

Author version : *Marine Pollution Bulletin*, vol.141; 2019; 398-403

Metagenomic analysis of tarball-associated bacteria from Goa, India

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Abstract

The beaches of Goa state in India are frequently polluted with tarballs, specifically during pre-monsoon and monsoon seasons. Tarballs contain hydrocarbons, including polycyclic aromatic hydrocarbons, which pose significant environmental risks. Microbes associated with tarballs reportedly possess capabilities to degrade toxic hydrocarbons present in tarballs. In this study, bacterial diversity associated with tarballs from Vagator and Morjim beaches of north Goa was analyzed based on V3–V4 regions of 16S rRNA gene sequenced using Illumina Miseq Platform. The Proteobacterial members were dominant in both Vagator ($\geq 85.5\%$) and Morjim ($\geq 94.0\%$) samples. Many of the identified taxa have been previously reported as hydrocarbon degraders (e.g. *Halomonas*, *Marinobacter*) or possible human pathogens (e.g. *Acinetobacter*, *Klebsiella*, *Rhodococcus*, *Staphylococcus*, *Vibrio*). This is the first study reported on a metagenomic analysis of bacteria associated with tarballs from Goa.

Keywords: Diversity, NGS, oil pollution, OTU, Petroleum hydrocarbons

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Introduction

The Goa state in India is a popular tourist destination, globally known for its beaches. In recent years, the southwest monsoon currents have been transporting tarballs to the Goan coast, thus polluting the beaches. A tarball is a semi-solid blob of crude-oil, and it is formed due to weathering of crude-oil in the ocean. Though the tarball pollution in Goa was first reported in the 1970s, its negative effects have been widely reported in recent decades (Shinde et al. 2017). Tarballs pollute marine environments, including pelagic, benthic and beach regions. Some tarballs may originate from natural oil sources present in the ocean (Itah et al. 2005; Hostettler et al. 2004; Lorenson et al. 2009).

It is well-documented that phylogenetically diverse microbes are associated with tarballs. These microbes may play an important role in degradation of hydrocarbons present in tarballs (Warnock et al. 2015; Sanyal et al. 2016; Shinde et al. 2017). In case of crude-oil, bacterial communities that degrade hydrocarbons considerably vary with hydrocarbon composition (Bacosa et al. 2010, 2012, 2015a, b). In case of microbial degradation of tarballs, source-oil type, associated microbes, and environmental conditions are believed to be crucial. Degradation of tarballs is presumably faster in a microbially-favourable warmer climate (Warnock et al. 2015; Bacosa et al. 2015a). This is because tarballs are less viscous at a higher temperature, thus greater surface area is made available for physical breakdown and degradation (Beazley et al. 2012). The by-products formed during tarball degradation support microbial growth (Shinde et al. 2017). Interestingly, tarballs may contain human pathogens such as *Vibrio*, which may not directly degrade hydrocarbons but get benefited from by-products affected by other microorganisms (Tao et al. 2011; Liu and Liu 2013).

Our understanding of microbial diversity associated with tarballs is inadequate and needs improvement (Shinde et al. 2018). Recent studies have reported culture-centric molecular characterization of tarball-associated bacteria from Malaysia (Nkem et al. 2016) and Goa (Shinde et al. 2018). Compared to culture-dependent approach, a metagenomic approach can provide a better understanding of bacterial diversity on/ in tarballs, including anaerobes and pathogens (Liu and Liu 2013; Bacosa et al. 2016; Lee et al. 2018; Shinde et al. 2018). Recently, Bacosa et al. (2016) reported characterization of bacteria associated with tarballs from Galveston and Mustang islands, Texas, USA, based on a metagenomic approach. They reported high numbers of alkane-degrading *Alcanivorax* and *Psychrobacter* in the Galveston tarballs, while Mustang Island tarballs contained high numbers of PAH-degrading *Pseudoalteromonas* and alkane-degrading *Psychrobacter*, *Oceanospirillales* and *Alcanivorax*.

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There has been no record of metagenomic analysis of tarball-associated microbes from Goa. To fill this research gap and obtain better understanding of bacterial diversity associated with tarballs from Goa, this study was initiated with two objectives: 1) to standardize the extraction of total DNA from tarball samples, and 2) to characterize bacteria associated with tarballs from two prime tourist beaches, Vagator and Morjim situated at northern Goa, based on analyses of 16S rRNA gene amplicons (V3 and V4 regions) in the Illumina Miseq Platform.

