

Isolation and Identification of
potential PAH degrading
Bacterial Species from the Diep-
and Plankenburg Rivers, Western
Cape, South Africa.



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Introduction

- Polycyclic Aromatic Hydrocarbons (PAHs) - persistent, semi-volatile organic pollutants that are ubiquitous in the environment

- Enter environmental matrices - natural and anthropogenic sources such as agricultural and industrial activities.



- They can cause harmful effects on ecosystems as well as human health, as many of them have been shown to be carcinogenic, teratogenic and mutagenic.



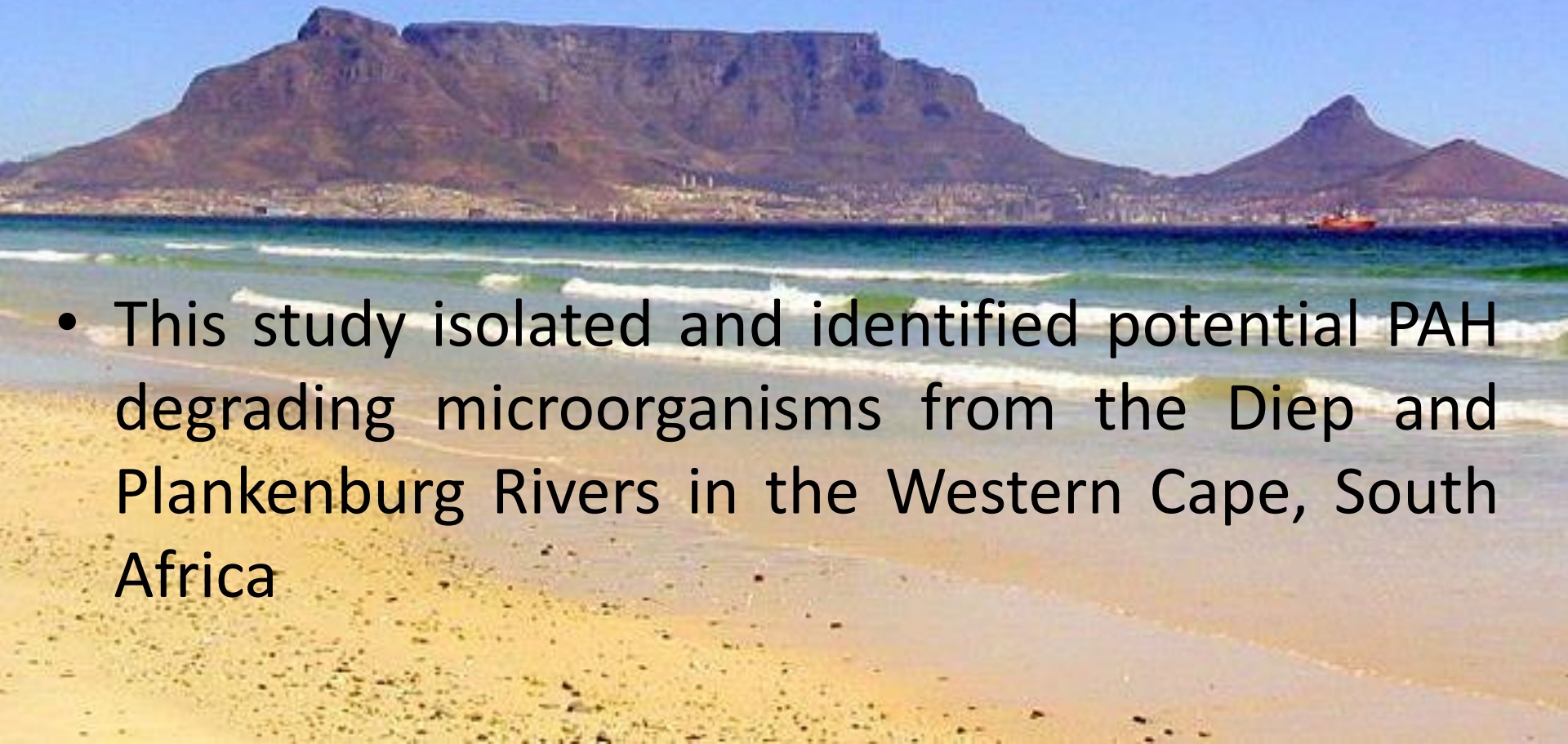
- Several approaches and strategies - physical, chemical and biological strategies have been developed and employed to remediate polluted sites.

- Bioremediation has been shown to be a cost effective and environmentally friendly approach to remediate contaminated sites.

- Several bacterial: *Pseudomonas*, *Alcanivorax*, *Microbulbifer*, *Sphingomonas*, *Micrococcus*, *Cellulomonas*, *Dietzia*, *Gordonia*, *Marinobacter* and fungal species: *Aspergillus* sp., *Trichocladium canadense*, and *Fusarium oxysporum* capable of degrading PAH compounds have been isolated and characterised.

- The PAH degrading capability of indigenous microorganisms isolated at significant point sources of pollution must therefore be investigated.

- This study isolated and identified potential PAH degrading microorganisms from the Diep and Plankenburg Rivers in the Western Cape, South Africa



Materials and Methods

Sampling Site Identification

- Plankenburg River:
 - ❖ Point A - Agricultural farming and Residential
 - ❖ Point B - Substation in Industrial Area
 - ❖ Point C - Informal Settlement of Kayamandi



