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Outlook on pollution effect and potential bioremediation pathways to overcome contamination problems on river nethravathy of dakshina kannada district, karnataka, india

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Abstract
Current study area covered a stretch of 25 km of the river Nethravathy starting from Kajoor at the foot hills of Western Ghats to the downstream of Dondole of Belthangady Taluk of Dakshina Kannada Dist., Karnataka state in India. The river receives untreated domestic sewage discharge at some locations. The human and animal excreta along with agricultural wastes were brought into the river with run off waters. Uncontaminated area known as Kajoor has been considered as first Contamination Station (S_1), non-discharge area at a place called Nidigal and bathing ghat near Dharmasthala, a famous pilgrimage centre in India were considered as second and third Contamination Stations respectively (S_2 and S_3). This study is completely focused on determination of specific hydrocarbon contaminants, and these are amenable to biological treatment and also to determine the time required for degradation. In the present investigation, microbes from samples collected at three study sites of river Nethravathy were isolated, characterized and tested for the *ex situ* biodegradation of two petroleum components- anthracene and Naphthalene with respect to time and concentration. From the results it is clear that among all, the microorganisms isolated from Station-III (S_3) sample showed better degrading activity compared to the other sites evaluated for the degradation of petroleum products. It is quite obvious that the microorganisms from these areas naturally depend on the petroleum products for their survival and only those can degrade can survive actively.

Keywords: *Nethravathy, Dakshina, Kannada district, Biodegradation, Hydrocarbon contaminants.*

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1. Introduction

Nethravathy is regarded as sacred river and people of Dakshina Kannada district worship it with supreme reverence. Hutments and human colonization are close to the river and there are no big industries situated on the bank of the river, particularly in the study sites. Hence till now, there has been no danger of industrial wastes getting into the river. Of course the river is used for bathing, washing cloths and cattle to dispose of the sewage throughout its stretch. People come to the river for performing last rites. The river is a focus of religious activity during special occasions of religious functions at Dharmasthala. The banks of the river in the rural stretch have dense pristine vegetation cover. Hence the bank aesthetics is very attractive. The river is a life line for over lakhs of people of Dakshina Kannada district.

The total channel length of this stream is 108 km. Rapid and waterfalls are the characteristic features of the upper channel. The bed material of the mainstream, at upper reaches consists of boulders – cobble assemblage of quartzite, phyllite gneiss and other metamorphic rocks. Charnockites, gneisses and coarse sand, gravel assemblage dominates in the middle part of the stream and the lower reaches of the channel has Gneiss rocks and coarse to fine sand as bed material (Bhat, 1992). Nethravathy is an eighth order stream. In its upper reaches it shows pebble – cobble assemblage along the channel, in the lower reaches shows channel bars mainly composed of sand. Nethravathy sub-basin has a total of 5166 streams. The longitudinal profiles of the Nethravathy shows a steep gradient in the initial stages i.e., to a distance of about 12 km and gradually flattens towards the mouth. Nethravathy drops from 1680m altitude to 200m within a span of about 8 km and shows a steep gradient of 185m. With respect to drainage pattern Nethravathy dendrite pattern, which is characterized by irregular branching in all directions with the tributaries joining the main streams at all angles and are sequent in origin. This is developed on the homogeneous lithological units like gneiss and charnockites.

Domestic wastes are let into the water bodies at Nidigal and bathing ghat near Dharmasthala by small open drainages from the banks and repeated vehicle washing. When the river is viewed in terms of its entire water shed rather than just in terms of the actual water body of water flowing in the channel we get a full picture of the sources that pose threats to the river in quality. The river banks and its basin actually form the generative environment for the river water quality (Unland *et al.,* 2014). The extent, the effects and the management of non-point sources of pollution are difficult to identify. Most public attention to water pollution is given to point source discharge (Williamson *et al.,* 1993). The provision of sewerage and sewage treatment and disposal still continues to remain a programmer of low priority (CUPS 1978-79). This together with the extensive surface runoff from the poorly managed drainage basins, remains as one of the main causes of pollution in India.