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Materials and methods

Sampling

Vagator and Morjim beaches are the prime tourist attractions in north Goa, India. The latter is a site of Olive Ridley turtle conservation. Tarball samples were collected during May 2017 from Vagator (15.602223 °N, 73.733781 °E) and Morjim (15.617607 °N, 73.732589 °E) beaches. Tarballs found on the beaches were randomly handpicked wearing sterile nitrile gloves, covered with aluminium foil and placed in zip-lock bags. The samples were kept in an icebox and carried to the laboratory. All samples were stored at 4 °C until further processing.

Sample processing and metagenomic DNA isolation

Tarball samples were processed in homogenization buffer based on Winogradsky's salt solution as described by Von Der Weid et al. (2008). 15 g of polyvinyl pyrrolidone (PVP) along with 10 g of 2 mm glass-beads were autoclaved at 121 °C for 15 min. Further, 10 g of tarball was added and incubated for 2 h on magnetic stirrer to dissolve the aggregated material. To collect the supernatant fraction, the sample was centrifuged at 4 °C for 10 min at 2500 rpm. This procedure was repeated thrice before all supernatants were pooled together and centrifuged at 4 °C for 5 min at 9500 rpm. The pellet was washed with Tris-EDTA buffer and centrifuged at 10,000 rpm. This was repeated until a clear supernatant was obtained. Metagenomic DNA was extracted from the pellet using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, USA). The metagenomic DNA was loaded on 1% agarose gel and viewed in a Bio-Rad Gel documentation system after staining with Ethidium bromide. DNA was stored at -20 °C.

Metagenomic analysis

The DNA samples were lyophilized and shipped to Eurofins Genomics India Pvt. Ltd. (Bengaluru, India) for metagenomic analysis. Amplicon library was prepared using Nextera XT index Kit (Illumina Inc.) as per the 16S rRNA gene metagenomic sequencing library preparation protocol. Amplification of the 16S rRNA gene, specifically targeting the V3-V4 region was carried out using the primer-set, 16S rRNA F: GCCTACGGGNGGCWGCAG and 16S rRNA R: ACTACHVGGGTATCTAATCC. Amplicons were then ligated with Illumina adaptors and amplified using i5 and i7 primers as per the standard Illumina protocol. The amplicon library was

purified by 1X AMPureXP beads and quantified by Qubit fluorometer. The amplified library was analysed on 4200 TapeStation system (Agilent technology) using D1000 Screen tape.

Bioinformatics analysis

Quantitative Insights into Microbial Ecology (QIIME) pipeline was used to analyze the sequence data from the samples (Wang et al. 2007). All the sequences were clustered into Operational Taxonomic Units (OTUs) at 97% threshold similarity using UCLUST algorithm (Edgar 2010). A representative sequence from each OTU was used to assign the taxonomic information using the Ribosomal Database Project (RDP) classification system. Diversity metrics (Shannon, Chao 1 and ACE) were also calculated by the QIIME. Distribution of the clustered OTUs was analysed by plotting rarefaction curves to observe species richness of the samples.

Results

Diversity of tarball-associated bacteria

The number of sequence reads from the Vagator and Morjim samples were 291,132 and 359,590, respectively (Supplementary Table 1). The OTUs recorded in the Vagator and Morjim samples were 1870 and 1703, respectively. The OTUs were used for generating rarefaction curves and for making calculations with the abundance-based coverage estimator ACE (Ckburg et al. 2005) and the Chao1 (Chao 1984) estimator of species diversity (Supplementary Table 2). Rarefaction curves of both samples showed the curve plateaus (Supplementary Fig. 1), which indicated that in both the samples the species richness had not approached completeness. Shannon index for the Vagator sample was 6.63, while that for the Morjim sample was 4.10. The Vagator sample supported more diverse bacterial diversity. The Chao1 and ACE species richness indices were also high in the Vagator sample (1964.1 and 2002.2), compared to those for the Morjim sample (1902 and 1908). This confirmed the presence of more diverse bacterial communities in the Vagator sample.

A total of 17 phyla were recovered from the Vagator sample. The Morjim sample comprised 19 phyla. The Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the top four abundant phyla, accounting for approximately 98.8% in the Vagator sample and 99% in Morjim sample. The Proteobacteria was the most abundant, both in the Vagator sample (85.5%) and Morjim (94%) sample. *Marinobacter* (7%), *Halomonas* (7%) and *Pseudomonas* (6%) were the major genera associated with the Vagator sample (Fig. 1). In the Morjim sample, *Halomonas* (7.5%), *Pseudomonas* (5.5%) and *Petrobacter* (3%) were the dominant genera (Fig. 2).

