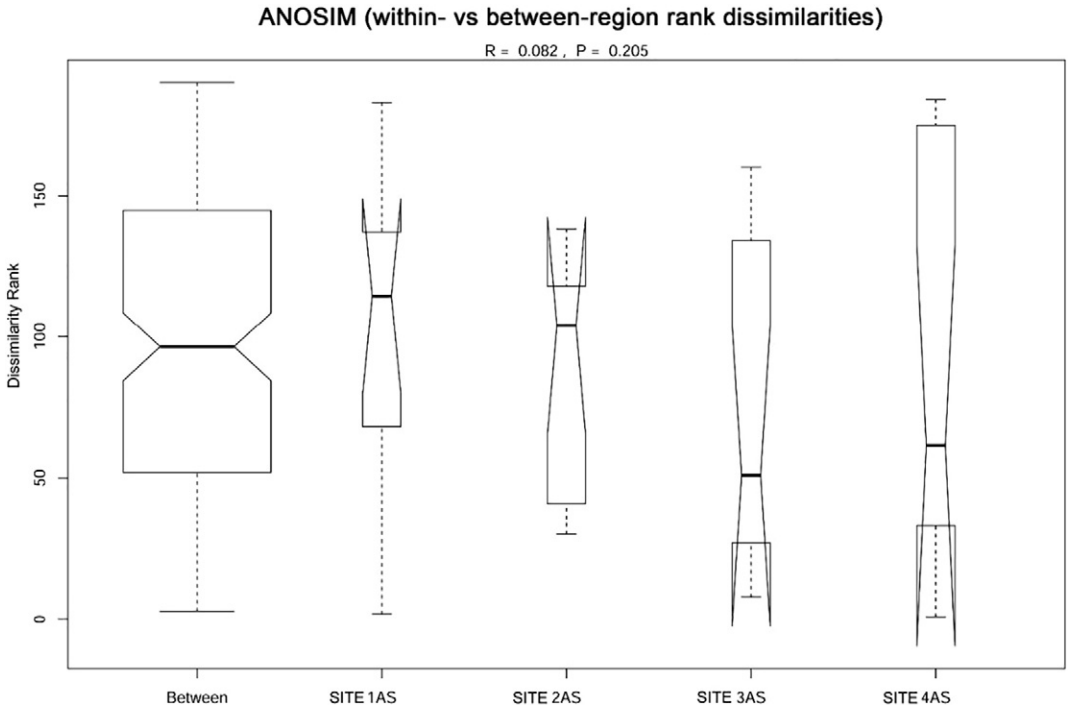

Fig. 3. ANOSIM results of the correlation of the phylum/class of the species cultured from the four different sites. Notched boxplots indicate the dissimilarity rank distributions for between and within sites presented in plots.

The 16S reads obtained at the 4 different sites were compared using biodiversity indices, based on two diversity measures (Shannon diversity indices and Simpson indices) (Table 2). Indicator of the diversity indices showed significant differences by site (for both Chao1 and Shannon's indices). Site 3AS (2.58 for Shannon and 131.75 for Chao1 indices), had the most diverse bacterial biofilm layer as compare to site 4AS.