Agricultural farming area adjacent to the Plankenburg River

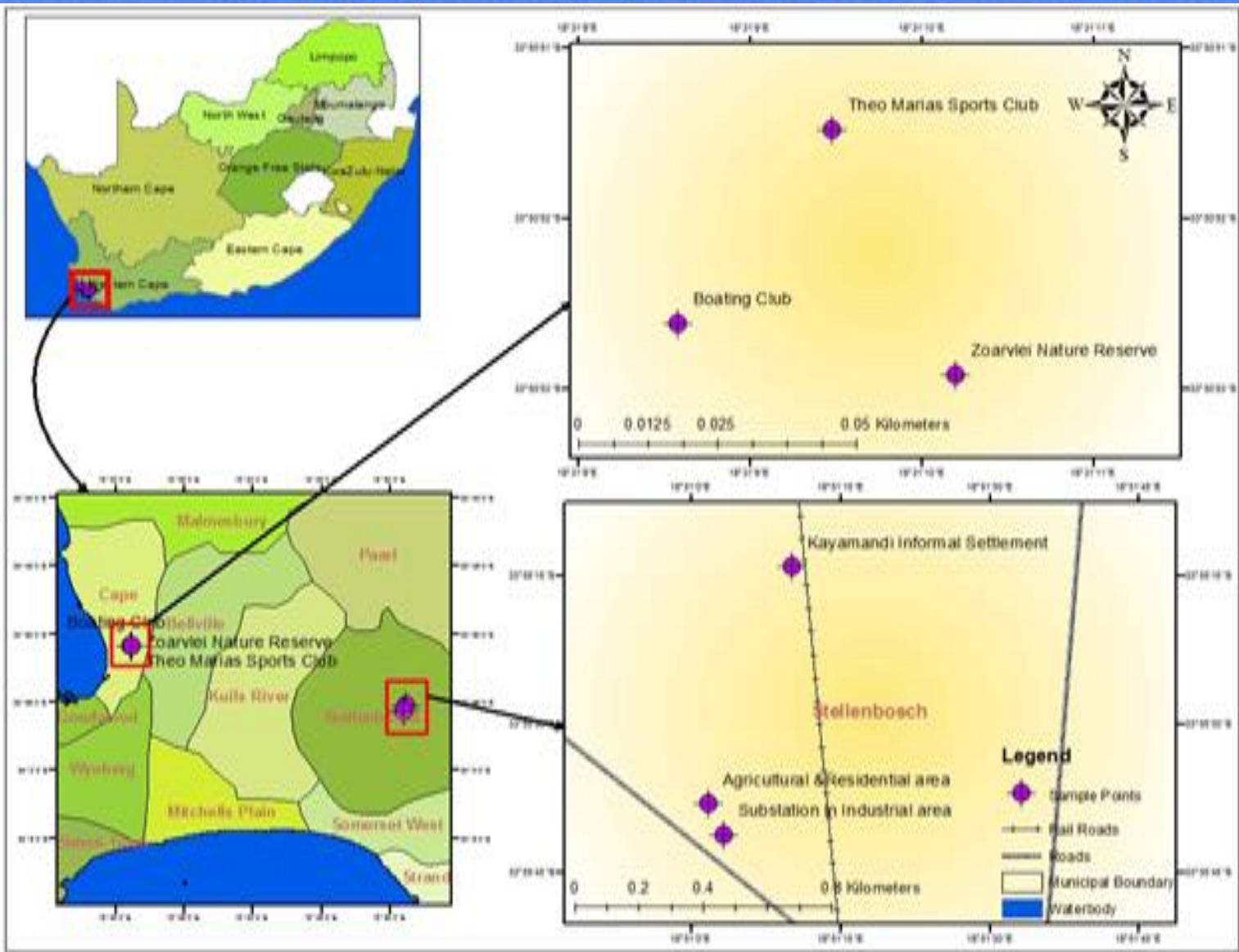
- Diep River:

- ❖ Point D - Zoarvlei Nature Reserve (Industrial as well as Residential)
- ❖ Point E - Theo Marias Sports club (Industrial and Residential Area)
- ❖ Point F - Rietvlei Boating club



Point E (Theo Marias Sports Club)





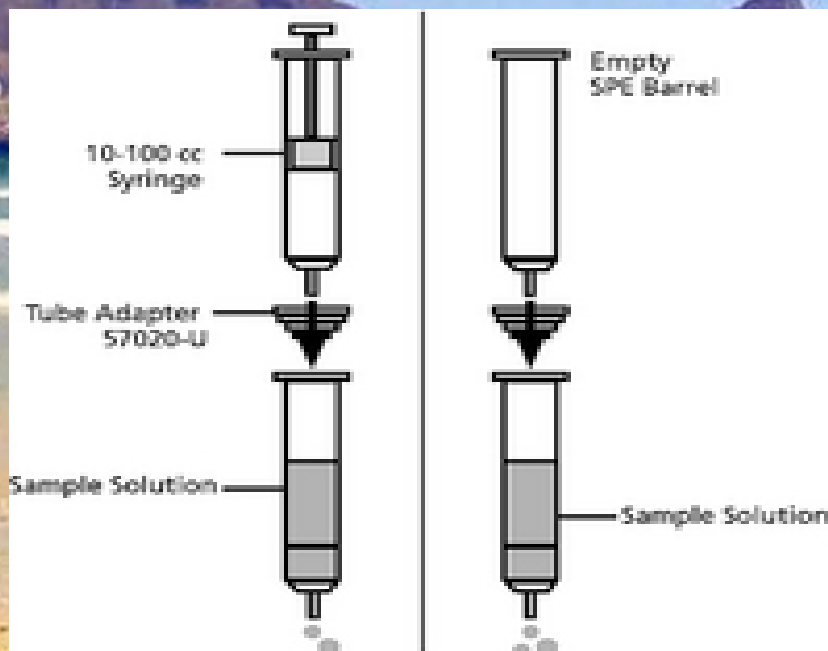
Sampling

- Water and sediment samples were collected once a month over a six month period.
- Water samples were collected in sterile amber bottles while sediment samples were collected using a sterile Ekman grab, placed in polypropylene containers and wrapped in aluminium foil.
- Both water and sediment samples were kept on ice at 4°C during transport to the laboratory for analyses.