Potential hydrocarbon degrading bacteria associated with tarballs

From the total/ overall bacterial diversity revealed in the Vagator sample, about 67% of the classified bacterial taxa, including 30 bacterial genera, were found to be reported for their hydrocarbon degrading abilities (Supplementary Table 3). Among the 30 reported potential hydrocarbon degrading bacterial genera, *Marinobacter*, *Halomonas*, *Pseudomonas* and *Glacieola* were dominant in the Vagator sample. In the Morjim sample, about 83% of the bacterial taxa, including 31 bacterial genera, were found to be previously reported for hydrocarbon-degrading capability (Supplementary Table 3). Among the 31 potential hydrocarbon degrading bacterial genera, *Halomonas*,

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Pseudomonas, *Petrobacter*, *Pseudoalteromonas* and *Cobetia* were dominant in the Morjim sample (Figs. 3–4).

Possible pathogens associated with tarballs

The bacterial taxa identified in the Vagator and Morjim samples were compared with relevant literature to obtain insights into their pathogenic abilities. Thirty-six bacterial genera from both Vagator and Morjim samples were found to be previously reported for their pathogenic life-style (Supplementary Table 4). Both Vagator and Morjim samples were dominated by *Halomonas* (48% and 34%) and *Pseudomonas* (36% and 28%), respectively (Fig. 5). The tarball samples collected from both the beaches contained some well-known pathogenic bacterial groups such as *Vibrio*, *Staphylococcus*, *Klebsiella*, *Rhodococcus* and *Acinetobacter* (Fig. 6).

Discussion

Tarballs are oily conglomerates which act as a unique substrate for diverse bacteria. The culture-dependent methods are inadequate to obtain a good representation of microbial diversity associated with tarballs (Shinde et al. 2018). Therefore, in this study we employed a metagenomic approach. The tarballs collected from Morjim and Vagator beaches were found to be dominated by the Proteobacteria. The phylum Proteobacteria includes heterotrophic organisms found in marine surface environment (Stevens et al. 2005). The diversity indices from this study suggested that the Vagator sample comprised a more diverse and even bacterial community structure, compared to the Morjim sample. However, in the latter, a large proportion of bacteria were unclassified at the genus level. Major genera present belonged to the γ -Proteobacteria. Similar results were reported for the dominance of γ -Proteobacteria during oil spills in Mexico beach (Kostka et al. 2011) and Norway (Brakstad et al. 2015).

Further, we compared our results with relevant published papers for hydrocarbon-degrading properties and pathogenic lifestyle of the bacteria. The Vagator sample revealed 30 bacterial genera which were previously reported for hydrocarbon degradation. Similarly, the Morjim sample contained 31 potential hydrocarbon degrading genera (Figs. 3–4). *Marinobacter*, *Pseudomonas* and *Halomonas* accounted for most abundant genera in the Vagator sample. These genera are well-known for their hydrocarbon degradation abilities. *Marinobacter* is known for its denitrification capacity and also for its versatility in utilizing a broad range of substrates including alkanes and PAHs (Head et al. 2006; Berlendis et al. 2010; Liu and Liu 2013; Al-Maillem et al. 2013; Gao et al. 2013). Previous reports suggest that *Marinobacter* species are effective in the mitigation of oil pollution since they are metabolically more versatile (Kostka et al. 2011).

Halomonas and *Pseudomonas* species are also known competent hydrocarbon degraders (Fathepure 2014; Mnif et al. 2009; Head et al. 2006). Bacosa et al. (2013) described *Pseudomonas* as one of the most dominant genera involved in heavy oil degradation, mainly responsible for the degradation of aliphatic and aromatic compounds. *Pseudomonas aeruginosa* was reported to be a potent organism for bioremediation of hydrocarbon-contaminated environments due to its versatility and hydrocarbon biodegradation capability (Mukherjee et al. 2010). *Halomonas* was reported from crude-oil-contaminated saline soil from Shengli oilfield, China which effectively degraded crude oil (Wang et al. 2007). Apart from these three genera, *Glacieola* also showed a significant abundance in the

Vagator sample. The role of *Glaciecola* in oil degradation is poorly understood, but there are reports suggesting its dominant presence in oil-contaminated cores of Arctic forged ice (Brakstad et al. 2008). However, in a study conducted by Chronopoulou et al. (2015) on a *Glaciecola* strain obtained from tetradecane-enrichments of the Antarctic did not support its direct participation in hydrocarbon degradation but inferred its ability to grow in the presence of alkanes.