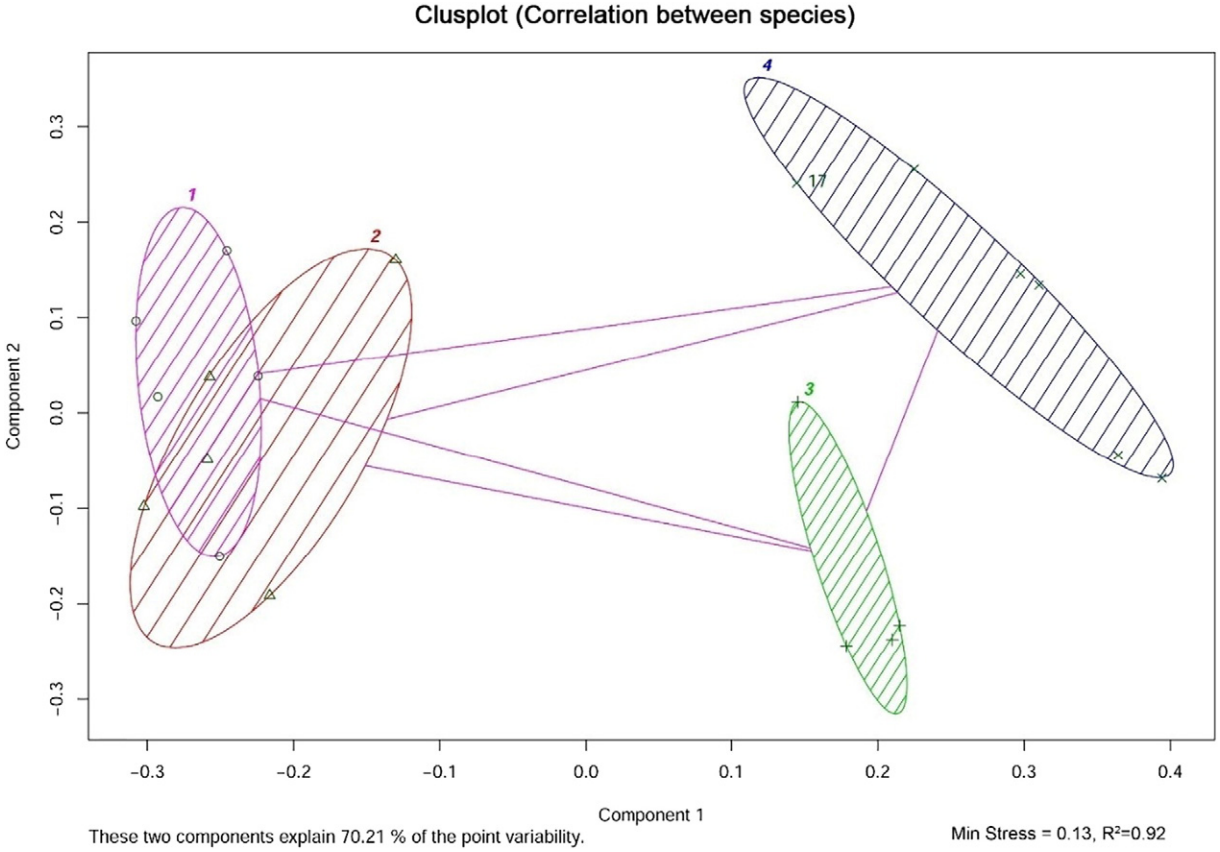

Fig. 4. Clusplot of the bacterial taxonomy in the different sites.

Table 3
Bacterial clusters from the clusplot.

Cluster	Phylum/class of species
1	Acidobacteria, Actinobacteria, Alphaproteobacteria, Chloroflexi, Gemmatimonadetes
2	Bacteroidetes, Chlamydiae, Gammaproteobacteria, Planctomycetes, Verrucomicrobia
3	Cyanobacteria, Deinococcus-thermus, Fibrobacteres, Lentisphaerae, Nitrospira, Spirochaetes
4	Betaproteobacteria, Deltaproteobacteria, Epsilonproteobacteria, Firmicutes

Fig. 3 indicate the Bray–Curtis dissimilarity rank distributions for between and within sites, after 999 permutations, presented in plots.

Further, ANOSIM boxplots (Fig. 3) for Site 1AS and 2AS had the highest dissimilarity rank while Sites 3AS had recorded the lowest dissimilarity, with an ANOSIM static R value of 0.062 with a significance of 0.205. Hence, 1AS and 2AS shared higher similarities in the taxonomy list as compared to sites 3AS.

Fig. 4 shows four elliptical clusters of the bacterial phylum/class (Table 3). The first two principal components derived from the data used concluded that almost half of the information about our multivariate data has been captured by the clusplot (Fig. 4).

Multivariate analysis demonstrated that the taxonomic structure of the bacterial communities within the biofilms differed significantly at 70.2% (Fig. 4), with an anosim R value of 0.062 ($P > 0.05$). The clusplot (Fig. 4) separates the bacterial phyla/class by the two principal components (Component 1 and Component 2). With $K\ means = 4$, the clustplot shows that species of cluster 1 and cluster 2 have an ambiguous relationship due to their Euclidean distance, compared to clusters 3 and 4. The plot shows that the majority of variation between the species was along the first principal coordinate axis (Fig. 4). Certain correlation patterns between different microbial groups were detected indicating either pairwise interactions between phyla or adaptation to similar environmental conditions. Chloroflexi, Gemmatimonadetes showed highest correlation.