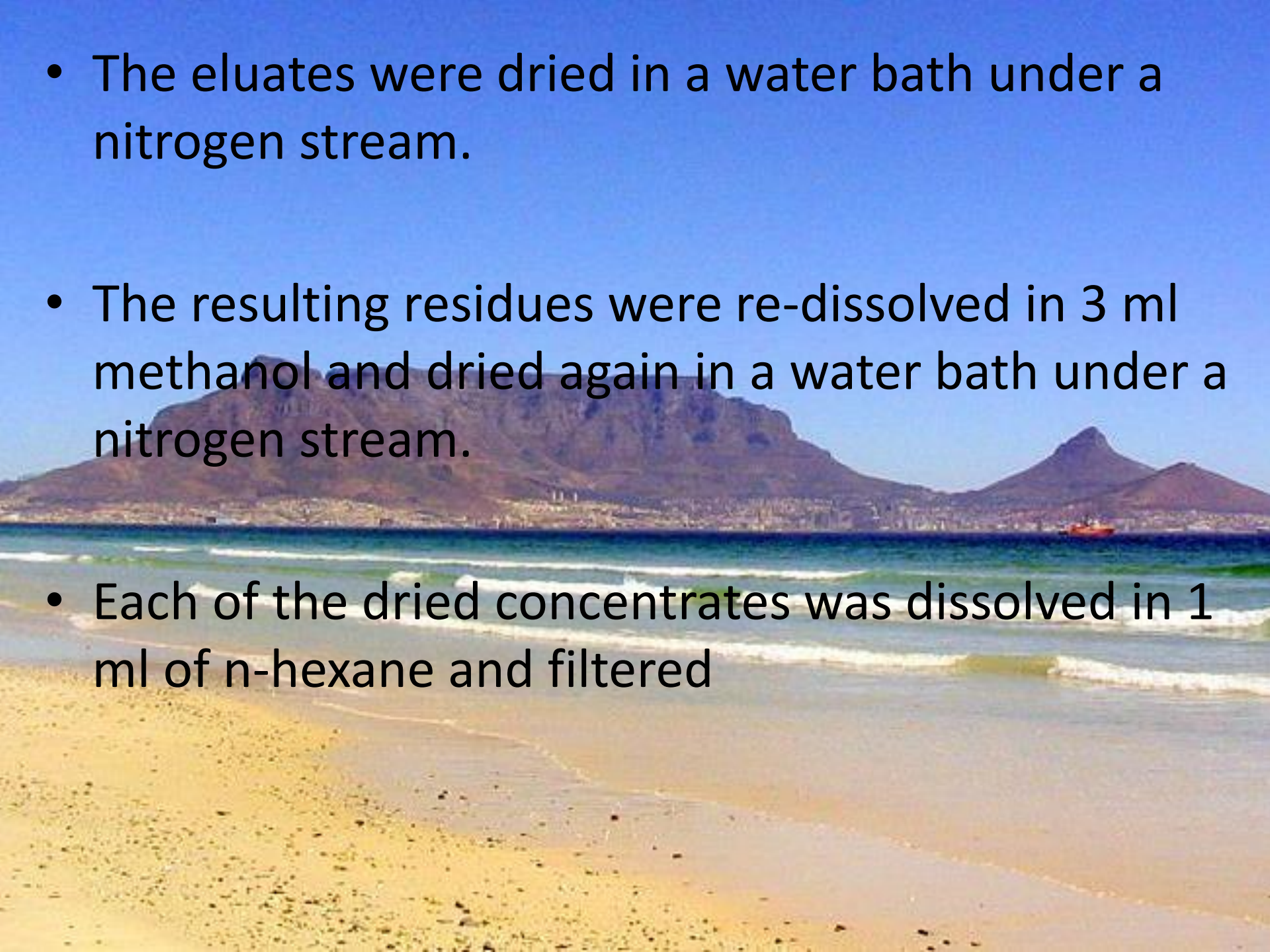
Determination of the presence and concentration of PAHs in the River systems

- Acenaphthene and Fluorene were extracted with 50 ml n-hexane and dichloromethane (DCM).
- The flask was left for 15 minutes at room temperature to allow for equilibration and efficient phase separation.
- This procedure was repeated thrice to ensure good PAH recovery (above 70%).
- The extracts were combined and dried in a water bath at 35°C under a stream of nitrogen.
- The dried samples were reconstituted to 2 ml with n-hexane.

- The extracts were cleaned using the solid phase extraction technique.
- Each PAH concentrate extract was then loaded on the SPE frit tubes and eluted with 70 ml of n-hexane.



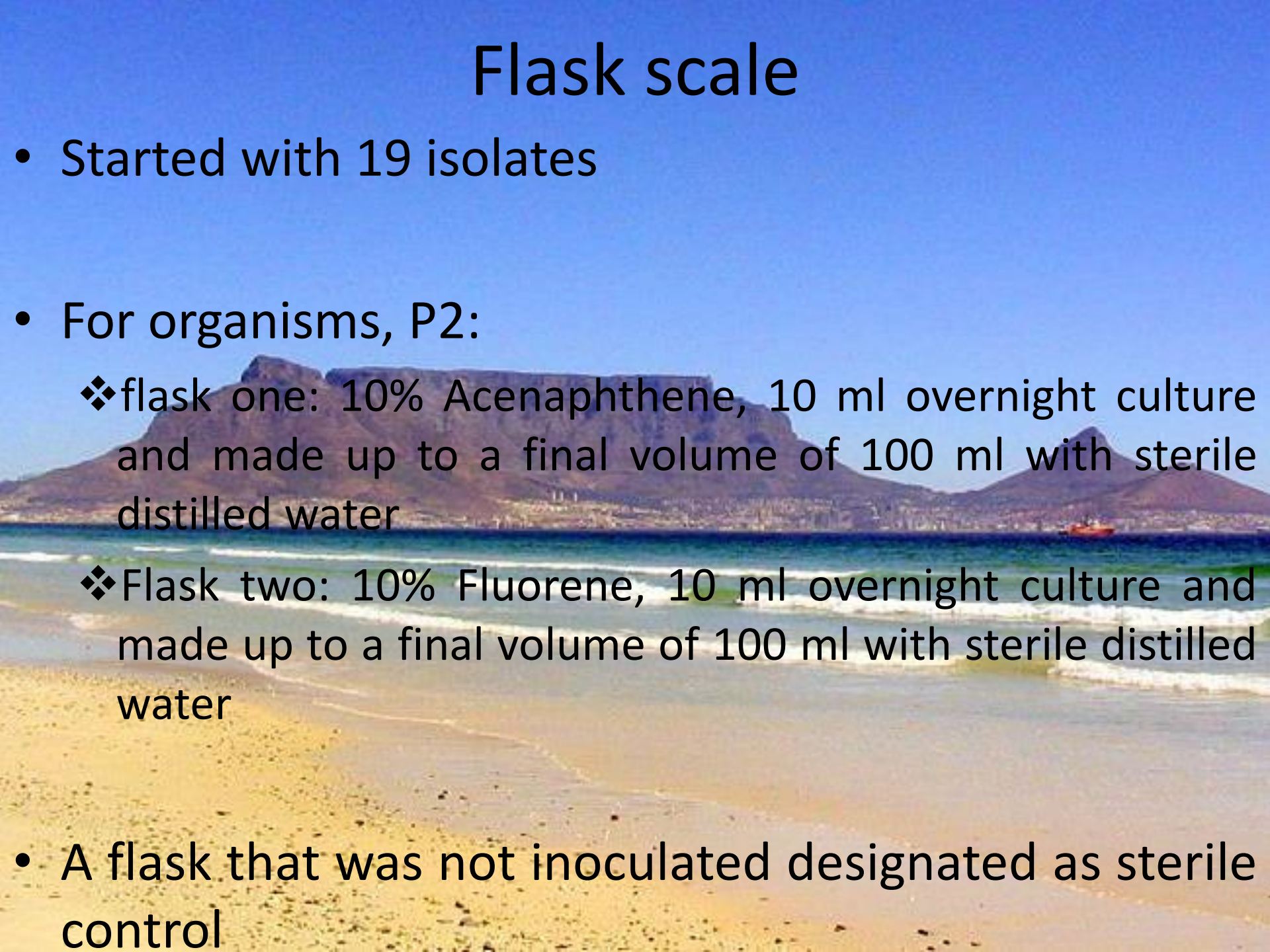
- The eluates were dried in a water bath under a nitrogen stream.
- The resulting residues were re-dissolved in 3 ml methanol and dried again in a water bath under a nitrogen stream.
- Each of the dried concentrates was dissolved in 1 ml of n-hexane and filtered