Petroleum hydrocarbons also known as crude oil which is a complex mixture of compounds which can be categorized into four fractions – saturates, aromatics, resins and asphaltene (Wang *et al.,* 2011). The seepage from natural deposits is one of the major routes by which petroleum oil enters marine environments (National Academy of Science, 1975). Bioremediation is defined as the process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state or to levels below concentration limits established by regulatory authorities (Kumar *et al*., 2011). By definition bioremediation is the use of living organisms, primarily microorganisms, to degrade the environmental contaminants into less toxic forms. The microorganisms may be indigenous to contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Biodegradation by naturally occurring populations of microorganisms is a major mechanism for the removal of petroleum from the environment (Das and Chandran 2010). Biodegradation of crude oil in natural ecosystem though complex has greatly enhanced the removal of harmful ions and solids from the environment by transformation into readily usable and less/non toxic materials by microorganisms (Regina *et al*., 2006). Current evidence suggests that in aquatic and terrestrial environments microorganisms are the chief agents for the biodegradation of molecules of environmental concern, including petroleum hydrocarbons (Okoh 2006; Swanell and Head, 1994). It is now generally accepted by the scientific community that no one species of microorganisms will completely degrade any particular oil (Maddela *et al.,* 2015). Degradation of both crude and refined oils seems to involve a consortium of microorganisms, including both eukaryotic and prokaryotic forms. The most common genera known to be responsible for oil degradation comprise mainly *Nocardia*, *Pseudomonas*, *Acinetobacter*, *Flavobacterium*, *Micrococcus*, *Arthrobacter*,

Corynebacterium, *Achromobacter*, *Rhodococcus*, *Alcaligenes*, *Mycobacterium*, *Bacillus*, *Aspergillus*, *Mucor*, *Fusarium*, *Penicillium*, *Rhodotorula*, *Candida* and *Sporobolomyces*, (Bensig *et al.,* 2014; Elkhir 2015; Sulaiman 2014).

2. Materials and Methods

2.1 *Bioremediation*

Initial water analyses of the total heterotrophic microbial counts and specific hydrocarbon degrading microbial counts in the contaminated water can provide useful information on biological activities and the extent to which the indigenous microbial population has acclimated to the site conditions. In this regard, the present study encompasses the initial microbial assessment of the contaminated site, monitoring the biodegradation of two petroleum components; Anthracene and Napthalene.

2.1.1 *Sample collection*

Water samples were collected from three study sites of River Nethravathy viz., S_1, S_2, S_3 and S_4. The sample collection was done from the surface to a depth of about 12 inches during summer.

2.1.2 *Microbial enumeration*

The total microbial count in representative water samples, using the standard serial dilution and nutrient agar- plate counting techniques (Lorch *et al*., 1995). The same was inoculated on to Sabouraud Dextrose Agar (SDA) to which 0.5ml antibacterial agent was incorporated. The plates were incubated at 30°C for 24 h and the colony counting done thereafter using electronic colony counter and the colony forming units (CFU) per ml of the original broth culture determined. Twenty different isolated colonies (six from each sample) were inoculated into the synthetic mineral salt media (SMS media) containing Anthracene at a concentration of 5mg/ml of acetone in 50 ml media. The procedure was repeated to check the activity against Naphthalene at same concentration. The activity was monitored for every 24 hours up to three days. The isolates showing maximum degradation were identified and taken for further analysis.

2.1.3 *Standardization of time and concentration*

The isolated organisms were inoculated into different aliquots ranging from 2 to 10mg/20ml SMS media. Activity on the compounds Anthracene and Naphthalene were checked by turbidometric measurement using Spectrophotometer. The absorbance was recorded at an interval of 24 hours for 7 days at 540nm. Different techniques are employed depending on the degree of saturation and aeration of an area. *In situ* techniques are defined as those that are applied to soil and groundwater at the site with minimal disturbance. *Ex situ* techniques are those that are applied to soil and ground water at the site which has been removed from the site via excavation (soil) or pumping (water). Bioaugumentation techniques involve the addition of microorganisms with the ability to degrade pollutants. Most often *in situ* bioremediation is applied to the degradation of contaminants in saturated soils and ground water. It is a superior method to cleaning contaminated environments since it is cheaper and uses harmless microbial organisms to degrade the chemicals. Chemotaxis is important to the study of *in situ* bioremediation because microbial organisms with chemotactic abilities can move into an area containing contaminants. So by enhancing the cells' chemotactic abilities *in situ* bioremediation will become a safer method in degrading harmful compounds. Characterization of isolates was done using tests and methods described by MacFaddin (1980) and Cruickshank *et al.,* (1975). Identification was based on the criteria of Bergey's manual of Determinative Bacteriology (Taddei *et. al.,* 2006).