To gain insights into the in situ metabolic functions of the biofilm community functional meta-analysis was carried out and annotations were performed using the KEGG Automatic Annotation Server [25]. Metagenomic and metatranscriptomic analysis identified core set of functional processes associated with biofilm formation, and functional involvement of genes associated with quorum sensing and cell motility were reported (Fig. 5 and Fig. 6).

The core metabolic functions included carbohydrate and energy metabolism, amino acid metabolism and nucleotide metabolism. Cellular processes, xenobiotic degradation and metabolism, membrane transport were also represented in the four datasets. The functional diversity however was highest at site 3AS as compared to the other sites. Further 24% of the identifiable genes in the 4 sites were associated with carbohydrate metabolism or energy metabolism, whereas genes associated to cell cycle were less frequent. Further the functional profile of the sites showed that genes were also involved in the quorum sensing and cell motility processes.

Genes related to quorum sensing and cell motility were further investigated (Fig. 6). Multiple genes were assigned to quorum-sensing circuits, virulence, protease activity and biofilm formation, Genes annotated using KEGG related to quorum sensing and cell motility was included (Fig. 6b). The gene pool was dominated by genes from Gammaproteobacteria and Alphaproteobacteria. Gene such as luxI and lacI, which are known to be critical for autoinducer, were assigned to in the quorum sensing pathway (Fig. 6b).

4. Discussion

The past years have witnessed an interesting number of researches in the microbial biofilm formation and factors mediating the structural

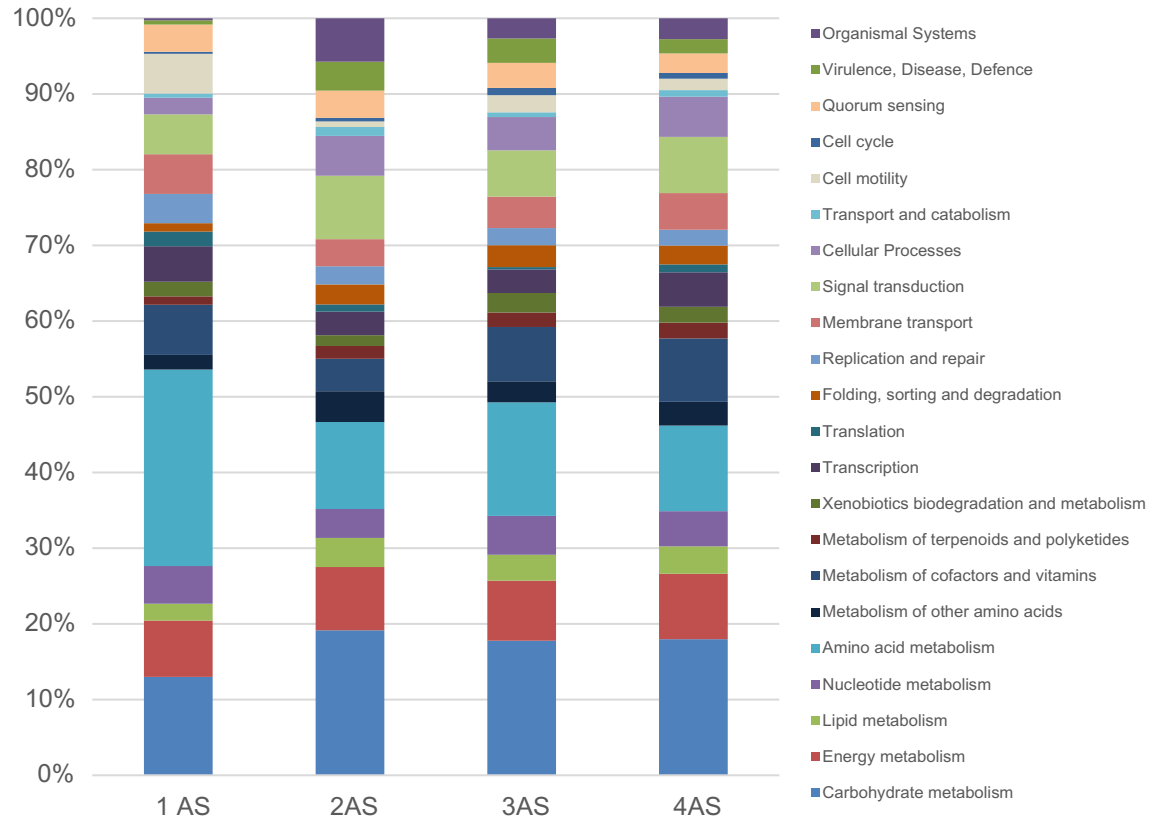

Fig. 5. Functional profiling of microbial communities based on KEGG functional processes. Twenty-two major processes have been highlighted.