Isolation and Identification of potential PAH degrading Bacterial Species using conventional techniques

- Standard cultural techniques
- Media such as Nutrient agar, Tryptic soy agar, Luria Bertani agar, MacConkey agar, *Pseudomonas* isolation agar base, *Aeromonas* isolation agar, etc.
- Phenotypic identification techniques such as staining and biochemical tests were conducted
- Isolates were selected on the basis of morphology and Gram reaction.

Flask scale

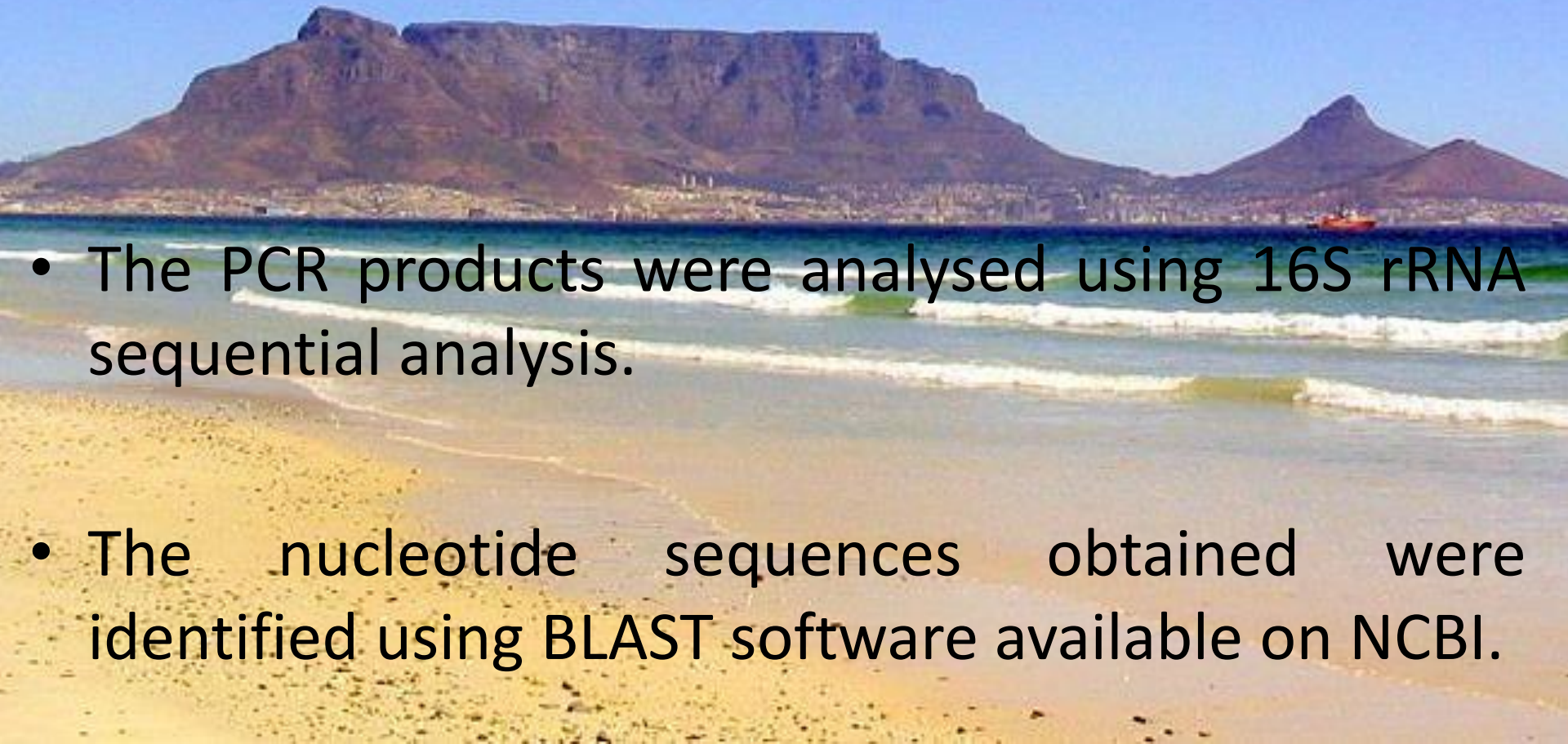
- Started with 19 isolates
 - For organisms, P2:
 - ❖ flask one: 10% Acenaphthene, 10 ml overnight culture and made up to a final volume of 100 ml with sterile distilled water
 - ❖ Flask two: 10% Fluorene, 10 ml overnight culture and made up to a final volume of 100 ml with sterile distilled water
 - A flask that was not inoculated designated as sterile control
- 
- A background image showing a sandy beach in the foreground, a blue ocean with white waves in the middle ground, and a large, flat-topped mountain range under a clear blue sky in the background.