3. Results and Discussion

River Nethravathy is a medium, perennial river, for most of the year, the river bed remains flowing thinly. The abundance of water during June to December off-sets the balance of water for all irrigation wells along the length of the river and prompted it as the chief source of drinking water Western Ghats, one of the 18 biodiversity hot spots of the world, together with the West Coast, form an important ecological region. It actually originates at Gangamoola in the Varaha Parvata (at an elevation of about 1,199m high) in the Western Ghats, in Chikmagalur district. Nethravathy is regarded as sacred river and people of Dakshina Kannada district worship it with supreme reverence. Rainfall is the most important exogenic process that shapes the landforms. The monsoon rains are unleashed when clouds strike against the relief's of the Western Ghats. The main rainy season is south west monsoon during which time about 86% of the annual rainfall occurs. Post monsoon season yields about 8% and the pre monsoon the barrons of the annual rainfall. During our study period it has been recorded that the December to March, the weather is usually dry whereas July to September are the wettest months.

3.1 *Microbial count*

The values for colony count after serial dilution of the five samples are tabulated (Table 1). The total colony forming units per ml increased consistently as the dilution increased from 10^{-3} to 10^{-6} with the values higher than 10^{9} in each case after maximum dilution.

Table 1. Colony count after serial dilution of four samples.

Dilutions	S1 (cfu/ml)	S2 (cfu/ml)	S3 (cfu/ml)	S4 (cfu/ml)
10^{-3}	2.82×10^{6}	2.94×10^{6}	3.04×10^{6}	2.70×10^{6}
10^{-4}	2.50×10^{7}	2.44×10^{7}	2.40×10^{7}	2.38×10^{7}
10^{-5}	1.94×10^{8}	1.80×10^{8}	1.96×10^{8}	2.00×10^{8}
10^{-6}	1.40×10^{9}	1.36×10^{9}	1.28×10^{9}	1.48×10^{9}

3.2 *Identification of microbes*

Among all, the microorganisms from sample collected at station $_3$ showed better activity against both Anthracene and Naphthalene (Table 2 and 3). All the six isolates from this sample were identified. Three bacterial isolates were identified on the basis of their cultural and biochemical characteristics and with reference to Bergey's Manual of Determinative Bacteriology (1992). The bacterial isolates were *Pseudomonas spp*., *Bacillus spp*. and *Flavobacterium spp*. Three fungal isolates *Aspergillus spp*., *Penicillium spp*. and *Candida spp*. were also identified.

Table 2. Activity of microorganisms from S_3 on Anthracene

	Incubation Time		
Strains isolated	**24 hrs**	**48 hrs**	**72 hrs**
Pseudomonas spp.	+	++	+++
Bacillus spp.	-	+	++
Flavobacterium spp.	+	+	++
Aspergillus spp.	+	++	++
Penicillium spp.	-	+	++
Candida spp.	+	++	-

Table 3. Activity of microorganisms from S_3 on Naphthalene

	Incubation Time		
Strains isolated	**24 hrs**	**48 hrs**	**72 hrs**

Pseudomonas spp.	-	++	+++
Bacillus spp.	-	+	++
Flavobacterium spp.	+	++	-
Aspergillus spp.	+	++	++
Penicillium spp.	+	+	++
Candida spp.	+	+	++

- : No inhibitory zone +: < 10 mm ++: 10-14 mm +++ : 15-20 mm

3.3 *Assessment of degrading activity*

The bacterial strains *Pseudomonas spp*. and *Bacillus spp*. showed the maximum optical density (O.D.) at 8mg concentration (Anthracene) and *Flavobacterium spp*. at a concentration of 6mg. Overall the maximum values were recorded on the fourth day. There was a decrease on fifth day and remained more or less constant thereafter. The two fungal strains *Aspergillus spp*. and *Penicillium spp*. recorded the maximum O.D at anthracene concentration of 6mg while *Candida spp*. showed highest O.D. at 8mg/20ml of SMS media. The values of absorbance are tabulated in Table 4 and 5.