development and functional properties of the biofilm matrix [14,27,28]. In general, the formation of bacterial biofilms complex begins when appropriate environmental signals are sensed by planktonic bacteria, hence shifting from motility-to-biofilm [29]. Recent studies of microbial biofilm community from natural and artificial substrates (ceramic, glass, plastic, aluminum, and coral skeleton) have been used to compare the community structure and diversity [30,31,32]. In doing so, these initiatives produced important insights into the microbial social interactions. Results from these studies indicated that the young bacterial biofilm community was led by Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Similar observations were made in this study as well, where the biofilm community drivers were Proteobacteria followed by Firmicutes Actinobacteria, and Bacteroidetes. Some studies have reported biofilms dominated by Alphaproteobacteria [33].

The most common bacteria in the microfilm were the Gammaproteobacterial groups. The dominance of Gammaproteobacteria within this study is in line with biofilm communities associated with carbohydrate metabolism and biofouling promotion [34] as well as a variety of stressed environments biofilms from the Antarctic [35]. The results further showed that the most common groups of Gammaproteobacteria were Alteromonadales and Oceanospirillales. Members of these two groups have been reported from young marine biofilms and are potentially known to be involved in tolerance of polysaccharide biodegradation and carbohydrate metabolism in the biofilm matrix [20]. Similarly the functional roles ascribed to