- The experiment was carried out in a shaking incubator at 150 rpm for 14 days at various temps.
- 25⁰C, 30⁰C, 35⁰C, 37⁰C, 38⁰C, 40⁰C and 45⁰C.
- All flasks were capped with cotton wool and covered with aluminium foil to minimise losses due to photo oxidation.



Molecular identification of PAH degrading bacterial isolates

- Polymerase chain reaction (PCR).
- The PCR products were analysed using 16S rRNA sequential analysis.
- The nucleotide sequences obtained were identified using BLAST software available on NCBI.



Results and Discussion

		Point A	Point B	Point C	Point D	Point E	Point F	
Winter	Water	0	0.004	0.0008	0	0	0.004	Ace
		0.0009	0.20	0.0007	0	0.0006	0.004	Flu
	Sediment	0.20	0.40	0.10	0	0.07	0.80	Ace
		0.60	0.90	0.60	0.40	0.80	0.90	Flu
Summer	Water	0	0.0006	0	0	0	0.0006	Ace
		0	0.003	0	0	0.0009	0.005	Flu
	Sediment	0.04	0.20	0.003	0	0.004	0.40	Ace
		0.04	0.70	0.002	0	0.004	0.60	Flu

- Results above showed that the highest concentrations were recorded in both water and sediment at substation at the Plankenburg River.



- This is an industrialised area surrounded by many different industries such as spray painting, mechanical workshops, etc.

Isolation and Identification of potential PAH degrading Bacterial Species using conventional techniques

Cultural Characteristics of bacterial isolates before degradation - four of the 19 isolated

Isolate	NA	AIA	PAB	GSP	BA	MAC
P2	Glistening, moist, Cream, round, elevated.	Small, yellow colonies, changes media colour from green to yellow	Round, cream colonies	Small, Purple, round, elevated	Cream, round, elevated	Round, pink colonies
AE1	Red, round, small, elevated colonies	–	–	–	Medium sized, buff coloured colonies	Colourless, translucent colonies
ISB2	White, large, round, vacuolated appearance,	–	–	–	Round, smooth, cream colonies	–
AE4	Large, Flat, Round, Cream coloured	Dark green opaque colonies with dark centres	–	Yellow colonies, starch hydrolysis (media colour change from red to yellow)	small, round, smooth, colonies	Pink colonies

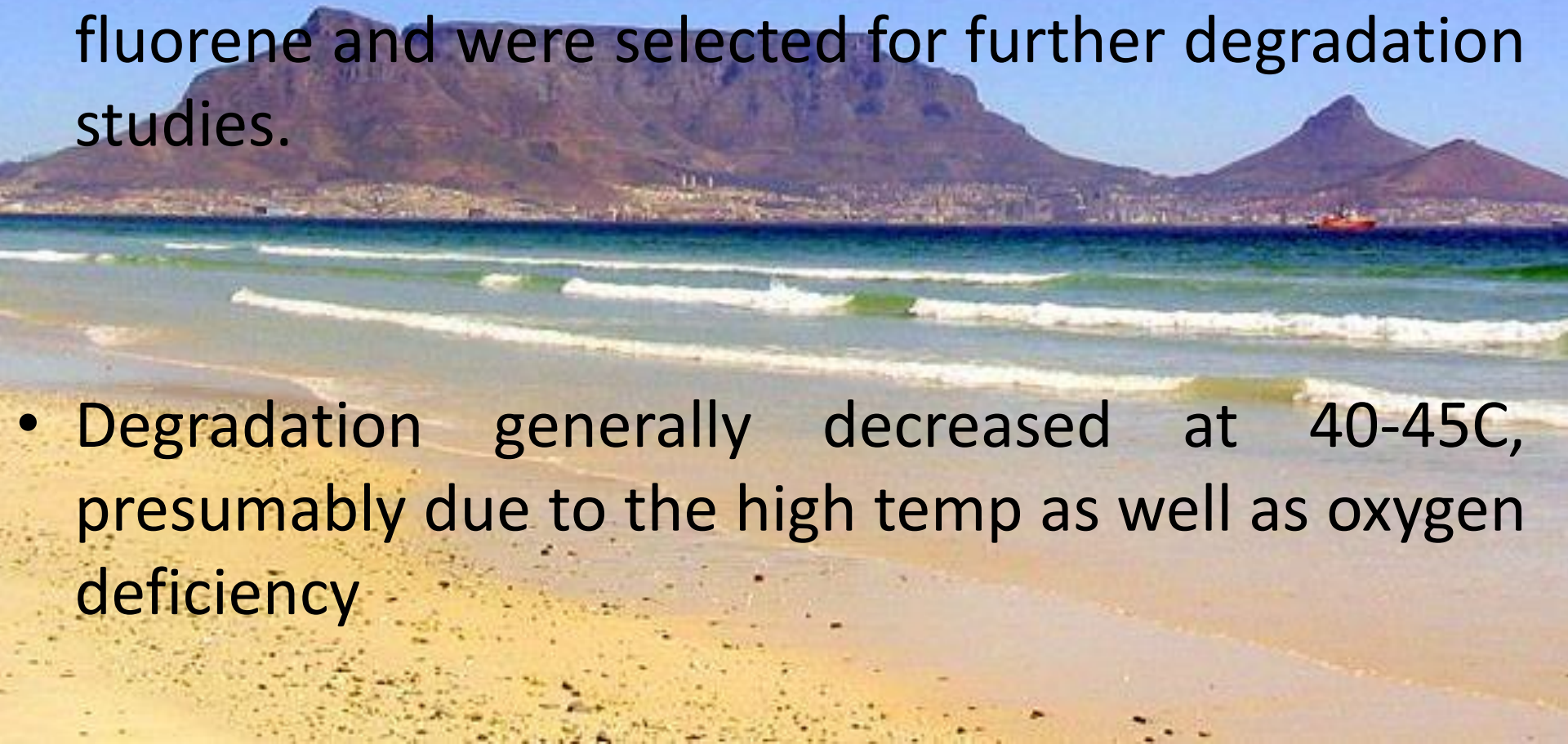
Key: NA: Nutrient Agar, AIA: Aeromonas Isolation Agar, PAB: Pseudomonas Agar Base, GSP: GSP Agar, BA: Blood Agar, MAC: Mac Conkey Agar, P2: *Raoutella ornithinolytica*, AE1: *Serratia marcescens*, ISB2: *Bacillus megaterium*, AE4: *Aeromonas hydrophila*