The Morjim sample showed the abundance of *Pseudomonas* and *Halomonas* along with *Petrobacter*, *Pseudoalteromonas* and *Cobetia* which were also revealed in the Vagator sample. *Petrobacter* comprises aerobic bacteria reported for its efficiency to degrade crude-oil hydrocarbons (Parnadi et al. 2007; De Vasconcellos et al. 2010). Many species of *Pseudoalteromonas* have been reported to possess catabolic ability to degrade PAHs (Hedlund and Staley 2006). Bacosa et al. (2016) reported the association of *Pseudoalteromonas* with tarballs in Mustang Island, Texas, USA. Their experiments indicated *Pseudoalteromonas* to be one of the potential candidates in oil degradation. *Cobetia* is not a well-known oil degrader, but there are reports indicating its potential in metabolizing hydrocarbons. Lee et al. (2018) reported that *Cobetia marina* plays a significant role in the degradation of PAHs in oil-contaminated sediment of Taean coast, Korea.

Apart from the presence of potential hydrocarbon degraders, the tarball samples from our study also revealed the presence of possible pathogenic bacteria in them. Bacterial genera like *Halomonas*, *Pseudomonas* and *Bacillus* were abundant in both Vagator and Morjim samples. Some of the species belonging to these genera, like *Halomonas venusta* and *Pseudomonas aeruginosa* are suggested to be human pathogens (Kim et al. 2013; Stevens et al. 2009; Thompson et al. 2005; Ahlén et al. 2000; von Graevenitz et al. 2000). *Bacillus* species are not well documented for their human pathogenicity from marine waters. However, there are a few species like *B. subtilis*, *B. sphaericus* and *B. anthracis* which reportedly cause serious and fatal infections in humans (Farrar 1963).

The Vagator and Morjim tarball samples showed the presence of *Rhodococcus* and *Acinetobacter* which usually prefer marine animals as their host, but certain species are identified to cause human infection (Thompson et al. 2005; Bergogne-Berezin and Towner 1996). *Rhodococcus equi* is reported to be an emerging human pathogen (Weinstock and Brown 2002). *Vibrio*, *Staphylococcus* and *Klebsiella* were abundantly observed in the tarball samples. Tarballs are reservoir of pathogenic bacteria (Tao et al. 2011; Shinde et al. 2017), *Vibrio vulnificus* being one of the dominant pathogenic

groups reported by Tao et al. (2011) in oil spillage samples of north-central Gulf of Mexico. *Vibrio* species are abundant in aquatic environments, and they may pose an indirect risk to susceptible humans. *Klebsiella* species are also known opportunistic pathogens, which are widely distributed and especially found in surface waters in the marine environment (Podschn and Ullmann 1998). To the best of our knowledge, there are no reports on *Staphylococcus* associated with tarballs, thus the observed abundance of *Staphylococcus* on tarballs from Morjim and Vagator beaches needs further investigation. It has been suggested that human activity is the source of *Staphylococcus* species at an Egyptian beach (El-Shenawy 2005), but there are also reports of *Staphylococcus* species as efficient hydrocarbon degraders (Shekhar et al. 2014).

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Conclusion

In this study, we investigated the diversity of bacteria associated with tarballs from Vagator and Morjim beaches of North Goa, India using a metagenomic approach. The Proteobacteria was found to be dominant in both Vagator and Morjim tarballs. The Proteobacteria, Actinobacteria and Firmicutes accounted for approximately $\geq 95.0\%$ in the Vagator sample. The Proteobacteria accounted approximately $\geq 94.0\%$ of the total phyla in the Morjim sample. This is the first report on the metagenomic analysis of bacterial diversity associated with tarballs from Goa, India. Further studies are required to obtain insights into hydrocarbon degrading capabilities of tarball-associated microbes. The association of pathogenic bacterial groups such as *Vibrio* and *Staphylococcus* reported from tarballs of Morjim and Vagator beaches needs detailed investigations as it could be a health concern.