Table 4. Absorbance recorded for bacterial strains at different concentrations of Anthracene.

Microorganisms	No. of days	Concentration of Anthracene per 20ml of SMS media				
		2mg	**4mg**	**6mg**	**8mg**	**10mg**
Pseudomonas spp.	1	0.245	0.298	0.350	0.387	0.341
	2	0.325	0.303	0.484	0.506	0.472
	3	0.384	0.466	0.647	0.771	0.689
	4	0.475	0.543	0.698	0.834	0.759
	5	0.446	0.504	0.605	0.790	0.677
	6	0.460	0.484	0.585	0.774	0.681
	7	0.434	0.519	0.615	0.760	0.660
Bacillus spp.	1	0.165	0.284	0.323	0.367	0.250
	2	0.290	0.344	0.416	0.480	0.364
	3	0.405	0.450	0.556	0.627	0.454
	4	0.534	0.616	0.595	0.817	0.601
	5	0.493	0.553	0.536	0.787	0.554
	6	0.501	0.584	0.583	0.768	0.574
	7	0.455	0.507	0.564	0.793	0.500
Flavobacterium spp.	1	0.188	0.210	0.591	0.510	0.462
	2	0.296	0.283	0.645	0.572	0.496
	3	0.384	0.413	0.696	0.605	0.528
	4	0.461	0.595	0.845	0.716	0.619
	5	0.428	0.523	0.760	0.684	0.600
	6	0.392	0.459	0.748	0.621	0.592
	7	0.305	0.422	0.705	0.635	0.435

Table 5. Absorbance for fungal isolates at different concentrations of Anthracene.

Microorganisms	No. of days	Concentration of Anthracene per 20ml of SMS media				
		2mg	**4mg**	**6mg**	**8mg**	**10mg**
Aspergillus spp.	1	0.246	0.264	0.414	0.380	**0.359**
	2	0.337	0.375	0.528	0.436	**0.484**
	3	0.422	0.464	0.641	0.507	**0.566**
	4	0.575	0.593	0.797	0.610	**0.628**
	5	0.530	0.605	0.706	0.580	**0.595**

	6	0.564	0.556	0.748	0.585	**0.55**
	7	0.554	0.580	0.716	0.605	**0.577**
Penicillium spp.	1	0.146	0.267	0.390	0.345	**0.289**
	2	0.268	0.329	0.504	0.475	**0.366**
	3	0.384	0.396	0.616	0.520	**0.427**
	4	0.478	0.504	0.754	0.677	**0.506**
	5	0.426	0.506	0.684	0.650	**0.478**
	6	0.414	0.481	0.671	0.626	**0.479**
	7	0.408	0.484	0.687	0.618	**0.501**
Candida spp.	**1**	**0.153**	**0.246**	**0.312**	**0.634**	**0.594**
	2	**0.269**	**0.298**	**0.351**	**0.682**	**0.616**
	3	**0.281**	**0.361**	**0.382**	**0.723**	**0.635**
	4	**0.347**	**0.388**	**0.480**	**0.748**	**0.700**
	5	**0.313**	**0.329**	**0.462**	**0.656**	**0.663**
	6	**0.305**	**0.322**	**0.451**	**0.697**	**0.659**
	7	**0.312**	**0.319**	**0.458**	**0.668**	**0.662**

The absorbance readings recorded for Naphthalene degradation indicate that the strains *Pseudomonas spp*., *Bacillus spp*. and *Flavobacterium spp*. showed highest O.D values at 6mg and 4mg and 8mg concentration of Naphthalene respectively (Table 6). Among the fungal strains *Aspergillus spp*. and *Candida spp*. recorded maximum value at a concentration of 8mg and *Penicillium spp*. at 10 mg/20ml of SMS media (Table 7). With respect to the days, maximum O.D i.e., the maximum degradation was recorded on the fourth day of incubation.

Table 6. Absorbance recorded at different concentrations of Naphthalene for bacterial strains.