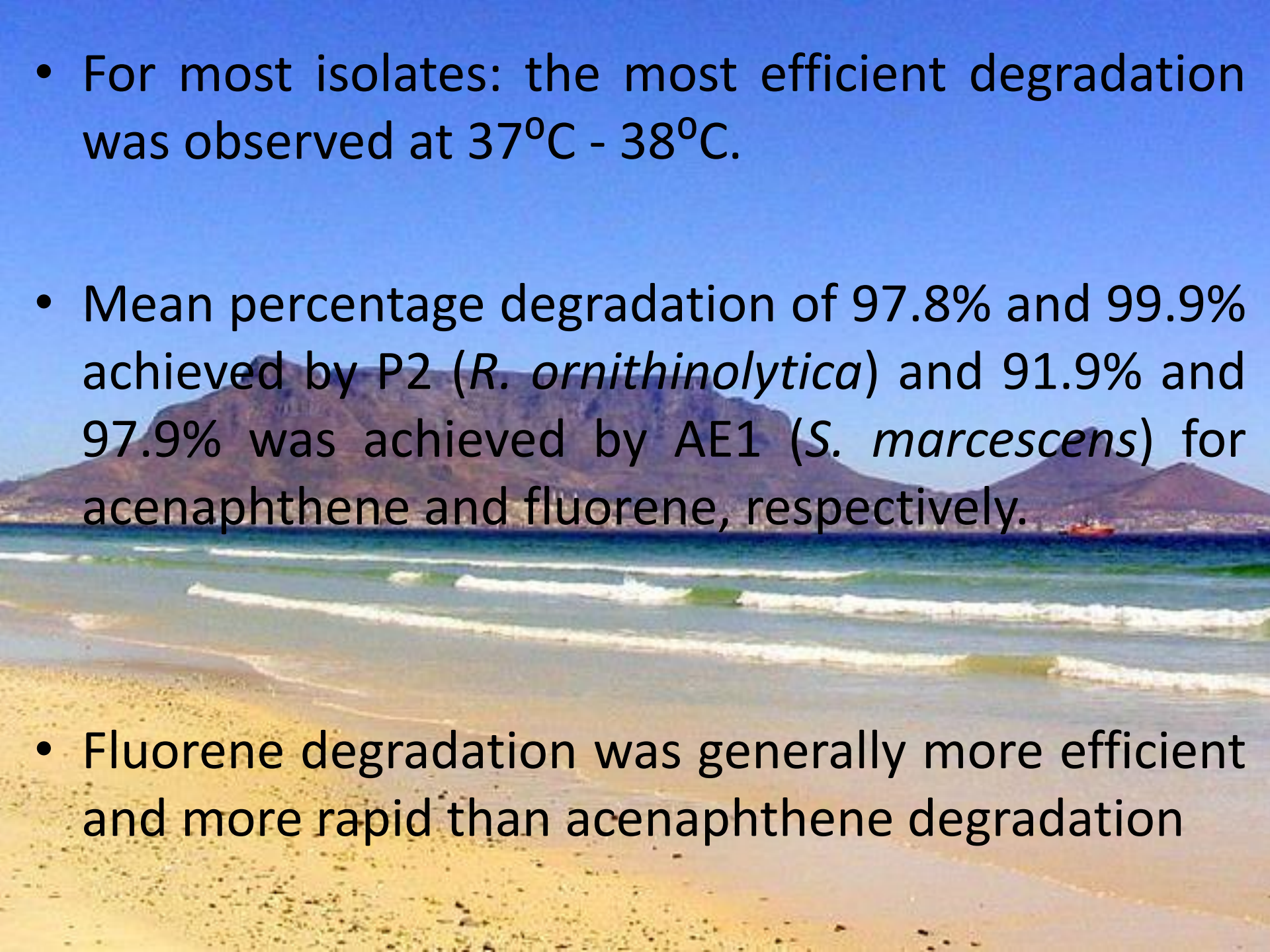
Biochemical and gram stains	Test Organisms			
Name of Test	P2	AE1	ISB2	AE4
Gram reaction	-	-	+	-
Endospore	-	-	+	-

- Isolate P2 grew on all media tested, while AE4 grew on all but one
- P2 was successfully cultured on *Pseudomonas* Agar base, a culture medium which is otherwise selective for *Pseudomonas spp* and certain members of *Burkholderia spp*
- *Aeromonas* isolation agar also allows growth of *Enterobacteriaceae* species so the medium is used as universal medium in the investigation of enteric disease.