Acknowledgements

The authors thank the Director, CSIR-National Institute of Oceanography, Goa (India) for providing necessary facilities. Authors thank the Department of Science, Technology & Environment (DST&E), Govt. of Goa for financial support under GAP 3129 project. This is NIO communication number

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Legends to Figures

Fig. 1 Prokaryotic community structure determined by dominant OTUs counted at the genus level from Vagator sample

Fig.2 Prokaryotic community structure determined by dominant OTUs counted at the genus level from Morjim sample

Fig. 3 Potential hydrocarbon degrading genera associated with Vagator sample

Fig. 4 Potential hydrocarbon degrading genera associated with Morjim sample

Fig. 5 Possible pathogenic bacterial genera dominantly associated with Vagator sample

Fig. 6 Possible pathogenic bacterial genera dominantly associated with Morjim sample

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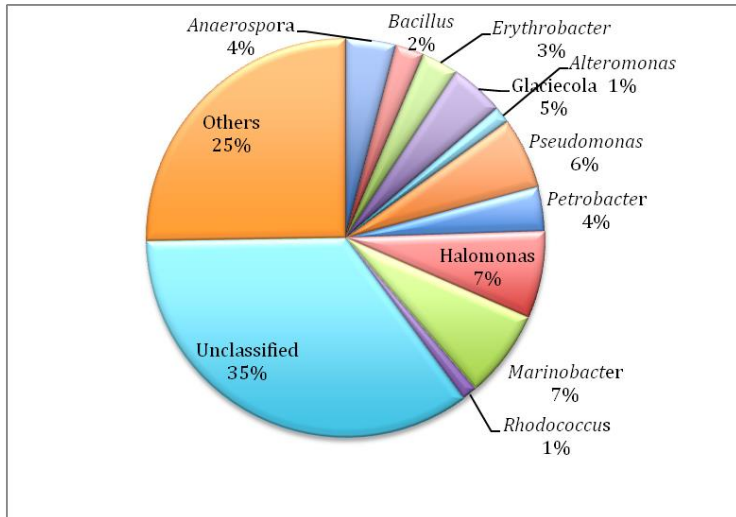