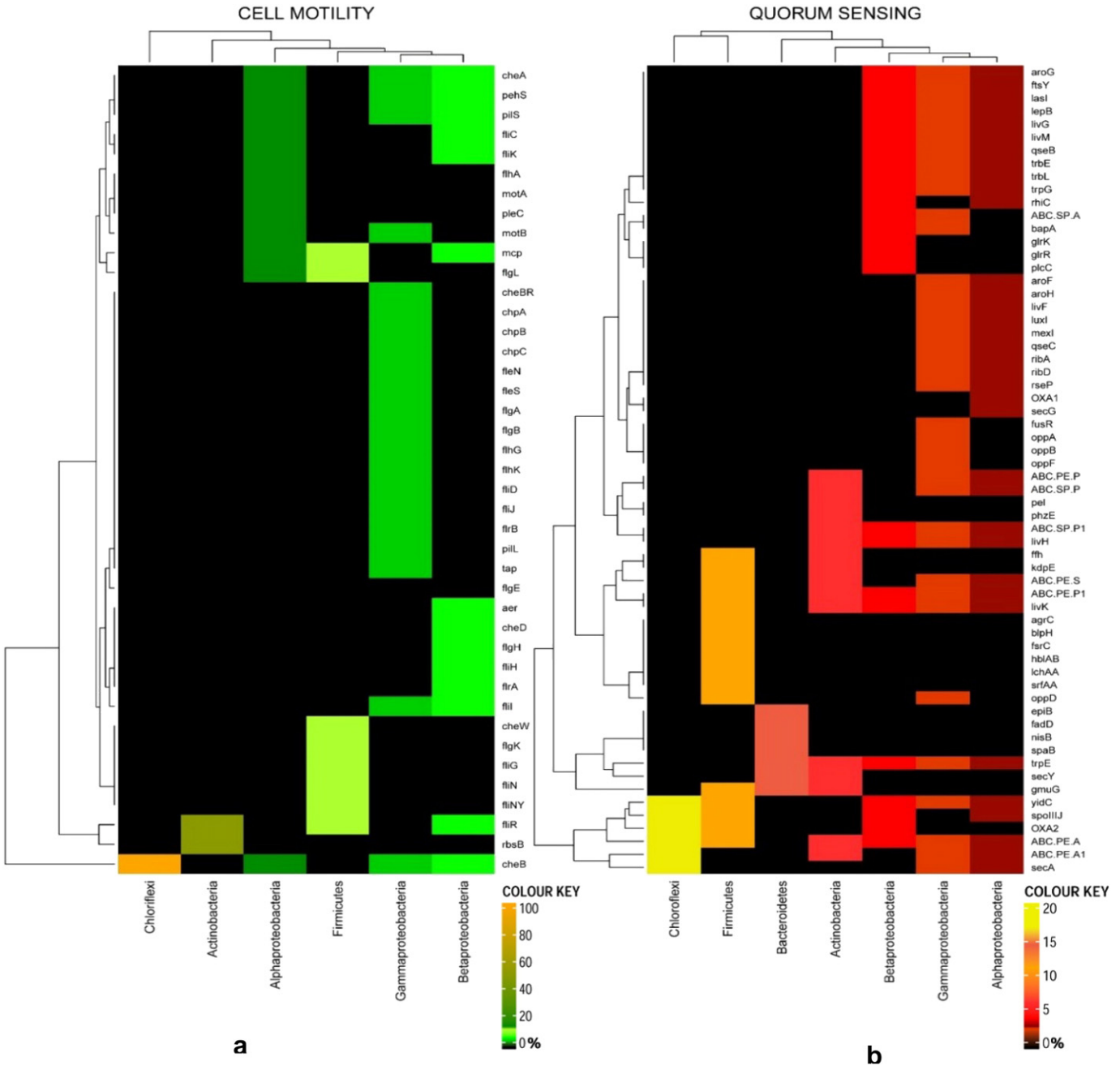

Fig. 6. Total reads assigned to genes within the functional class of cell motility and quorum sensing pathways relative to the different taxonomic groups were presented the two above heat maps.

Actinobacteria, Verrucomicrobia and Flavobacteriaceae, as observed in this study, have been reported in nitrogen fixation, carbon degradation and production of exopolysaccharides [20].

Among predominant genera, *Alteromonas* spp. (found at relative abundances between 3.6 to 7.0% in different samples) were important primary colonizers of the submerged marine substrata. *Alteromonas* spp. are thought to be involved in the protection of a biofilm against land-based contaminants [5]. Genera such *Streptococcus*, *Actinomyces Actinobacteria* and *Neisseria* were also observed, all being well known partners for co-aggregation [8,9]. Congregation of bacteria community overcomes many environmental challenges by using various adaptations and strategies to form the biofilm layer [36]. Mutualistic adaptations benefit bacterial biofilm community by increasing biofilm formation from environmental changes, this may explain the co-existence of *Nitrospira* and *Pseudomonas*as observed [37]. Furthermore, a growing number of human pathogens have been reported from the marine environments [38]. Ortega-Morales et al. [37] described pathogenic microbes as competitive, opportunistic and rapid colonizers and may thus explain their presence in young biofilms. During this study, human pathogens such as *Exiguobacterium* (up to 3.7%) and *Vibrio* (up to 5.8%) [39] were recorded, at all the four sampled sites.

It is important to understand quorum sensing signals as well as the motility responses systems of bacteria in natural environments as their influences on the biofilm formation have been reported previously [40]. Functional genes such as lasI, luxI and agrC, all known for their central roles in quorum sensing were assigned to Gamma/Alpha Proteobacteria and Firmicutes. The genes involved in control quorum-sensing-mediated biofilm development were similar in the natural marine coastal waters. Genes recovered in this study correspond to flagellar and twitching motility of bacteria and are essential for the initiation of biofilm. motA/motB gene is known to be involved in the assembly of the flagellar filament, fliL for swimming, flgN a flagellar chaperone and motB for motility [29]. Laboratory based studies have also demonstrated the involvement of these genes in motile bacterial [41].

5. Conclusions

This study provides the first in-depth metagenomics and metatranscriptomics information of early bacterial biofilm forming community and metabolic functions in the coastal waters of Mauritius within 24 h. The functional analysis was successful in identifying the natural early bacterial biofouling community along with an understanding of prominent activities and involvement at the sampled sites. Moreover, insights on gene involved in quorum sensing and cell motility in the coastal waters were described. This approach therefore provides information on bacterial gene involved in the early biofilm formation in environmental sample which may be of potential use in the development of new antifouling strategies.

Disclosure statement

No potential conflict of interest was reported by the authors.

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