Flask scale

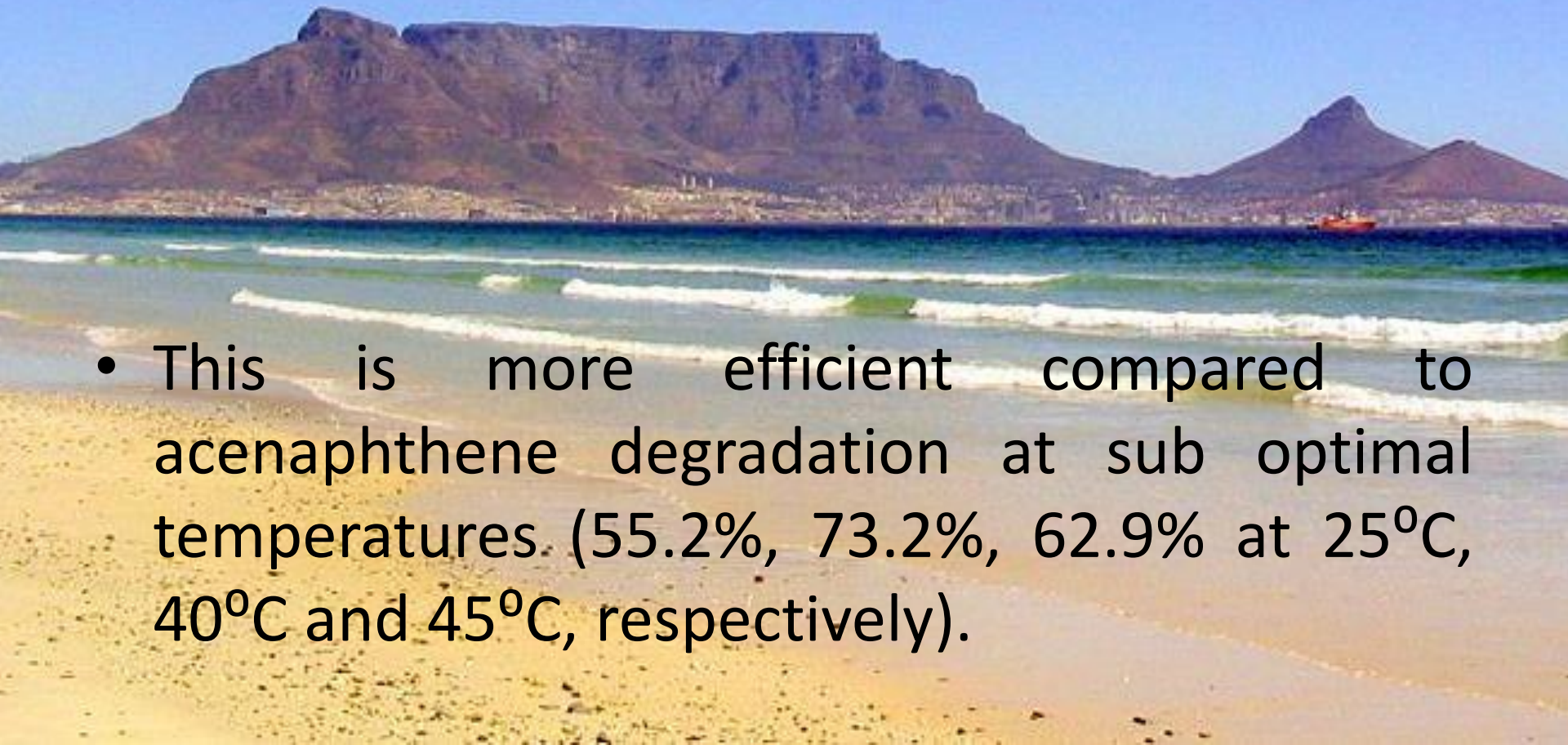
- After subjecting all the isolated species to screening, four of the isolates successfully degraded above 75% of acenaphthene and fluorene and were selected for further degradation studies.
- Degradation generally decreased at 40-45C, presumably due to the high temp as well as oxygen deficiency



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- For most isolates: the most efficient degradation was observed at 37°C - 38°C.
 - Mean percentage degradation of 97.8% and 99.9% achieved by P2 (*R. ornithinolytica*) and 91.9% and 97.9% was achieved by AE1 (*S. marcescens*) for acenaphthene and fluorene, respectively.
 - Fluorene degradation was generally more efficient and more rapid than acenaphthene degradation

- *Raoultella ornithinolytica* degraded above 91% of fluorene at all temperature values tested (25°C - 45°C).

- This is more efficient compared to acenaphthene degradation at sub optimal temperatures (55.2%, 73.2%, 62.9% at 25°C, 40°C and 45°C, respectively).



Flasks	Temperature (°C)	Initial conc (ppm)	Residual conc (ppm)	Percentage degradation (%)
A (Ace + <i>R. ornithinolytica</i>)	25°C	50	22.38	55.20
	37°C	50	1.09	97.80
	45°C	50	18.56	62.90
B (Ace + <i>S. marcescens</i>)	25°C	50	27.49	45
	37°C	50	4.00	91.90
	45°C	50	13.16	73.70
C (Ace + <i>B. megaterium</i>)	25°C	50	17.58	64.80
	37°C	50	18.62	62.80
	45°C	50	19.50	60.90
D (Ace + <i>A. hydrophila</i>)	25°C	50	12.29	75.40
	37°C	50	0.42	99.20
	45°C	50	22.38	55.20
(Ace + cocktail)	25°C	50	13.10	73.80
	37°C	50	0.42	99.20
	45°C	50	4.78	90.40

Flasks	Temperature (°C)	Initial conc (ppm)	Residual conc (ppm)	Percentage degradation (%)
J (fluorene + <i>R. ornithinolytica</i>)	25°C	50	4.22	91.60
	37°C	50	0.02	99.90
	45°C	50	4.33	91.60
K (fluorene + <i>S. marcescens</i>)	25°C	50	18.87	62.30
	37°C	50	1.06	97.90
	45°C	50	38.40	23.20
L (fluorene + <i>B. megaterium</i>)	25°C	50	2.68	94.60
	37°C	50	3.87	92.30
	45°C	50	3.08	93.80
M (fluorene + <i>A. hydrophila</i>)	25°C	50	12.70	74.40
	37°C	50	0.73	98.50
	45°C	50	21.36	57.30
N (fluorene + cocktail)	25°C	50	0.69	98.60
	37°C	50	0.29	99.40
	45°C	50	0.25	99.50

Isolation and Identification of potential PAH degrading Bacterial Species using conventional techniques

- 19 bacterial isolates were obtained from the sampling points
- Most of the organisms that showed resistance belonged to the Gram negative group
- Gram negative organisms have been shown before to be more resistant to antibiotics, etc.
- Reason for this is that they have a thinner peptidoglycan layer and thus do not absorb contaminants as readily as the Gram positive organisms