Fig.1

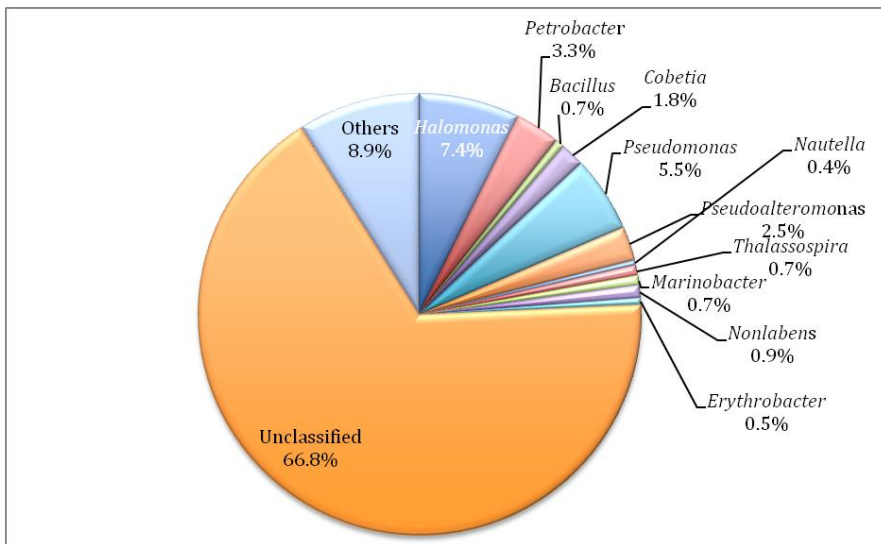


Fig.2

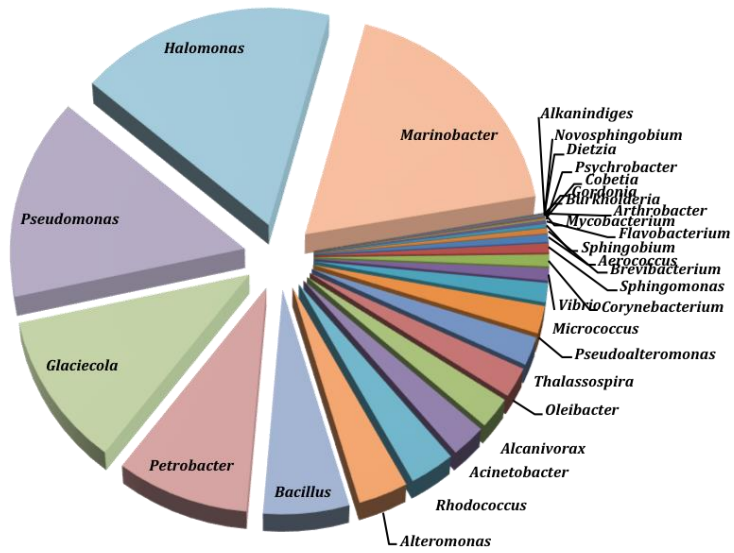


Fig. 3

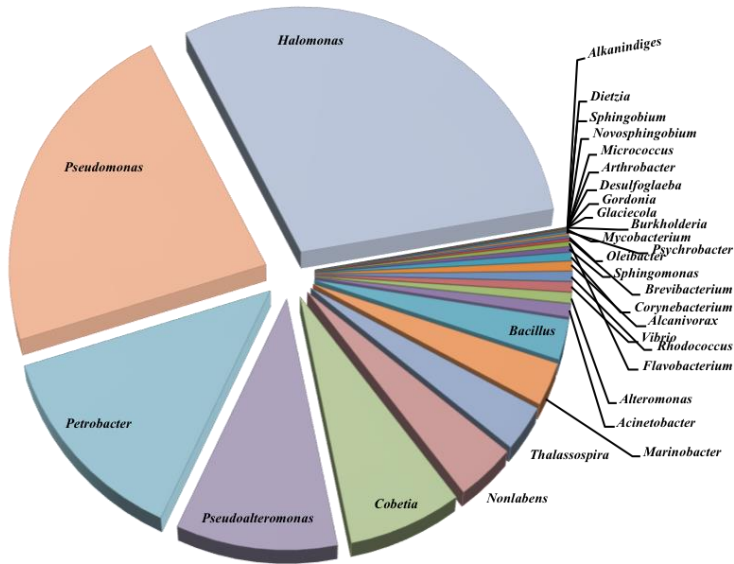


Fig. 4

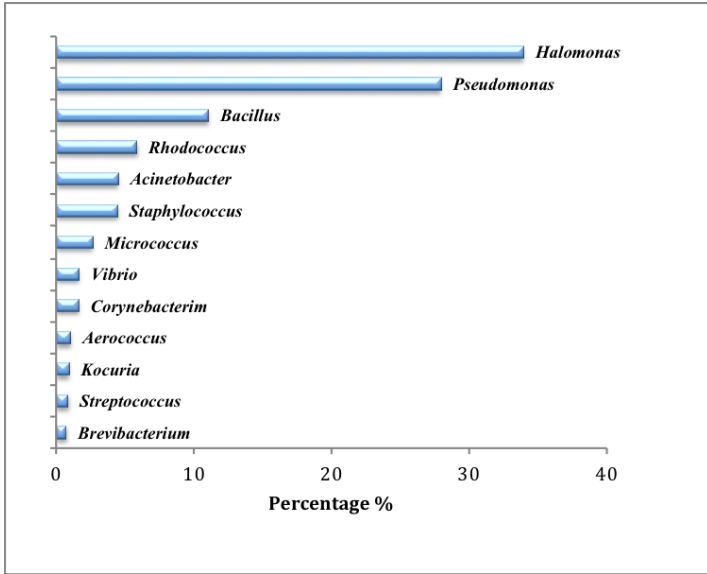


Fig. 5

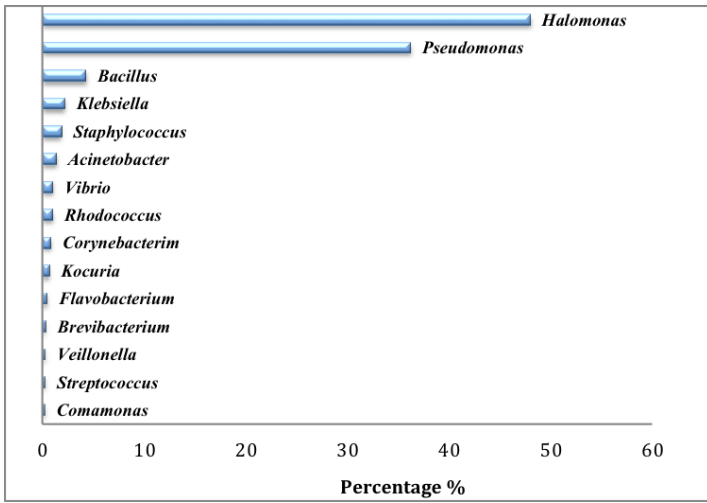


Fig. 6