Microorganisms	**No. of days**	**Concentration of Naphthalene per 20ml of SMS media**				
		2mg	**4mg**	**6mg**	**8mg**	**10mg**
Pseudomonas spp.	1	0.184	0.200	0.405	0.325	**0.284**
	2	0.242	0.308	0.512	0.436	**0.360**
	3	0.391	0.437	0.580	0.525	**0.398**
	4	0.463	0.512	0.749	0.670	**0.555**
	5	0.468	0.504	0.715	0.659	**0.450**
	6	0.453	0.510	0.658	0.603	**0.458**
	7	0.450	0.492	0.707	0.620	**0.421**
Bacillus spp.	1	0.105	0.395	0.330	0.318	**0.264**
	2	0.287	0.452	0.351	0.416	**0.370**
	3	0.372	0.537	0.462	0.438	**0.455**
	4	0.493	0.712	0.610	0.584	**0.508**
	5	0.472	0.686	0.602	0.550	**0.493**
	6	0.453	0.697	0.591	0.540	**0.498**
	7	0.426	0.684	0.599	0.545	**0.480**
Flavobacterium spp.	1	0.156	0.178	0.210	0.485	0.326
	2	0.228	0.216	0.295	0.496	0.394
	3	0.259	0.320	0.316	0.526	0.465
	4	0.384	0.415	0.345	0.612	0.416
	5	0.335	0.422	0.405	0.597	0.548
	6	0.296	0.396	0.384	0.505	0.506
	7	0.288	0.348	0.296	0.491	0.353

Table 7. Absorbance recorded at different concentrations of Naphthalene for fungal isolates.

Microorganisms	No. of days	Concentration of Naphthalene per 20ml of SMS media				
		2mg	4mg	6mg	8mg	10mg
Aspergillus spp.	1	0.115	0.220	0.245	0.372	0.301
	2	0.263	0.345	0.304	0.463	0.420
	3	0.354	0.396	0.412	0.540	0.478
	4	0.472	0.485	0.532	0.690	0.600
	5	0.451	0.457	0.502	0.623	0.586
	6	0.450	0.421	0.495	0.608	0.550
	7	0.428	0.436	0.505	0.602	0.558
Penicillium spp.	1	0.090	0.185	0.203	0.284	0.360
	2	0.204	0.271	0.295	0.350	0.455
	3	0.331	0.364	0.338	0.469	0.588
	4	0.470	0.525	0.561	0.632	0.769
	5	0.451	0.507	0.500	0.618	0.704
	6	0.412	0.470	0.523	0.604	0.708
	7	0.438	0.450	0.500	0.582	0.689
Candida spp.	1	0.124	0.229	0.320	0.358	0.345
	2	0.206	0.261	0.328	0.364	0.350
	3	0.180	0.354	0.390	0.432	0.405
	4	0. 345	0.480	0.564	0.645	0.362
	5	0.248	0.356	0.368	0.492	0.420
	6	0.256	0.330	0.470	0.501	0.458
	7	0.310	0.365	0.488	0.540	0.426

From the results it is clear that among all, the microorganisms isolated from Station-III sample showed better degrading activity compared to the other sites evaluated for the degradation of petroleum products. It is quite obvious that the microorganisms from these areas naturally depend on the petroleum products for their survival and only those can degrade can survive actively.

4. Conclusion

Microbial degradation of crude oil and its derivatives is an important field of biotechnological/bioremediation research because of the impact of oil spills on the environment. Biodegradation of crude oil in natural ecosystems is quite complex as it occurs relatively slow. Bioremediation is emerging as a promising technology particularly very effective in dealing with petroleum hydrocarbon contamination. It is a cost-effective and environmentally sound remediation technology, particularly for dealing with petroleum hydrocarbon contamination. However, feasibility studies are required prior to full scale remediation. The goal of such studies is to determine if specific hydrocarbon contaminants are amenable to biological treatment and also to determine the time required for degradation. In the present investigation, microbes from samples collected at three study sites of river Nethravathy were isolated, characterized and tested for the *ex situ* biodegradation of two petroleum components- anthracene and Naphthalene with respect to time and concentration. The intent of the present study is to present a broad and updated overview of the microbial ecology of hydrocarbon degradation, emphasizing both environmental and biological factors which are involved in determining the rate at which and extent to which hydrocarbons are removed from the environment by biodegradation.

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