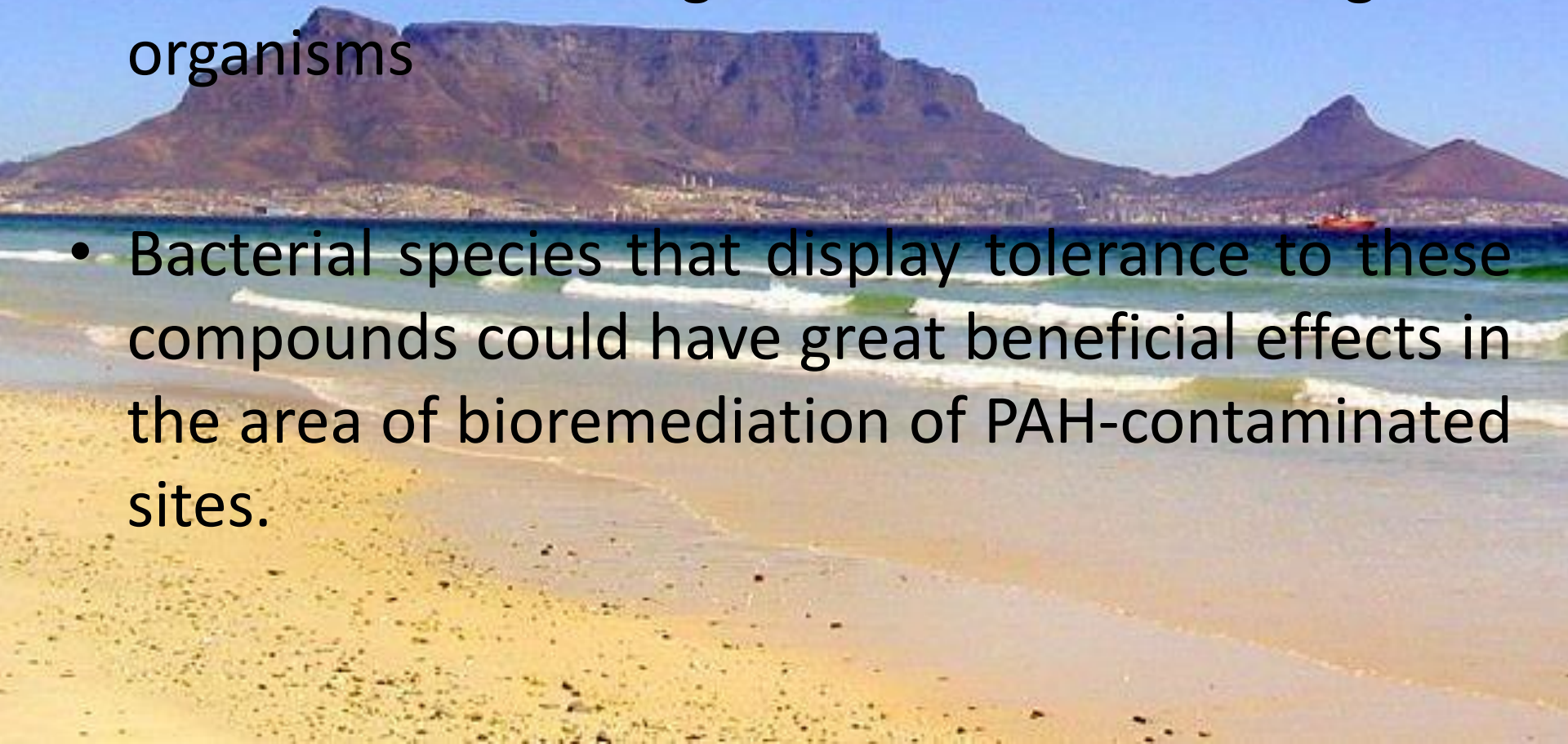
Identified organisms using Blast

S/N	Isolate	BLAST ID	Accession number
1	DR	<i>Raoultella ornithinolytica</i>	KT239136
2	DB	<i>Bacillus megaterium</i>	KT239138
3	PA	<i>Pseudomonas aeruginosa</i>	KT282224
4	LC	<i>Raoultella planticola</i>	KT282225
5	LP	<i>Klebsiella oxytoca</i>	KT282226
6	MA	<i>Escherichia coli</i>	KT282227
7	DL	<i>Enterobacter cloacae</i>	KT282228
8	PY	<i>Exiguobacterium acetylicum</i>	KT282229
9	PR	<i>Acinetobacter</i> sp.	KT282230
10	XP	<i>Exiguobacterium</i> sp.	KT282231
11	RC	<i>Bacillus</i> sp.	KT282232
12	SE	<i>Serratia marcescens</i>	KT239137
13	AH	<i>Aeromonas hydrophila</i>	KT239139
14	BA	<i>Bacillus aryabhatai</i>	KT003255
15	TA	<i>Bacillus aquimaris</i>	KJ009414
16	QO	<i>Bacillus marisflavi</i>	KR016603
17	ST	<i>Staphylococcus saprophyticus</i>	KR023561
18	KP	<i>Citrobacter freundii</i>	KT315777
19	DG	<i>Exiguobacterium undae</i>	AB669472

Conclusion

- Acenaphthene and fluorene were isolated in the highest concs from the substation, where waste from spray painting and mechanical works flow in
- The predominant degradative species identified included *Raoutella ornithinolytica*, *Serratia marcescens*, *Bacillus megaterium* and *Aeromonas hydrophila*.
- *Raoutella ornithinolytica* is a member of the *Enterobacteriaceae* while *Aeromonas sp.* closely resembles *Enterobacteriaceae*, which could account for its growth on most media.

- These organisms optimally degraded between 37-38°C
- Three of these organisms are Gram negative organisms
- Bacterial species that display tolerance to these compounds could have great beneficial effects in the area of bioremediation of PAH-contaminated sites.



Future work

- Characterise these organisms further and determine what makes them resistance
- Optimise these organisms for remediation purposes



Thank you

